Differential Expression of Estrogen Receptors in Women with Systemic Lupus Erythematosus

VIRGINIA RIDER, XIAOLAN LI, GREG PETERSON, JOYCE DAWSON, BRUCE F. KIMLER, and NABIH I. ABDOU

ABSTRACT. ***Objective.*** Systemic lupus erythematosus (SLE) is an autoimmune disease primarily affecting women. T cell activation markers (calcineurin, CD154) increase in SLE T cells cultured with estradiol 17-ß. Biological effects of estradiol are mediated through 2 receptor proteins, estrogen receptor-α (ER-α) and estrogen receptor-ß (ER-ß). We compared the amount of estrogen receptor subtypes in T cells and measured the ability of receptor agonist-specific ligands to activate marker gene expression.

Methods. T cells were isolated from 22 female patients with SLE and 17 control women. The amount of ER subtypes was measured by immunoblotting. Some T cells were cultured with ER-α or ER-ß-specific agonists. Receptor activation was measured by increased expression of the T cell activation markers CD154 and calcineurin.

Results. Although the amount of ER-α appeared to be less in SLE T cells than in control T cells, the difference was not statistically significant ($p = 0.081$). The quantity of ER-ß was similar in SLE and control T cells. The expression of ER-α or ER-ß was independent of menstrual cycle phase, age, or SLE disease activity. Calcineurin and CD154 expression increased in SLE T cells cultured in medium containing ER-α and ER-ß agonists.

Conclusion. These data indicate that both ER subtypes activate calcineurin and CD154 in SLE but not in normal T cells. Variation in the amount of ER-α in SLE T cells suggests this receptor subtype participates in the sensitivity of SLE T cells to estrogen. (J Rheumatol 2006;33:1093–101)

Key Indexing Terms:

SYSTEMIC LUPUS ERYTHEMATOSUS | AUTOIMMUNITY | ESTRADIOL
ESTROGEN RECEPTOR-α | ESTROGEN RECEPTOR-ß | T CELLS

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that is characterized by abnormal immunoregulation[1-3]. Although the acquisition of SLE is linked to genetic susceptibility[4], there are diverse environmental agents such as dietary factors, infectious agents, and drugs[5] that act on genetically susceptible individuals and promote SLE onset. SLE occurs during the child-bearing period 9 times more frequently in women than in men, and flares of disease are associated with certain phases of the menstrual cycle[6,7]. Ovulation induction by hormones and procedures that prepare women for *in vitro* fertilization increases estradiol in circulation and can correlate with *de novo* SLE onset, stimulating disease flares[8]. In the SLE mouse model, NZB × NZW F1 female mice develop SLE and die at an earlier age than their male counterparts. Administration of exogenous estrogen to female mice enhances SLE disease and increases anti-DNA antibody levels[9]. Castrated male F1 mice treated with estrogen die at an earlier age than untreated males, whereas survival is prolonged in ovariectomized female mice treated with androgens[9].

Two specific receptor proteins termed estrogen receptor-α (ER-α) and estrogen receptor-ß (ER-ß)[10] mediate estrogen action on target cells. Separate genes code for these receptors, although the proteins share a high degree of identity in the DNA-binding domains (~96%). Differences in the ligand-binding domains between the 2 receptor subtypes are proposed to contribute to selective affinities for certain ligands. The mechanisms by which estrogens affect disease activity in SLE are under investigation in our laboratory and by others[11–23]. Overlap in the expression of both ER-α and ER-ß is reported for some target tissues including T cells[11,12] and B cells[11,13,14], which express transcripts for both receptor subtypes. Evidence for functional ER in T cells has been shown by increased expression of T cell activation markers[15,16] and increased production of immunoglobulin and anti-double-stranded DNA antibodies in B cells exposed to estradiol in culture[17]. Evidence suggests that the sensitivity of SLE T cells

From the Department of Biology, Pittsburg State University, Pittsburg, Kansas, USA; Department of Dermatology and Rheumatology, Kunming Medical College, Kunming, People's Republic of China; Department of Radiation Oncology, University of Kansas Medical Center; and Center of Rheumatic Diseases, Allergy-Immunology, Kansas City, Missouri, USA.

Supported in part by NIH grant AI49272 and RR-16475 from the INBRE Program of the National Center for Research Resources; the Evans Memorial fund; and private funds from the Center for Rheumatic Diseases.

V. Rider, PhD, Department of Biology, Pittsburg State University; X. Li, MD, Department of Biology, Pittsburg State University, and Department of Dermatology and Rheumatology, Kunming Medical College; G. Peterson, MS; J. Dawson, MSN, Department of Biology, Pittsburg State University; B.F. Kimler, PhD, Department of Radiation Oncology, University of Kansas Medical Center; N.I. Abdou, MD, PhD, Center of Rheumatic Diseases, Allergy-Immunology.

Address reprint requests to Dr. V. Rider, Department of Biology, Pittsburg State University, Pittsburg, Kansas 66762. E-mail: vrider@pittstate.edu

Accepted for publication February 24, 2006.

to estradiol is mediated through the ER because cells cultured with the ER antagonist ICI 182,780 do not show increased expression in T cell activation markers[18]. Further, ER antagonists delay disease progression in SLE mouse models[19,20], suggesting that estrogen action is mediated through one or both of the ER proteins.

We are interested in the molecular basis underlying the increased sensitivity of SLE T cells to estradiol[21]. We have shown[15,16] that estradiol increases T cell activation marker gene expression, but the mechanism does not involve mRNA stabilization[22]. Further, although alternative splicing of ER-α primary transcripts occurs[11], no particular variant is associated with SLE T cells, suggesting alternative splicing may not account for increased SLE T cell sensitivity to estradiol. We found that the binding affinity of estradiol to ER-α is similar between SLE and normal T cells[23]. Studies by others[24] have shown that the amount of estradiol in circulation in women with SLE does not correlate with enhanced cytokine production. Although abnormal estradiol metabolism has been reported in some patients with SLE[25], SLE T cells cultured in the poorly metabolized 2-fluorestradiol show increased expression of the T cell activation markers calcineurin and CD154, presumably in the absence of estradiol metabolites[16]. Therefore, to gain additional insight into the molecular mechanisms underlying the sensitivity of SLE T cells to estradiol we have now measured and compared the amount of ER-α and ER-ß proteins in SLE and normal T cells. We have also utilized receptor-specific ligands[26,27] to test if activation of ER-α or ER-ß can stimulate increased expression of calcineurin and CD154 in cultured SLE T cells. These results are the first to show that T cell activation markers calcineurin and CD154 increase in response to activation through both ER-α and ER-ß in SLE T cells. The results further indicate that there is greater variation in the amount of ER-α between SLE and normal T cells. Altered expression or turnover of ER-α may therefore sensitize T cells to estradiol and contribute to altered immune function in women with SLE.

MATERIALS AND METHODS

Study participants. The participants enrolled in this study included 22 female patients with SLE and 17 age-matched female controls. The age of participants ranged between 21 and 46 years with a median age for the SLE patients of 39 years. Thirty-seven of the participants were Caucasian and 2 were African-American. Twenty of the patients had regular menstrual cycles. Two of the patients with active disease ceased menstruating during the time of this study. SLE patients met at least 4 of the criteria of the American College of Rheumatology for classification of SLE[28]. Their disease activity varied from mild to active, with SLE Disease Activity Index (SLEDAI) scores at the time blood was drawn between 2 and 28 (median score of patients assayed for ER-α, 9; median score of patients assayed for ER-ß, 6). Nine patients were receiving prednisone at a mean dose of 8.2 mg/day. Five of the patients were taking mycophenolate (1–2 g daily), 11 were taking hydroxychloroquine, and 15 patients were taking nonsteroidal antiinflammatory drugs. No participant was taking hormone replacement therapy, oral contraceptives, or had a history of other collagen vascular disease. Mean duration of lupus in the patients selected for the study was 10 years (median duration 10.2). Seven patients had lupus nephritis and finished a 2-year course of pulsed intravenous cyclophosphamide. Three of the 18 patients had central nervous system disease. No patient at the time of the study had active renal or central nervous system disease.

Collection of T cell enriched peripheral blood mononuclear cells. T cell enriched mononuclear cells were separated from blood samples (~90 ml) by density gradient (Histopaque, Sigma, St. Louis, MO, USA). The leukocytes were removed, washed in serum-free medium (RPMI 1640, Fisher Scientific, Hanover Park, IL, USA), and residual red blood cells were lysed (H-Lyse buffer, R&D Systems, Minneapolis, MN, USA). T cells were purified by negative selection through T cell isolation columns (Human T Cell Enrichment Columns, R&D Systems). T cell purity was greater than 95% (95.9% ± SEM 1.4) as assessed by flow cytometry (data not shown).

T cell extracts. Cell extracts were prepared by incubating T cells for 30 min on ice in a lysis solution (10 mM Tris-HCl, pH 7.5, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate), containing protease inhibitors as described[16,29]. Lysates were clarified by centrifugation at 10,000 ×g for 10 min at 4°C. Purified T cell extracts were stored at –80°C.

Western blots. T cell extracts were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The extracts were analyzed for ER-α and ER-ß proteins using 12% and 15% polyacrylamide gels, respectively. T47D breast cancer cell extract (100 μg) was electrophoresed on the gels as a positive control for ER-α, while human umbilical vein endothelial cell (HUVEC) extract (100 μg) was used as a positive control for ER-ß[10,18]. Prestained molecular size markers (BioRad, Hercules, CA, USA) were electrophoresed on the gels to determine the molecular mass of reactive proteins. Fractionated proteins were transferred to nitrocellulose membranes using standard methods[29]. The membranes were reacted with ER-α (ER-α Ab-15, NeoMarkers, Freemont, CA, USA) and ER-ß (ER-ß 51-7700, Zymed, San Francisco, CA, USA) antibodies (1:500 dilution) at 22°C for 60 min. The membranes were washed and incubated for 60 min with species-specific peroxidase-conjugated secondary antibodies (1:25,000, Pierce Biotechnology, Rockford, IL, USA). The blots were incubated with a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) for 5 min. The blots were exposed to x-ray film for 1 to 5 min to visualize chemiluminescent proteins. The membranes were stripped and reacted with a ß-actin antibody (1:50,000, ß-actin AC-15, Sigma). The membranes were washed and incubated for 60 min with anti-mouse-specific peroxidase-conjugated secondary antibody (1:50,000, Pierce). Scanning densitometry was used to determine the relative amount of ER-α and ER-ß. The quantity of ER-α and ER-ß in each sample was normalized to ß-actin on the same blot by dividing the optical density of the receptor by the optical density of ß-actin in the same sample, and expressing the value as a ratio.

Statistical analyses. Power analysis indicated that a sample size of n = 10 would achieve a power of 0.95, p = 0.05, for each test. T cell samples from a minimum of 10 patients or controls were measured to quantitate each of the receptor proteins. Because the entire content of a blood draw was required for an individual assay, samples from each subject enrolled in this study were not tested in all of the assays. However, 7 controls and 7 SLE patients were assessed for both ER-α and ER-ß. The amount of receptor expression was compared by nonparametric Kruskal-Wallis test with $p < 0.05$ considered statistically significant.

Cell cultures and ER selective ligand activation. SLE T cell enriched mononuclear cells were cultured in serum-free medium (QBSF 56 Serum-Free Medium, Sigma), and serum-free medium containing 2,3-bis (hydroxyphenyl)-proprionitrile (DPN, 10^{-7} M, Tocris, Ellisville, MO, USA) or 1,3,5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, 10^{-7} M, Tocris) for 18 h at 37°C. DPN is an ER-ß-specific agonist, while PPT is an ER-α-specific agonist[26,27]. Some T cells cultured with PPT were cultured with 10-fold molar excess of 1,3-bis (4-hydroxyphenyl)-4methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride [MPP, Tocris]. MPP is an ER-α-specific antagonist. Some T cells were cultured with DPN and 10-fold molar excess of ICI 182,780 (Tocris). ICI 182,780 is a pure anti-estrogen.

Measurement of calcineurin and CD154 mRNA. Semiquantitative polymerase

chain reaction (PCR) using a Peltier Thermocycler (MJ Research, San Francisco, CA, USA) was used to measure calcineurin and CD154 mRNA. We showed previously that there is correspondence between increased calcineurin mRNA and phosphatase activity in SLE T cells[15]. There is also correspondence between increased CD154 mRNA and cell-surface expression of the protein on SLE T cells[16]. Total RNA was isolated from cultured SLE T cells after appropriate treatments. To ensure removal of genomic DNA, samples were treated with DNase I according to the manufacturer's protocol (DNA-free, Ambion, Austin, TX, USA). cDNA were synthesized in 20 μl from 4 μg of RNA using an oligo (dT) primer (0.5 μg/μl) and MMLV reverse transcriptase (RT) as described[16,22]. The primers for glyceraldehyde phosphate dehydrogenase (G3PDH, sense, 5'-GAG TCA ACG GAT TTG GTC GT-3'), calcineurin (sense, 5'-TTG ATT GCC ACT GTA GTT TGG T-3'), and CD154 (sense, 5'-ACA TAC AAC CAA ACT TCT CC-3') have been verified in our laboratory by sequence analysis of the resulting PCR products[16,22]. PCR reactions for CD154 were amplified at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 24 cycles. PCR reactions for calcineurin were carried out at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 26 cycles. PCR amplification was within the linear range under the conditions used. Control reactions were amplified without template. Amplified products were separated on 2% agarose gels containing ethidium bromide. A 100 base pair DNA size standard (Promega, Madison, WI, USA) was electrophoresed on the same gel to determine product size. The gel was photographed and the amount of calcineurin and CD154 from each sample was analyzed by scanning densitometry. To normalize the results, the amount of calcineurin and CD154 was divided by the amount of G3PDH amplified from the same template.

RESULTS

Expression of ER-α and ER-β proteins in human T cells. Purified human T cell extracts contained a protein consistent with the size (65 kDa) of ER-α (Figure 1A, upper panel). This

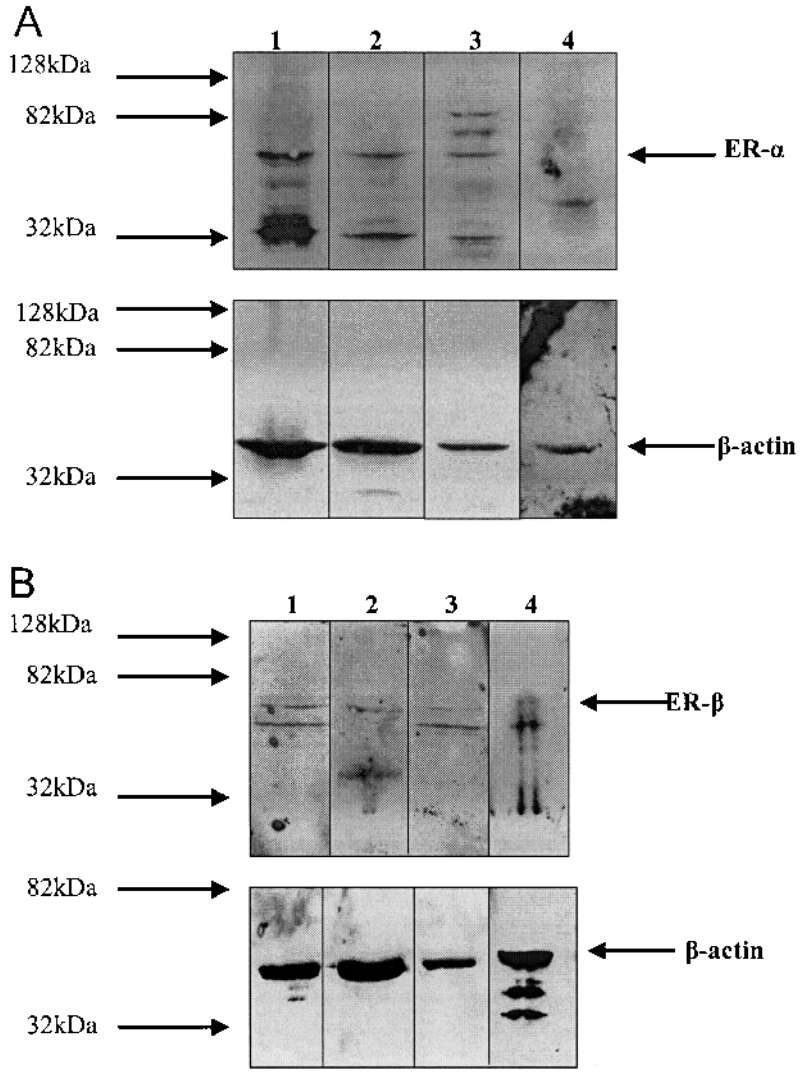

Figure 1. Identification of estrogen receptor subtypes in T cells. T cells were purified as detailed in the text and extracts were prepared and size fractionated by SDS-PAGE. The proteins were electrophoretically transferred to a nitrocellulose membrane and reacted with receptor-specific antibodies. The blots were stripped and reacted with a ß-actin antibody to normalize the receptor data. A. Estrogen receptor-α (ER-α) migrated with a molecular mass of about 65 kDa (upper panel). Lane 1, extract from female control T cells; lane 2, extract from female SLE T cells; lane 3, extract from T47D breast cancer cells (positive control); lane 4, extract from female SLE T cells without primary antibody (negative control). The lower panel shows ß-actin in these same samples. B. Estrogen receptor-ß (ER-ß) migrated with an apparent molecule mass of 56 kDa (upper panel). Lane 1, extract from female control T cells; lane 2, extract from female SLE T cells; lane 3, extract from HUVEC cells (positive control); lane 4, extract from female SLE T cells without primary antibody (negative control). The lower panel shows ß-actin in these same samples.

protein was present in T cell extracts (lanes 1 and 2) and in T47D breast cancer cell extract (lane 3, positive control). Incubation of a Western blot without primary antibody eliminated the 65 kDa band in T cell extract, verifying this protein as ER-α (lane 4). The Western blot was stripped and reacted with a ß-actin antibody to normalize the ER-α data (Figure 1A, lower panel).

Purified human T cell extracts expressed a protein consistent with the molecular mass (56 kDa) of ER-ß (Figure 1B, upper panel). This protein was present in T cell extracts (lanes 1 and 2) and in HUVEC cell extracts (lane 3, positive control). Incubation of a Western blot without primary antibody eliminated this band in T cell extracts, indicating it was ER-ß protein (lane 4). A cross-reacting protein with a molecular mass of about 50 kDa was observed in some protein extracts. This protein did not appear to be an ER-ß variant, since it was detected on Western blots in the absence of primary antibody (lane 4). This same Western blot was stripped and reacted with a ß-actin antibody to normalize the ER-ß values (Figure 1B, lower panel).

Expression of ER-α in SLE and normal T cells. The amount of ER-α was measured in normal and SLE T cell extracts. Although the amount of ER-α appeared to be less in SLE T cells than in control T cells (Figure 2), a statistically significant difference was not indicated ($p = 0.081$). This was despite the fact that ER-α levels in 7 of the 13 SLE T cell samples were below the lowest amount of receptor measured in the normal T cells. The median amount (0.66) of ER-α in T cells from controls was numerically greater than the median value (0.50) for SLE T cells. There also appeared to be greater variation in the amount of ER-α among SLE T cells than in normal T cells (Figure 2).

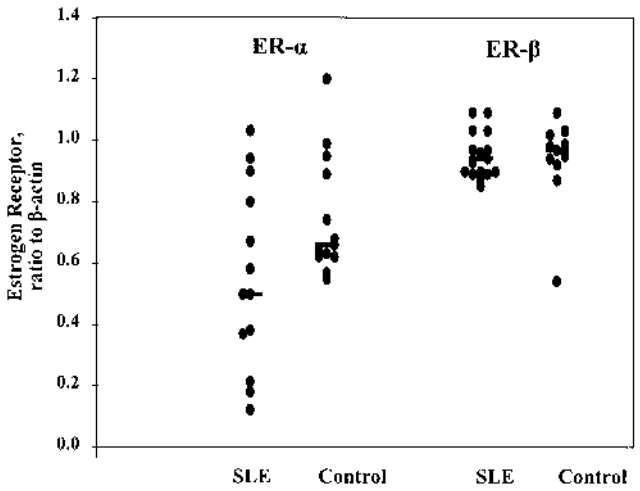

Figure 2. Measurement of estrogen receptors in human T cells shows greater variation in the amount of ER-α in SLE compared with normal T cells. Data shown are the amount of ER-α and ER-ß in T cell extracts, expressed as a ratio to the amount of ß-actin in the same samples. Horizontal lines are the median values from 13 and 16 SLE patients and 13 and 11 normal controls for ER-α and ER-ß, respectively.

Expression of ER-ß in SLE and normal T cells. The amount of ER-ß was measured in normal and SLE T cell extracts (Figure 2). There were no differences in the quantity of ER-ß in SLE (median = 0.94) compared with normal T cells (median = 0.97). It is also of interest that the amount of ER-ß in SLE T cells was less variable as compared with ER-α (compare ER-α and ER-ß values in Figure 2).

Receptor levels are not menstrual cycle-dependent. It was important to determine if the levels of ER varied with the stage of the menstrual cycle. We compared the amount of ER-α and ER-ß in SLE T cells purified from patient's blood drawn during the follicular or luteal phases of the menstrual cycle (Table 1). The amount of both receptor subtypes was not significantly different between the follicular and luteal phases of the menstrual cycle. A similar result was obtained from T cell extracts prepared from blood drawn from the control females at the follicular or luteal phases of the menstrual cycle.

ER-α and ER-ß expression does not decline with age in cycling females. In order to rule out that differences in ER-α were due to declining expression levels in older females, we compared the amount of both ER-α and ER-ß versus ages for the patients enrolled in this study (Figure 3). There was no apparent decline in ER-α (Figure 3A) or ER-ß (Figure 3B) as the age of the patients increased. Similarly, no decline in the expression levels of either receptor subtype in T cells was apparent with increasing age in control females with regular menstrual cycles (Figure 3).

Disease activity does not correlate with ER-α or ER-ß expression. Most of the patients enrolled in this study were classified with moderate disease activity (SLEDAI > 4 and ≤ 12). To determine if the decrease in ER-α correlated with disease activity, the amount of ER-α in SLE T cells was compared from patients with mild (SLEDAI ≤ 4), moderate (SLEDAI > 4 and ≤ 12), or active (SLEDAI > 12) disease (Table 2). Although the number of patients is small, there was no apparent association between decreased ER-α expression and disease activity. The amount of ER-ß was also not affected by disease activity (Table 2).

Ligand-selective receptor agonists suggest both ER-α and ER-ß can stimulate expression of SLE T cell activation markers. In order to test if one receptor subtype was responsible for increased expression of the T cell activation markers calcineurin and CD154, SLE and normal T cells were cultured in medium containing ligand-selective receptor agonists (Figures 4, 5). The response of SLE T cells to the ER-α agonist PPT varied from a 15% to 56% increase in T cell activation marker expression compared with T cells from the same patient cultured without PPT (Figure 4A). T cells from healthy controls showed no more than a 10% increase in calcineurin and CD154 expression (Figure 4B). Activation of SLE T cell marker gene expression increased from 9% to 62% after culture in the ER-ß agonist DPN (Figure 5A). T cell marker gene

Table 1. Effect of menstrual cycle on estrogen receptor (ER) values. The amount of ER-α and ER-ß is not dependent on menstrual cycle phase. T cells were purified from blood drawn from SLE patients and normal controls at the follicular or luteal phases of the menstrual cycle. Data shown are the median and range of ER-α and ER-ß expressed as a ratio to ß-actin in the same samples.

Cohort	Menstrual Cycle Phase	ER-α Ratio n	ER-α Ratio median (range)	ER-ß Ratio n	ER-ß Ratio median (range)
SLE	Follicular	6	0.44 (0.12–0.94)	9	0.97 (0.87–1.09)
	Luteal	6	0.74 (0.21–1.03)	5	0.90 (0.85–1.09)
Controls	Follicular	7	0.63 (0.55–0.99)	3	0.99 (0.98–1.03)
	Luteal	6	0.70 (0.62–1.20)	8	0.95 (0.54–1.09)

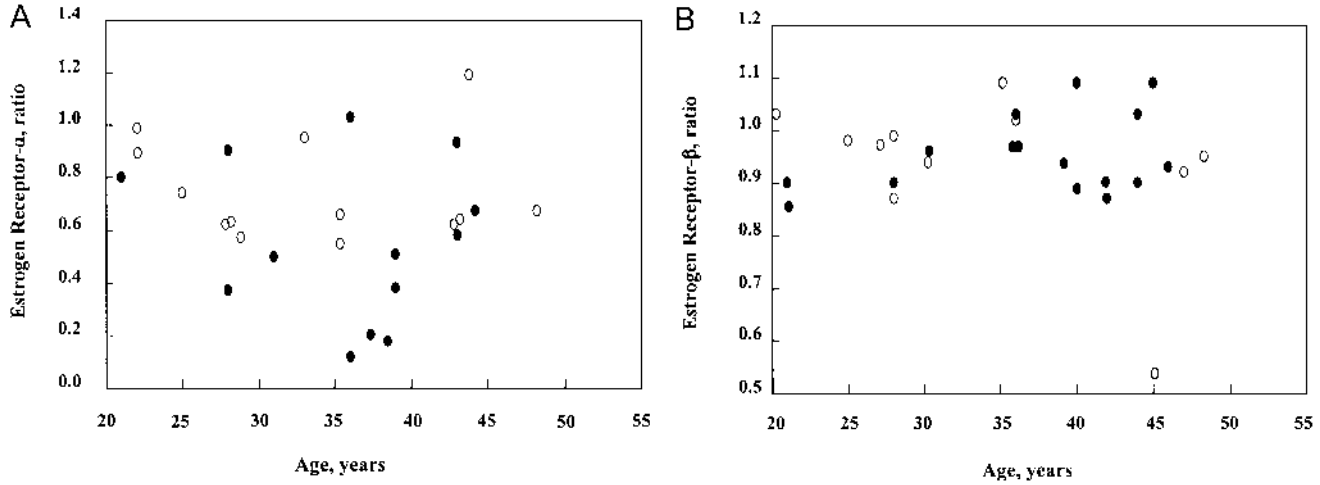

Figure 3. Variation in the amount of estrogen receptors does not correlate with increasing age in SLE patients (●) and control females (○). Data shown are the relative amounts of estrogen receptor to ß-actin measured in T cell extracts plotted against age. A. The amount of ER-α varied among the SLE patients, but a lower level of ER-α did not correlate with increasing age. B. The amount of ER-ß was relatively consistent in the T cell extracts from patients and control females across all ages.

Table 2. Effect of SLEDAI on estrogen receptor (ER) values for SLE participants. Levels of ER do not correlate with disease activity. T cell extracts were prepared from patients with SLE with different disease activity as assessed by SLEDAI at the time blood was drawn. Data shown are the median values and range of ER-α and ER-ß expressed as a ratio to ß-actin in the same samples.

SLEDAI Category	ER-α Ratio n	ER-α Ratio median (range)	ER-ß Ratio n	ER-ß Ratio median (range)
≤ 4	3	0.58 (0.21–0.80)	5	1.03 (0.90–1.09)
> 4 and ≤ 12	6	0.44 (0.12–1.03)	8	0.95 (0.85–1.03)
> 12	4	0.70 (0.38–0.94)	3	0.90 (0.89–0.90)

expression did not change in response to the ER-ß agonist in the control T cells, except for one sample (Con 2) in which there was about a 20% increase in CD154 compared with the same T cells cultured without agonist (Figure 5B).

Ligand-specific activation of ER-α and ER-ß is inhibited by receptor antagonists. Receptor antagonists were utilized to determine if the increased expression of T cell activation marker genes could be suppressed by addition of antagonists (Figure 6). T cells were cultured with the ER-α agonist PPT, and some cultures contained 10-fold molar excess of the ER-α antagonist MPP (Figure 6A). MPP is an antagonist primarily for ER-α because it exhibits greater than 200-fold selectivity for ER-α over ER-ß. Addition of MPP to SLE T cells cultured with the ER-α agonist reduced target gene expression (Figure 6A). While selective ER-ß antagonists have been reported[26], many of these have agonist activity on ER-α. To test the specificity of the ER-ß agonist DPN, we utilized ICI 182,780 that is a pure anti-estrogen (Figure 6B). Addition of ICI to SLE T cell cultures reduced target gene expression in response to ER-ß activation.

DISCUSSION

Emerging evidence suggests that steroid receptor subtypes impart distinct transcriptional activity when the receptors are coexpressed in the same cells[30-32]. The glucocorticoid[30], progesterone[31], and estrogen receptor[32] subtypes have been shown to possess distinct functional activities consistent with the postulate that the quantity and distribution of receptor subtypes could be major determinants of the biological effects. Our study was undertaken to measure the amount of ER-α and ER-ß proteins in SLE T cells in order to explore the possibility

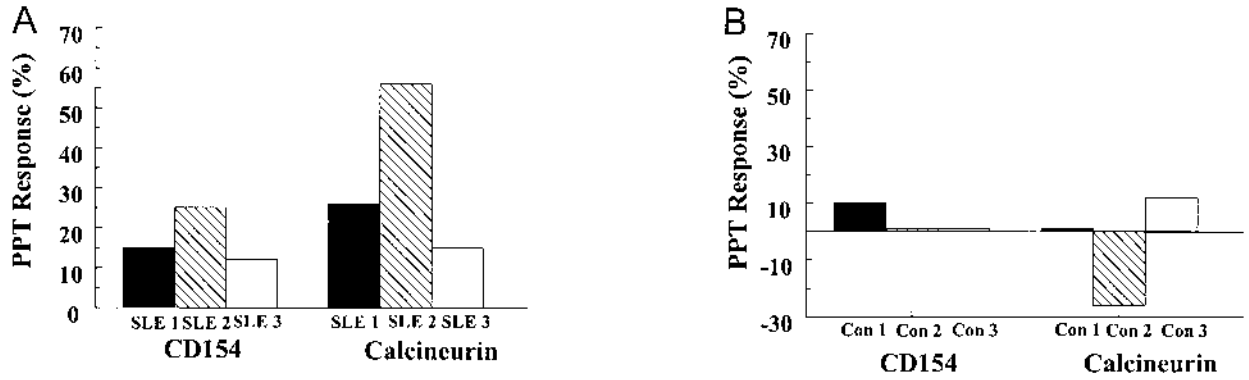

Figure 4. Ligand-selective estrogen receptor agonist suggests ER-α can be active in SLE T cells. T cells were cultured in medium containing the ER-α selective agonist PPT (10^{-7} M) or in QBSF medium without agonist. At the end of culture total RNA was isolated, converted into cDNA, and amplified by PCR using gene-specific primers for CD154 and calcineurin. The PPT response (%) was calculated by comparing the normalized amount of CD154 and calcineurin in T cells from cultures without and with agonist from the same person. A. Data shown are the response to PPT from 3 SLE patients. B. The response to PPT from 3 control females.

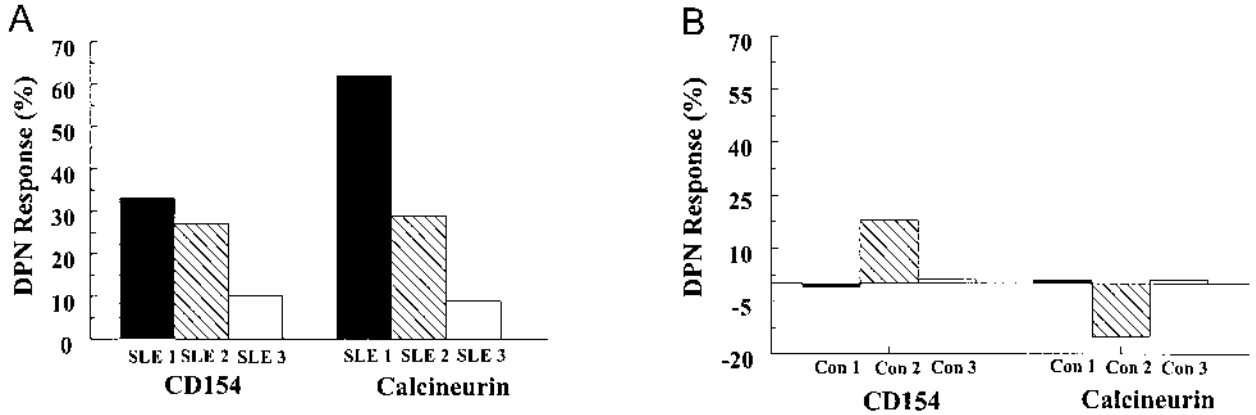

Figure 5. Ligand-selective estrogen receptor agonist suggests ER-ß can be active in SLE T cells. T cells were cultured in medium containing the ER-ß selective agonist DPN (10^{-7} M) or in QBSF medium without agonist. At the end of culture total RNA was isolated, converted into cDNA, and amplified by PCR using gene-specific primers for CD154 and calcineurin. The DPN response (%) was calculated by comparing the normalized amount of CD154 and calcineurin in T cells from cultures without and with DPN from the same person. A. Data shown are the response to DPN from 3 SLE patients. B. The response to DPN from 3 control females.

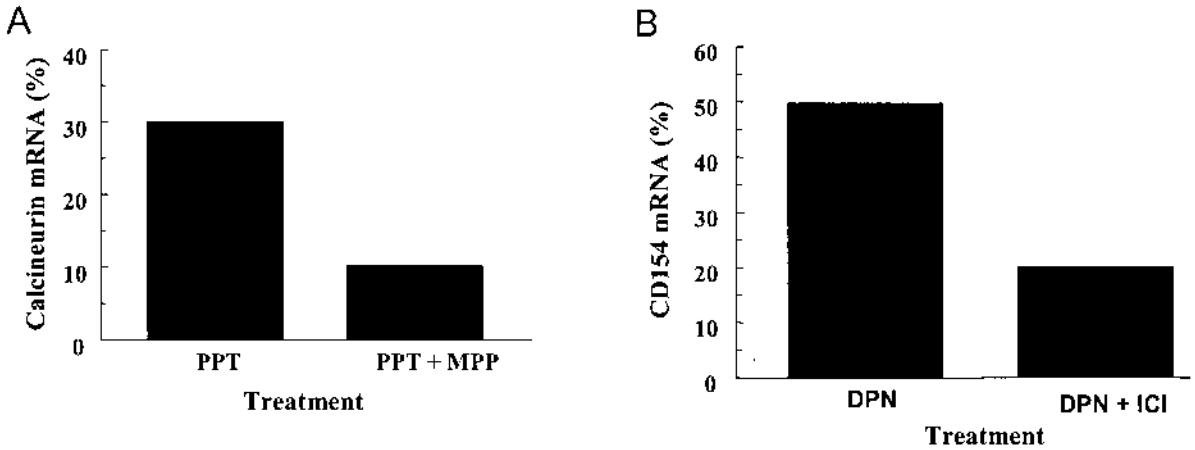

Figure 6. Receptor antagonists antagonize ligand-specific activation of estrogen receptors. SLE T cells were cultured in medium containing selective agonists and 10-fold molar excess of antagonists. At the end of culture total RNA was isolated, converted into cDNA, and amplified by PCR using gene-specific primers for CD154 and calcineurin. The response (%) was calculated by comparing the normalized amount of CD154 and calcineurin in T cells from cultures in medium without any additions. Data are representative of 2 independent experiments. A. Activation of ER-α using PPT was antagonized by the addition of 10-fold molar excess of the ER-α antagonist MPP. B. Activation of ER-ß with DPN was antagonized by addition of 10-fold molar excess of the pure estrogen antagonist ICI 182,780.

that differences in the amount of receptor subtype expression could account for the increased sensitivity of SLE T cells to estradiol. While the results suggest that there is less ER-α in SLE compared with normal T cells, the differences were not statistically significant. Moreover, there was considerable variation in the amount of ER-α in SLE compared with normal T cells. The 7 patients with the lowest levels of ER-α shared in common moderate disease activity (SLEDAI) and moderate elevation of anti-double-stranded DNA antibodies. There was no evidence among these patients that specific organ involvement was associated with low ER-α. We anticipated that low levels of ER-α might allow for unusual ER-ß activity, resulting in increased SLE T cell activation of marker gene expression. To our surprise, both ER-α and ER-ß-selective ligands increased expression of CD154 and calcineurin, which we showed previously are estrogen-responsive genes in SLE T cells[15,16,18].

The biological effects of estradiol are mediated through several different pathways[33]. The classical ligand-dependent pathway involves receptor-binding and dimerization to target gene promoters, resulting in altered rates of transcription[34]. Cells that express both ER subtypes have been shown to utilize heterodimers of ER-α and ER-ß to regulate target gene expression[32,35]. Moreover, the identification of additional ER-ß isoforms that preferentially heterodimerize and inhibit ER-α activity suggests ER-ß may modulate ER-α-dependent transcriptional activity[36]. Our data suggest that ER-ß synthesis and/or turnover is unlikely to account for the estradiol sensitivity in SLE T cells, since the quantity of ER-ß is similar in both patient and normal T cells. Nevertheless, the results show that the ER-ß-specific agonist DPN increases T cell activation gene expression in SLE T cells. This indicates that ER-ß can be functional. Our results also indicate that the level of ER-α is variable in SLE T cells. Our initial interpretation of these data was that ER-ß activity and not ER-α activation would stimulate SLE target gene expression. To our surprise, however, an ER-α selective agonist increased calcineurin and CD154 expression, clearly showing that ER-α can be functionally active in SLE T cells. Recent evidence[37] suggests that low levels of ER-α are not necessarily indicative of receptor inactivity, but rather are consistent with increased ER-α action in target cells. Continuous turnover of ER-α through the proteasome-mediated degradation pathway is linked to transcriptional activity. The cyclic turnover of ER-α may permit cells to respond continuously to changes in estradiol[37]. Thus, the low level of ER-α in SLE T cells is consistent with increased turnover and transcriptional activity of ER-α dependent gene regulation. If this interpretation is correct, then increased ER-α activation may underlie the sensitivity of SLE T cells to estradiol. Alternatively, SLE is a disease characterized by the overproduction of autoantibodies[38-40]. The low level of ER-α may arise from the production of ER-α antibodies that destroy the receptor and contribute to the lower level of this receptor subtype in SLE T cells. Inhibition of proteasome activity in SLE T cells is necessary to clarify this point, and those experiments are currently in progress.

Although the number of patients in whom both receptor subtypes were analyzed was small, there was no suggestion of a correlation between ER-α and ER-ß. This is not unexpected, given the tight clustering of values for ER-ß (Figure 2). Interestingly, there was a significant ($p = 0.005$) inverse correlation between ER-α and ER-ß in the 7 normal controls for which both assays were performed. The observation should be viewed with caution since this result was dependent upon a single subject that displayed extreme values for both ER-α (1.2) and ER- ß (0.54; see Figure 2). Exclusion of this subject resulted in no correlation between the 2 receptor subtypes.

Using a transient transfection system, Hall and McDonnell[32] showed that ER-α-dependent reporter gene expression is unaffected by increasing levels of ER-ß when HepG2 cells are cultured in medium containing 100 nM estradiol. However, at low levels of estradiol (100 pM), increasing amounts of ER-ß inhibited ER-α-dependent reporter gene expression. Their research[32] suggests that ligand concentration affects receptor subtype activity and, therefore, the concentration of estradiol in circulation may be a major mechanism for controlling cellular sensitivity to estradiol. Results from our study support the postulate that circulating levels of estradiol, rather than changes in receptor levels, may determine the cellular sensitivity to estradiol. This follows from the present data showing that the amount of both receptor subtypes in SLE and normal T cells does not vary significantly between the follicular and luteal phases of the menstrual cycle. In addition, our earlier studies indicate that SLE T cells isolated from patient's blood drawn during either the follicular or luteal phases of the menstrual cycle respond with similar magnitude to physiological amounts of estradiol[15,16,21]. Together, these results support the concept that sensitivity of SLE T cells may occur by changes in the bioavailability of estradiol.

Ligand-binding studies show that human ER-α and ER-ß bind equally well to estradiol[41,42]. If the pool of ER-α is reduced in SLE T cells, then it follows that at low concentrations of estradiol, ER-ß may be preferentially activated. Additional studies with different concentrations of receptor-selective agonists are required to test if the ER-ß agonist increases T cell activation gene expression at a lower concentration than the ER-α agonist. Another potential complication regarding the estrogen sensitivity of SLE T cells relates to reports of abnormal estradiol metabolism[25,43]. Little is known about the interaction of estradiol metabolites with ER. It is possible that abnormal metabolites preferentially activate or stimulate turnover of one ER subtype over the other. It is now important to explore the possibility that preferential activation or turnover of one receptor subtype in SLE T cells stimulates abnormal gene transcription.

The molecular mechanism by which estradiol increases CD154 and calcineurin expression is not known[18,22]. The acti-

vation of T cell marker gene expression by both receptor agonists is consistent with a transcriptional regulatory mechanism, since PPT and DPN function as full agonists on their preferred receptor subtype[26]. However, a search for estrogen response elements (ERE) in the promoter regions of CD154[16] and calcineurin (unpublished data) did not reveal any consensus ERE. However, these ER-selective agonists have been shown to regulate genes that are stimulated by direct binding of ER to DNA and genes for which the ER is tethered to the DNA by binding to other proteins[26]. Therefore our results are consistent with a transcriptional regulatory mechanism, since ligand preferences appear to be independent of the presence of ERE. The possibility that other, nonclassical signal transduction pathways are involved must still be considered.

Gene expression profiling[44] and genetic analyses[45] reveal there are numerous candidate genes whose altered expression may contribute to SLE pathogenesis. Not surprisingly, many of the identified genes are involved in regulating inflammation, apoptosis, signal transduction, and the cell cycle. Estrogen has been shown to regulate many of these processes in other target cells such as the uterus and breast[46], but similar functions in cells that make up the immune system are still under investigation[47,48]. Recently, data have emerged that indicate regulation of signal transduction by estrogen is more complicated than originally thought[33,34,49]. The biological effects of estradiol and its receptors are exerted through at least 4 rather than one defined ER pathway. The identity of more than one receptor for estradiol has complicated our understanding of estrogen action in target cells in which both receptor subtypes are expressed. The results from our study suggest that ER-α and ER-ß can increase expression of activation genes in SLE T cells. Further understanding of the molecular basis of estradiol action and ER subtype activation in SLE T cells is expected to contribute new information about endocrine-immune interactions. This knowledge should provide new approaches to treat gender-biased autoimmune diseases such as SLE.

ACKNOWLEDGMENT

We are grateful to the volunteers for donating blood and for the excellent skills of Cindy Greenwell, RN, for recruiting, organizing, and monitoring the blood donors. We thank Jim Swafford for help with the illustrations.

REFERENCES

1. Kyttaris VC, Tsokos GC. T lymphocytes in systemic lupus erythematosus: an update. Curr Opin Rheumatol 2004;16:548-52.
2. Qing X, Putterman C. Gene expression profiling in the study of the pathogenesis of systemic lupus erythematosus. Autoimmun Rev 2004;3:505-9.
3. Lang TJ. Estrogen as an immunomodulator. Clin Immunol 2004;113:224-30.
4. Nath SK, Kilpatrick J, Harley JB. Genetics of human systemic lupus erythematosus: the emerging picture. Curr Opin Immunol 2004;16:794-800.
5. Mongey AB, Hess EV. The role of environment in systemic lupus erythematosus and associated disorder. In: Wallace DJ, Hahn BH, editors. Dubois' lupus erythematosus. Baltimore: Williams and Wilkins; 1997:31-7.
6. Garovich M, Agudelo C, Pisko E. Oral contraceptives and systemic lupus erythematosus. Arthritis Rheum 1980;23:1396-8.
7. Cooper GS, Dooley MA, Treadwell EL, St. Clair EW, Parks CG, Gilkeson GS. Hormonal, environmental, and infectious risk factors for developing systemic lupus erythematosus. Arthritis Rheum 1998;41:1714-24.
8. Casoli P, Tumiati B, LaSala G. Fatal exacerbation of systemic lupus erythematosus after induction of ovulation. J Rheumatol 1997;24:1639-40.
9. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. J Exp Med 1978;147:1568-83.
10. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Nat Acad Sci USA 1996;93:5925-30.
11. Suenaga R, Rider V, Evans M, Mitamura K, Abdou NI. Peripheral blood T cells and monocytes and B cell lines derived from patients with lupus express estrogen receptor transcripts similar to those of normal cells. J Rheumatol 1998;25:1305-12.
12. Kassi EN, Vlachoyiannopoulos PG, Moutsopoulos HM, Sekeris CE, Moutsatsou P. Molecular analysis of estrogen receptor α and ß in lupus patients. Eur J Clin Invest 2001;31:86-93.
13. Suenaga R, Evans MJ, Rider V, Abdou NI. In vitro-activated human lupus T cells express normal estrogen receptor proteins, which bind to the estrogen response element. Lupus 2001;10:116-22.
14. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis. J Clin Invest 2002;109:1625-33.
15. Rider V, Foster RT, Evans M, Abdou NI. Gender differences in autoimmune diseases: estrogen increases calcineurin expression in systemic lupus erythematosus. Clin Immunol Immunopathol 1998;89:171-80.
16. Rider V, Jones S, Evans M, Bassiri H, Afsar Z, Abdou NI. Estrogen increases CD40 ligand expression in T cells from women with systemic lupus erythematosus. J Rheumatol 2001;28:2644-9.
17. Kanda N, Tsuchida T, Tamaki K. Estrogen enhancement of anti-double stranded DNA antibody and immunoglobulin production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Arthritis Rheum 1999;42:328-37.
18. Rider V, Jones SR, Evans M, Abdou NI. Molecular mechanisms involved in the estrogen-dependent regulation of calcineurin in systemic lupus erythematosus. Clin Immunol 2000;95:124-34.
19. Apelgren LD, Bailey DL, Fouts RL, et al. The effect of a selective estrogen receptor modulator on the progression of spontaneous autoimmune disease in MRL lpr/lpr mice. Cell Immunol 1996; 173:55-63.
20. Wu WM, Lin BF, Su YC, Suen JL, Chiang BL. Tamoxifen decreases renal inflammation and alleviates disease severity in autoimmune NZB/W F1 mice. Scand J Immunol 2000;52:393-400.
21. Rider V, Abdou NI. Gender difference in autoimmunity: Molecular basis for estrogen effects in systemic lupus erythematosus. Int Immunopharmacol 2001;1:1009-24.
22. Rider V, Keltner S, Abdou NI. Increased estrogen-dependent expression of calcineurin in female SLE T cells is regulated by multiple mechanisms. J Gend Specif Med 2003;6:14-21.
23. Suenaga R, Mitamura K, Evans MJ, Abdou NI. Binding affinity and quantity of estrogen receptor in peripheral blood monocytes of patients with systemic lupus erythematosus. Lupus 1996;5:227-31.
24. Verthelyi D, Petri M, Ylamus M, Klinman DM. Dissociation of sex hormone levels and cytokine production in SLE patients. Lupus 2001;10:352-8.
25. Lahita RG, Bradlow HL, Kunkel HG, Fishman J. Increased 16 alpha hydroxylation of estradiol in systemic lupus erythematosus. J Clin Endocrinol Metab 1981;53:174-8.

26. Harrington WR, Shubin S, Barnett DH, Petz LN, Katzenellenbogen JA, Katzenellenbogen BS. Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol Cell Endocrinol 2003;206:13-22.
27. Manas ES, Unwalla RJ, Xu ZB, et al. Structure-based design of estrogen receptor-ß selective ligands. J Am Chem Soc 2004;126:15109-19.
28. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
29. Rider V, Thomson E, Seifert C. Transit of rat uterine stromal cells through G1 phase of the cell cycle requires temporal and cell-specific hormone-dependent changes on cell cycle regulators. Endocrinology 2003;144:5450-8.
30. Oakley RH, Sar M, Cidlowski JA. The human glucocorticoid receptor ß isoform. Expression, biochemical properties, and putative function. J Biol Chem 1996;271:9550-9.
31. Giangrande PH, Pollio G, McDonnell DP. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. J Biol Chem 1997;52:32889-900.
32. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ER beta) of the human estrogen receptor modulates ER alpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999;140:5566-78.
33. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 2001;276:36869-72.
34. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 1999;20:321-44.
35. Cowley SM, Hoarse S, Mosselman S, Parker MG. Estrogen receptors alpha and beta form heterodimers on DNA. J Biol Chem 1997;272:19858-62.
36. Hanstein B, Liu H, Yancisin MC, Brown M. Functional analysis of a novel estrogen receptor-beta isoform. Mol Endocrinol 1999;13:129-37.
37. Reid G, Hubner MR, Metivier R, et al. Cyclic proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. Mol Cell 2003;11:695-707.
38. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. Semin Arthritis Rheum 2004;34:501-37.
39. Dorner T, Lipsky PE. Molecular basis of immunoglobulin variable region gene usage in systemic autoimmunity. Clin Exp Med 2005;4:159-69.
40. Waldman M, Madaio MP. Pathogenic autoantibodies in lupus nephritis. Lupus 2005;14:19-24.
41. Kuiper GG, Carlsson B, Grandian K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor alpha and beta. Endocrinology 1998;138:863-70.
42. Harris HA, Bapat AR, Gonder DS, Frail DE. The ligand binding profiles of estrogen receptors α and ß are species dependent. Steroids 2002;67:379-84.
43. Lahita RG. Sex steroids and SLE: metabolism of androgens to estrogens. Lupus 1992;1:125-7.
44. Rus V, Chen H, Zernetkina V, et al. Gene expression profiling in peripheral blood mononuclear cells from lupus patients with active and inactive disease. Clin Immunol 2004;112:231-4.
45. Tsao BP. Update on human systemic lupus erythematosus genetics. Curr Opin Rheumatol 2004;16:513-21.
46. Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic mice. Annu Rev Physiol 2005;67:285-308.
47. Thongngarm T, Jenkins JK, Ndebele K, McMurray RW. Estrogen and progesterone modulate monocyte cell cycle progression and apoptosis. Am J Reprod Immunol 2003;49:129-38.
48. McMurray RW, May W. Sex hormones and systemic lupus erythematosus: review and meta-analysis. Arthritis Rheum 2003;48:2100-10.
49. Nilsson S, Gustafsson J-A. Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. Breast Cancer Res 2000;2:1-9.