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Estradiol Differentially Regulates Calreticulin: A Potential Link with Abnormal T Cell Function in Systemic Lupus Erythematosus?

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Abstract

Objective—Systemic lupus erythematosus (SLE) is an autoimmune disease that affects women nine times more often than men. The present study investigates estradiol-dependent control of the calcium buffering protein, calreticulin, to gain further insight into the molecular basis of abnormal T cell signaling in SLE T cells.

Methods—T cells were purified from blood samples obtained from healthy females and SLE patients. Calreticulin expression was quantified by real time polymerase chain amplification. Calreticulin and estrogen receptor- α were co-precipitated and analyzed by Western blotting to determine if the proteins associate in T cells.

Results—Calreticulin expression increased ($p = 0.034$) in activated control T cells, while estradiol decreased ($p = 0.044$) calreticulin in resting T cells. Calreticulin expression decreased in activated SLE T cell samples and increased in approximately 50% of resting T cell samples. Plasma estradiol was similar ($p > 0.05$) among SLE patients and control volunteers. Estrogen receptor- α and calreticulin co-precipitated from nuclear and cytoplasmic T cell compartments.

Conclusions—The results indicate that estradiol tightly regulates calreticulin expression in normal human T cells and the dynamics are different between activated and resting T cells. The absence of this tight regulation in SLE T cells could contribute to abnormal T cell function.

Keywords

SLE; human T cells; estradiol; calreticulin; estrogen receptor- α

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Conflict of interest statement.

The authors declare that there is no conflict of interest.

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that affects women nine times more often than men.¹ A fundamental problem in SLE appears to be peripheral T cell signaling defects leading to a reduction in activation-induced cell death, the persistence of autoreactive T cells and abnormal cytokine production.^{2, 3} The female sex hormone, estradiol, alters signal transduction pathways in SLE T cells. Some of these pathways are considered signature pathways for abnormal SLE T cell signaling, yet the gene targets involved remain largely unknown.⁴

Activation of the T cell receptor (TCR) results in a rapid and sustained increase of intracellular calcium in normal and SLE T cells.⁵⁻⁷ However, TCR engagement in SLE T cells stimulates an initial calcium response of greater magnitude and longer duration than the initial response in normal T cells. By contrast, the sustained calcium response is shorter in SLE T cells compared with normal T cells. The concentration of cytoplasmic calcium regulates many signaling pathways that control the activity of transcription factors, enzymes, phosphatases, and ion channels⁸⁻¹⁰ suggesting that this difference in calcium signaling in SLE T cells may have broad reaching effects and contribute to disease pathogenesis. Rapamycin, a drug that controls calcium fluxing, reduces active disease symptoms in human SLE T cells¹¹ while dipyrindamole, an inhibitor of calcineurin-nuclear factor of activated T cells (NFAT) interaction, reduces calcium fluxing in activated T cells in a MRL/lpr lupus mouse model.¹²

In the classical mechanism of steroid hormone action, estradiol diffuses into target cells and binds to estrogen receptors located in the nucleus. The ligand activated receptors interact at specific DNA sites, termed estrogen response elements, along target genes and alter the rate of transcription.¹³ Estradiol can both activate and repress genes within a given signal transduction pathway contributing to abnormal signal transduction in SLE T cells.⁴ Previous experiments in our laboratory indicated increased expression of calcineurin and CD154 in human SLE T cells cultured with estradiol, while these genes in normal T cells were unaffected by estradiol.^{14, 15} We hypothesized that upregulation of these genes in SLE T cells could enhance calcium-calcineurin-NFAT signaling leading to exaggerated help to B cells and hypersecretion of autoantibodies. Consistent with this postulate was an improvement of disease activity and, a reduction in the expression of these T cell activation markers (calcineurin and CD154) in female SLE patients treated with the estrogen receptor antagonist, Faslodex.¹⁶ More recently, direct evidence for the importance of early calcium signaling via this pathway was reported in a lupus mouse model, in which T cells from MRL/lpr lupus prone mice responded robustly to activation and provided help to B cells in a calcineurin-dependent fashion.¹² Further, T cells treated with dipyrindamole, a drug that blocks early calcium dependent signaling in SLE T cells by inhibiting calcineurin-NFAT interaction suppressed T cell function and improves disease activity in lupus prone mice.¹²

Calreticulin is a major calcium buffering protein in the endoplasmic reticulum.¹⁷ Mice that are deficient in calreticulin do not survive because heart formation is disrupted owing to a reduced amount of calcium released from the endoplasmic reticulum.¹⁸ In calreticulin-deficient mice, the nuclear translocation of a number of transcription factors, including

NFAT, is abnormal. Overexpression of activated calcineurin can rescue calreticulin deficient mouse embryos and re-establish the nuclear localization of NFAT.¹⁹ Overexpression of calreticulin in transformed human embryonic kidney cells increases calcium flux across the endoplasmic reticulum but decreases the amount of mitochondrial calcium and the resting membrane potential of the mitochondrial membrane.²⁰ SLE patients treated with rapamycin show an improvement in disease activity that correlates with normalization of T cell activation-induced calcium fluxing.¹¹

Aside from its critical role in calcium homeostasis, calreticulin serves as a molecular chaperone and ensures proper folding and quality control of glycoproteins. Changes in the cellular environment can stress the protein folding machinery of the endoplasmic reticulum and activate an intracellular signaling pathway called the unfolded protein response (UPR). Activation of the UPR stimulates expression of chaperone protein genes and stimulates endoplasmic reticulum-associated protein degradation.^{21, 22} Activation of the UPR occurs in various inflammatory diseases and endoplasmic reticulum stress proteins are postulated to play a role in inflammatory processes and autoimmune responses.²³ Calreticulin influences MHC class 1 antigen processing, apoptosis, and clearance of apoptotic debris in T cells.^{24,25} In SLE patients calreticulin is a prevalent autoantigen with up to fifty percent of the sera samples from SLE patients containing antibodies to calreticulin. Calreticulin associates with the Ro/SSA ribonucleoprotein (RNP) complex, where it may serve as a molecular chaperone and aid in the formation of the complex.^{26, 27} Calreticulin recognizes the peptide sequence, KLGFFKR, which is similar to the peptide sequence, KVFFKR, found in the DNA binding domain of glucocorticoid and other steroid hormone receptors.²⁸ Calreticulin shuttles glucocorticoid receptors from the nucleus to the cytosol affecting glucocorticoid receptor distribution and turnover. Calreticulin inhibits glucocorticoid receptor binding to DNA in gel shift assays and binds to the glucocorticoid receptor in a calcium dependent manner leading to receptor export from the nucleus to the cytosol.²⁹ The KAFFKR motif in the first zinc finger domain of the estrogen receptor is essential for DNA binding and could be a site for interaction with calreticulin, although this postulate in relation to nuclear export has not been formally tested. Recently, the role of calreticulin in export of the androgen receptor has been questioned.³⁰

We showed previously that the calcium signaling pathway was affected by estradiol in SLE T cells.⁴ When the estradiol responsive genes were positioned in the calcium signaling pathways using the Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA) calreticulin emerged as one of several estrogen-responsive targets (data not shown). The purpose of this study was to investigate further, the estrogen-dependent control of calreticulin expression in SLE T cells and normal T cells. A second goal of the study was to test whether calreticulin binds to estrogen receptor- α . Such binding could affect nuclear-cytoplasmic shuttling, receptor turnover³¹ and contribute to lower estrogen receptor- α protein in SLE T cells compared with normal T cells.³²

Methods

Study Participants

This study was approved by the St. Luke's Hospital Institutional Review Board and the Committee for the Protection of Human Research Subjects at Pittsburg State University. All subjects provided written informed consent prior to participation. Twelve female patients who met the American College of Rheumatology (ACR) criteria for classification of SLE³³ were enrolled in the study. The patient's disease activity ranged from mild to active with a median SLEDAI value of 4 (range 2–16) at the time of blood draw. The age of the patients at the time of enrollment ranged from 33 to 51 years with a median age of 42 years. The SLE patients were taking various medications including mycophenolate mofetil (n=4), hydroxychloroquin (n=7) and prednisone (n=10). Fifteen healthy control females were enrolled in the study. The control volunteers were between the ages of 29 and 48 with a median age of 46 years. None of the patients or control females was taking oral contraceptives or hormone therapy at the time of blood draw.

T Cell Isolation and Culture

Blood samples (~80 ml) were drawn and T cell enriched mononuclear cells were separated by density gradient using Histopaque (1077, Sigma, St. Louis, MO). Residual red blood cells were lysed (H-Lyse buffer, R & D Systems, Minneapolis, MN). T cells were purified by negative selection using human T cell isolation columns (Human T Cell Enrichment Columns, R & D Systems, Minneapolis, MN). T cells were cultured at 37° C under 5% CO₂ in serum-free medium (Hybridoma medium, Sigma, St. Louis, MO) supplemented with L-glutamine (200 mM). Three physiological levels of estradiol (10⁻⁹ M, 10⁻⁸ M and 10⁻⁷ M) were tested at various time points to determine if calreticulin mRNA and protein were regulated in a dose- and time-dependent manner. Subsequent experiments utilized 10⁻⁷ M estradiol since this concentration of estradiol was used in the microarray study that identified the calcium signaling pathways as an estradiol target in SLE T cells.⁴ Phorbol 12 myristate 13-acetate (10 ng/ml, PMA, Sigma, St. Louis, MO) and ionomycin (0.5 µg/ml, Sigma, St. Louis, MO) were used to activate T cell samples during the final 4 h of culture.

T47D Cell Culture

T47D cells (ATCC, HTB-133, Manassas, VA), a breast cancer cell line, were cultured to confluence at 37° C under 5% CO₂ in T47D medium [RPMI (ATTC, Manassas, VA) with 200 mM L-glutamine, 10% Fetal Bovine Serum (009501, Harlan Bioproducts For Science, Madison, WI), penicillin (100 units/ml)-streptomycin (100 µg/ml, Hyclone, Logan, UT), and bovine insulin (0.2 units/ml, Sigma, St. Louis, MO)].

Nuclear and Cytosolic Extracts

For nuclear and cytosolic extracts, T47D cells and human T cells were homogenized as described in detail by us elsewhere.³⁴ Briefly, the cells were homogenized in 5 volumes of homogenization buffer (0.3 M sucrose, 10 mM Tris-HCL, pH 7.5, 3 mM MgOAC, 1mM DTT, 0.4 M KCl) containing phenylmethylsulfonyl fluoride (PMSF, 1 mM) and phosphatase inhibitor cocktail (PIC) 1 (leupeptin 1 mg/ml, antipain 2 mg/ml, and

benzamidin 10 mg/ml dissolved in aprotinin stock) and PIC II[chymostatin, 1 mg/ml, (Peninsula Labs, San Carlos, CA), pepstatin A 1 mg/ml (Peninsula Labs), dissolved in DMSO] added on the day of use. The samples were centrifuged at 1600 x-g for 10 min and the cytosolic fraction was removed. The nuclear pellet was suspended in homogenization buffer made 1.9 M sucrose. Samples were layered onto a 2 M sucrose cushion and centrifuged at 20,000 x-g for 90 min. Nuclear pellets were suspended in nuclear extract buffer (0.4 M KCL, 20 mM Tris-HCL, pH 7.85, 0.25 M sucrose, 1.1 mM MgCl₂, 10 mM 2-mercaptoethanol) on ice for 10 min. The nuclear proteins were collected by centrifugation at 1600 x-g for 10 min at 4° C. Cytosolic and nuclear extracts were divided into aliquots and stored at -70° C until assay. Protein concentration was measured using the Bradford Reagent (BioRad Laboratories, Hercules, CA).

RNA Purification

RNA was isolated from T cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) and the Phase Lock Heavy Gel System (Eppendorf, Fisher Scientific, Pittsburgh, PA). Total RNA was precipitated with isopropanol (0.5 ml) and glycogen (10 µg, Ambion, Austin, TX) at -20° C for 18 h. Total RNA was treated with DNase I according to the manufacturer's protocol (DNA-free, Ambion, Austin, TX). RNA concentration and purity was assessed by absorbance. For the dose-response and time-course analysis, total RNA and proteins were sequentially separated from the same T cell samples by column purification (Norgen Biotek, Thorold, ON, Canada).

Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized with 1–4 µg of RNA using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA).

Real-time Polymerase Chain Reaction Amplification

Real-time PCR (Step-one, Applied Biosystems) was carried out according to the manufacturer's protocol. Templates of calreticulin were quantified using a Taqman probe and a calreticulin primer (CALR, Hs00189032, Applied Biosystems) specific for the calreticulin gene. A Taqman probe and glyceraldehydes 3-phosphate dehydrogenase (GAPDH, Hs99999905, Applied Biosystems) specific gene primers were used for the internal control. In each cycle, fluorescent signals for the target gene and GAPDH were collected from triplicate samples. The relative quantity (RQ values) was calculated and compared with cDNA synthesized from a T47D breast cancer cell line, which served as the positive control. Samples without template were included in triplicate on each plate as a negative control. The fold change in expression was calculated by dividing the sample RQ values obtained from T cells cultured without and with estradiol from the same individual. For the time-course and dose-response studies, the relative quantity (RQ values) was compared to a T cell sample that was cultured without estradiol.

Measurement of plasma estradiol

Plasma samples were collected from seven control and eight SLE patients at the time of blood draw. Estradiol levels were measured by duplicate using a commercial ELISA plate

(Estradiol ELISA 11-ESTHU-E01, ALPCO Diagnostics, Salem, NH). The amount of estradiol was determined by absorbance at 450 nm from a standard curve. The intra-assay coefficient of variation across all samples was 5.85%.

Measurement of SSA/Ro

Plasma samples were collected from the SLE patients and normal controls. The amount of human SSA/Ro was measured in duplicate using a commercial ELISA plate (SSA/Ro ELISA, Genway Biotech #521216, San Diego, CA), which quantitates IgG class antibodies directed against the SSA/Ro antigen in human plasma. The positive control AB Index was 1.78 and the negative control AB index was 0.04. Values < 0.9 are interpreted to not have detectable antibody while values > 1.1 contain detectable antibody to SSA/Ro by ELISA. The coefficient of variation across all samples was 4.22%.

Measurement of plasma calreticulin

Plasma samples were collected from the SLE patients and normal controls. Calreticulin protein in plasma samples was measured in duplicate using a human calreticulin ELISA (Aviscera Bioscience, Inc. Santa Clara, CA, #SK00016-01). The coefficient of variation across all samples was 6.0%.

Immunoprecipitation Assay

Nuclear and cytosolic extracts were immunoprecipitated using either a calreticulin (H-170, Santa Cruz Biotechnology) or an ER- α (61035, Active Motif) antibody at 4° C for 18 h. Protein A/G PLUS agarose (20 μ l, SC2003, Santa Cruz Biotechnology) was added to the extracts for 18 h at 4° C. The samples were washed four times with PBS-0.1 M NaCl and suspended in sodium dodecyl sulfate (SDS) sample buffer (20 μ l).

Western Blot Analysis

Samples were heated at 95° C for 5 min. Proteins were separated by electrophoresis through 10% SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes in Transblot Buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol). After the protein transfer, non-specific protein binding sites were blocked with Superblock (37515, Thermo Scientific, Rockford, IL) containing 0.02 % Tween 20 (Sigma) at 4° C for 18 h. For the dose response and time course studies, the blots were incubated with a calreticulin antibody (1:7,000, Santa Cruz H170) followed by goat anti-rabbit peroxidase conjugated secondary antibody (1:6,000 Thermo Scientific, 32460). The blots were stripped and reacted with β -actin (1:6,000, Sigma, A5441) followed by goat anti-mouse peroxidase conjugated secondary antibody (Thermo Scientific, 32430). To detect ER- α , the blots were incubated with a mouse monoclonal antibody (1:500, Active Motif, 61035) followed by a goat anti-mouse secondary (1:6,000, Thermo Scientific 32430). To detect calreticulin, the blots were incubated with a rabbit polyclonal antibody (1:700, H-170, Santa Cruz Biotechnology) followed by a goat anti-rabbit secondary (1:6,000 Thermo Scientific, 32460). Blots were washed 4 times (5 min each) with 100 ml of wash buffer (PBS containing 0.05% Tween-20). Binding was visualized using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, 34096). The blots were exposed to

autoradiography film (30 sec to 5 min) to visualize reactive protein species. To assess nuclear protein enrichment, a blot was reacted with a histone deacetylase 1 (HDAC-1) antibody (1:500, Santa Cruz, sc-8410). The amount of protein in each sample was determined using scanning densitometry (Kodak Gel Logic). The quantity of calreticulin protein was determined by dividing the optical density of calreticulin by β -actin in the same sample. The fold change in expression was calculated by comparing T cell samples cultured with estradiol to T cell samples culture without hormone (dose response) or time in culture (time course). Results were obtained from two independent experiments.

Identification of Putative Estrogen Receptor Binding Sites in the Calreticulin Gene

The University of California Santa Cruz (UCSC) Genome Browser was used to search for putative estrogen receptor binding sites known as estrogen response elements, in the regulatory regions of the calreticulin gene. The promoter and enhancer regions and sequence within the gene were searched for consensus sequences to the canonical estrogen response element: 5'-GGTCANNNTGACC-3'.¹³

Statistical Analysis

Effects of estradiol on calreticulin expression were analyzed using the non-parametric Mann-Whitney and Wilcoxon signed rank tests (SPSS statistical software). P values less than 0.05 (two-sided) were considered statistically significant.

Results

Calreticulin expression increased in a dose-dependent manner to estradiol in activated T cells

To extend our previous findings,⁴ T cells were cultured for 24 h at three physiological concentrations of estradiol (Figure 1). In order to compare the dose response results to the microarray profiles generated in our previous study⁴ the T cells were activated during the last 4 h of culture. At the three concentrations of estradiol, calreticulin mRNA increased over a range of 2.5 to 3-fold compared to T cells cultured without estradiol. Calreticulin protein increased over a range of 1.1 to 1.9 fold compared with T cells cultured without estradiol. Although the 10^{-8} M concentration resulted in slightly higher calreticulin mRNA (3-fold) and protein (1.9- fold) we chose 10^{-7} M estradiol (2.5-fold mRNA increase and 1.6-fold protein increase) for further studies since our microarray profiling analysis was conducted using this estradiol concentration.

Calreticulin expression increased in a temporal-dependent manner to estradiol in activated T cells

To gain additional insight into the kinetics of estradiol regulation of calreticulin, T cells were stimulated with estradiol and calreticulin expression was measured at 0, 6 and 24 h post stimulation (Figure 2). In order to compare the time-course results to the microarray profiles generated in our previous study⁴ the T cells were activated during the last 4 h of culture. Calreticulin mRNA (Figure 2a) and protein (Figure 2b) increased at 6h post estradiol stimulation and, at 24 h, remained elevated over that in T cells cultured without estradiol. There was approximately a 3-fold increase in calreticulin mRNA at 6 h post

estradiol stimulation and mRNA levels remained elevated at 24 h (2.5-fold) compared with T cells cultured without estradiol. There was a modest change (1.7) in calreticulin protein at 6 h and the amount of protein remained greater (1.8-fold) in T cells cultured for 24 h compared with T cells cultured without estradiol.

Estradiol increased calreticulin mRNA expression in activated control T cells

We next tested whether changes in calreticulin expression without and with estradiol were significantly different. Control T cell samples were cultured without and with estradiol and the T cells were activated during the last 4 h of culture. Expression of calreticulin mRNA was compared between estradiol-treated and untreated T cells from the same individuals (Figure 3). The fold change in expression ranged from 0.81 to 2.3 (median 1.3). Estradiol increased expression in 7 of the 10 T cell samples, and decreased expression in 3 samples (Figure 3). The preponderance of increased expression over decreased expression with addition of estradiol was statistically significant ($p = 0.028$; Wilcoxon signed rank test) in activated T cell samples.

Estradiol effects on calreticulin mRNA expression were variable in activated SLE T cells

Comparison of calreticulin expression in activated SLE T cell samples in response to estradiol revealed no significant differences ($p = 0.44$). The fold change in activated SLE T cell samples ranged from 0.31 to 4.6 (median 0.98). Calreticulin expression increased in 4 SLE T cell samples, did not change in 1 sample, and decreased in 5 SLE T cell samples (Figure 5). For the SLE T cell samples that exhibited increased expression, the median fold change was 2.9, while for those samples that either decreased or did not change in response to estradiol the median fold change was 0.53. The variation in calreticulin expression did not seem to be due to differences in disease activity because the mean fold change values were similar between patients with mild disease (SLEDAI 4, $n = 6$, mean fold change = 1.85) versus those with moderate to active disease ($n = 4$, mean fold change = 1.6).

Calreticulin expression increased in a dose-dependent manner to estradiol in resting T cells

In order to test whether the estradiol-dependent regulation of calreticulin was different between activated and resting T cells, we conducted a time-course of estradiol effects in resting control T cells. At 6 and 12 h post estradiol addition calreticulin mRNA increased 4- and 4.7-fold, respectively (Figure 5a). However, unlike the results obtained from the activated T cells, at 24h post estradiol addition, calreticulin mRNA decreased (0.79 fold) compared to samples cultured without estradiol. A modest increase in calreticulin protein (1.7-fold) was measured in these same samples at 6 and 12 h post estradiol stimulation (Figure 5b). The amount of protein in T cells cultured for 24 h declined (0.8fold), in line with the mRNA decrease at this same time.

The dynamics of estradiol-dependent control of calreticulin mRNA expression was different in resting T cells

In order to gain insight into the kinetics of calreticulin regulation in resting T cells, we measured calreticulin expression in additional resting T cell samples. With the exception of two subjects, baseline expression of calreticulin was similar among resting T cell samples obtained from control females (Figure 6). Estradiol returned to baseline in 7 samples, had no effect on 1 sample, and slightly increased calreticulin expression in 2 samples. The fold change in expression of calreticulin in response to estradiol at 24 h ranged from 0.35 to 1.28 (median 0.81). The addition of estradiol resulted in more overall reductions ($p=0.044$; Wilcoxon signed rank test) of calreticulin expression in resting control T cell samples. Comparison of calreticulin expression in resting T cells with that in activated T cells obtained from the same individuals (albeit at different blood draws) revealed no statistically significant response to activation. However, comparison of the estradiol effect (+E/-E) on calreticulin expression between activated and non-activated control T cells approached significance ($p = 0.080$), with 4 of the 5 samples exhibiting a substantial activation effect.

Estradiol effects on calreticulin mRNA expression were variable in resting SLE T cells

Estradiol reduced calreticulin expression in 3 SLE T cell samples, had no effect on 1 sample, and increased expression in 6 SLE T cell samples (Figure 7). The fold change in SLE T cell samples ranged from 0.45 to 2.1 (median 1.2). While overall there was no statistically significant effect of estradiol on calreticulin expression in resting SLE T cells ($p = 0.77$; Wilcoxon signed rank test), it is notable that the response to estradiol was dissimilar in that approximately one-half of the patient T cell samples responded robustly to estradiol (median fold change of 1.69), while the other one-half of the samples exhibited decreased expression or no response to estradiol (median fold change of 0.53). The variation in calreticulin expression did not seem to be due to differences in disease activity because the mean fold change values were similar between patients with mild disease (SLEDAI ≤ 4 , $n = 5$, mean fold change = 1.2) versus those with moderate to active disease (≥ 18 , $n = 5$, mean fold change = 1.2).

Plasma estradiol is similar between SLE patients and control females

In order to test whether plasma estradiol levels in circulation could account for the differential response in SLE T cell samples, plasma estradiol was measured at the time of blood draw by ELISA and individual measurements are provided in Tables 1 and 2. The mean plasma estradiol level in the SLE patients was 120.2 pg/ml (± 12.9 pg/ml SEM, $n = 8$). The mean plasma estradiol level in the control females (Table 2) was similar to the patients with a mean value of 133 pg/ml (± 11 pg/ml SEM, $n = 7$). The plasma estradiol measurements for both the SLE patients and control females were within the expected range for healthy females with menstrual cycles.

Detection of SSA/Ro antibodies

The mean plasma antibody index for SSA/Ro antibodies in the patients was 0.29 (± 0.129 , SEM) compared with a mean index of 0.129 (± 0.19 , SEM) for the control samples (Tables 1 and 2). The antibody index revealed one positive subject (indicated by asterisk) among the

patients (Table 1). None of the control plasma samples (Table 2) was considered positive according the antibody index interpretation for this assay.

Measurement of calreticulin protein in plasma

The mean amount of calreticulin protein in SLE patient plasma was 7.29 pg/ml ($\pm$ 1.4 pg/ml, SEM). The amount of calreticulin in the SLE patients ranged from 4.6 pg/ml to 20.03 pg/ml (Table 1). The mean amount of calreticulin in the control samples was 7.0 pg/ml ($\pm$ 0.65 pg/ml, SEM). The amount of calreticulin protein in the control plasma samples ranged from 4.56 pg/ml to 11.35 pg/ml, Table 2).

Estrogen receptor binding sites in the calreticulin gene

In order to explore the possibility that estradiol could regulate calreticulin expression directly by binding at the estrogen response elements, we looked for consensus response elements within the gene. Three potential response elements were identified. One was positioned downstream from the transcriptional start site at position +495 (5'-CAGCCGCTCTGACCT-3'). The other two response elements were in the 5' flanking DNA at positions -52 (5'-AAACCAACCTGACCC-3') and -3,665 (5'-AGACCAGCCTGACCT-3'). These three putative response elements shared 90% homology to the canonical ERE (5'-GGTCANNNTGACC-3').¹³

Calreticulin binds estrogen receptor- α

In order to assess whether calreticulin associated with estrogen receptor- α in vivo, nuclear and cytosolic extracts were prepared from T47D breast cancer cells and from normal T cells. Western blot analysis with a HDAC1 antibody indicated enriched separation of nuclear and cytosolic fractions since HDAC1 localized primarily in the nuclear extract, as expected (Figure 8a). Precipitation of T47D nuclear and cytosolic extracts, using a calreticulin antibody and subsequent Western blot analysis using an estrogen receptor- α antibody, revealed estrogen receptor- α protein precipitated with calreticulin (Figure 8b). Estrogen receptor- α in extracts precipitated with calreticulin antibody localized primarily to the nuclear extract of T47D cells (Figure 8b).

Precipitation of T47D nuclear and cytosolic extracts using the estrogen receptor- α antibody, and subsequent Western blot analysis using calreticulin antibody, revealed calreticulin protein precipitated with estrogen receptor- α (Figure 8c). Analysis of calreticulin in extracts precipitated using estrogen receptor- α antibody from resting control T cell samples revealed estrogen receptor- α protein precipitated with calreticulin in both the cytosolic and nuclear compartments, and slightly more receptor was in the nuclear extract (Figure 8d). It is of interest that the immunoreactive protein at approximately 120 kDa is consistent with the size expected for estrogen receptor dimers.

Discussion

Calreticulin is a multi-functional protein that is central to calcium homeostasis and protein integrity. Calreticulin is also implicated in many important T cell functions including MHC class I antigen processing, apoptosis, clearance of apoptotic debris and inflammatory

diseases.^{23,35–37} In the present study, we have investigated the regulation of calreticulin by the female sex steroid, estradiol. The results suggest that calreticulin is tightly regulated by estradiol in normal T cells. In normal resting T cells, calreticulin expression decreases at 24 h but in activated normal T cells, calreticulin expression stays significantly elevated. Although these estradiol-dependent changes are modest, previous studies suggest a 1.6-fold increase of calreticulin protein expression can increase intracellular calcium storage and decrease store-operated calcium influx.³⁸ The present results extend our previous findings⁴ and suggest one of the early estradiol gene targets in the calcium signaling pathway, calreticulin, is tightly regulated in normal T cells. This regulation is abnormal in SLE T cells.

In approximately one-half of the resting or activated SLE T cell samples, estradiol is stimulatory while in the other one-half, there is little response to estradiol. The basis for this difference is not known but it is unlikely due to differences in circulating estradiol since plasma estradiol levels are similar between control females and patients with SLE. Similarly, this differential regulation does not appear to be associated with disease activity since the mean-fold change in calreticulin expression in response to estradiol was similar between patients with mild to moderate disease compared with patients with more active disease. The patients enrolled in the study were taking medications and we cannot exclude the possibility that medications influence differential calreticulin regulation. However, there was no consistent drug effect on calreticulin expression in that the T cell response differed (responder vs. non-responder) between patients taking the same medications. There is no clear association of the differential calreticulin response with the presence of SSA-Ro antibodies or calreticulin protein in the plasma. Taken together, the results suggest that differential regulation of calreticulin in SLE T cells may be due to altered regulation of the calreticulin gene by estrogen receptors.

Numerous signaling abnormalities are reported in SLE T cells following TCR-engagement.³⁹ Changes in the expression of calreticulin correlate directly with changes in calcium signaling.⁴⁰ Calreticulin is positioned upstream of the calcium-calcieneurin-NFAT signal suggesting differential regulation of this gene could underlie changes in calcium signaling and cytokine transcription in SLE T cells.^{10, 41} Unexpectedly, the results show that the kinetics of a sustained estrogen-dependent response may be required for the persistent increase in cytosolic calcium that is characteristic in the normal activation response and deficient in SLE T cells.^{5–6}

Ro is a RNP complex containing one of two isoforms of the Ro protein and one of four noncoding cytoplasmic RNAs.²⁶ Anti-Ro antibodies are found in approximately 50% of SLE patients compared with 20% in normal individual's sera.⁴² Interestingly, our results indicate differential regulation of calreticulin by estradiol in approximately one-half of the SLE patients enrolled in the study. However, only one of the patients tested by ELISA was positive for SSA-Ro antibodies. The low number of SSA-Ro positive patients may be due to the small sample size. Calreticulin is proposed to be a human autoantigen that is released from cells in apoptotic blebs, which contain high concentrations of Ro, La and calreticulin.²⁷ Release of these proteins from membrane bound vesicles at sites of high inflammation could trigger autoimmune responses, particularly if clearance of apoptotic cells is impaired. The

mean amount of calreticulin protein in the plasma of SLE patients and normal individuals was not significantly different in this study. It is noteworthy that plasma calreticulin does not measure calreticulin bound to cell surfaces and other proteins. Calreticulin could function as an endogenous adjuvant and promote inflammation through its binding and chaperone activity. This mechanism could contribute to inflammation in SLE owing to the abnormal regulation of calreticulin by estradiol.

Calreticulin expression increases in activated T cells and estradiol augments calreticulin expression over that of activation alone. We identified three putative estrogen receptor binding sites with 90% or greater homology to the consensus estrogen response element. Two of these putative elements are located in the promoter/enhancer regions, while the other is downstream of the transcriptional start site. During cardiac development, calreticulin is down-regulated after birth and failure to suppress calreticulin expression in newborns leads to severe cardiac pathology. The cardiac specific transcription factor *tinman* (Nkx2.5) increases calreticulin expression in the heart while chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) binds to the Nkx2.5 binding site and suppresses transcription from the calreticulin promoter.^{43,44} In the present study, calreticulin expression markedly decreases at 24 h of estradiol stimulation suggesting this decline is due to the presence of an inhibitory factor. While downregulation of the estrogen receptor itself could result in decreased expression this interpretation is less likely since estradiol maintains calreticulin expression for 24 h in activated T cells. It is tempting to speculate that COUP-TF1, an established suppressor of steroid receptor binding^{21,45} inhibits estrogen-dependent activation of calreticulin in resting T cells. We postulate that in activated T cells COUP-TF1 is either not expressed or is unable to bind to regulatory regions of the calreticulin gene. Experiments to test these postulates are in progress. Analysis of the human calreticulin-1 gene promoter also revealed four specificity protein 1 (SP-1) sites and a single activator protein 1 (AP-1) site. Estrogen receptors can be tethered to transcriptional regulatory sites through protein-protein interactions with DNA bound SP-1 and AP-1 proteins. The receptor does not actually interact with the DNA but rather stabilizes the protein complex and helps recruit additional transcriptional regulators.^{46,47} Estrogen upregulates SP-1 in human T cells and increases SP-1 binding to the cyclic AMP response element modulator α .⁴⁸

Results from the present study suggest that estradiol regulates calreticulin expression in normal T cells and this regulation is altered in SLE T cells. Estradiol increased calreticulin mRNA significantly, while changes in calreticulin protein were more modest. However, previous studies suggest a 1.6-fold increase of calreticulin expression can increase intracellular calcium storage and decrease store-operated calcium influx.³⁸ Calreticulin is upregulated by estradiol during activation and we hypothesize that this prepares T cells for the sustained calcium elevation that follows antigen encounter.^{5,8} Deregulation of calreticulin is expected to affect signal transduction and cytokine profiles in SLE T cells. Activation of the mitogen activated protein kinase (MAPK) by extracellular signal-regulated kinase 1/2 (ERK1/2) is abnormal in SLE T cells^{49,50} and mouse T cell clones that lack calreticulin exhibit prolonged ERK activation.⁴¹ Abnormal regulation of calcium homeostasis in SLE T cells could alter the turnover of signaling proteins in the calcineurin-NFAT pathway.⁵¹ In addition, our results indicate that calreticulin and estrogen receptor- α

associate in normal T cells. This study did not determine whether calreticulin and estrogen receptor- β can also associate in T cells and future experiments are required to test this possibility. Calreticulin may serve as a molecular chaperone for estrogen receptor- α and deregulation of calreticulin may result in a defective receptor shuttling mechanism. Alternatively, the binding of estrogen receptor- α with calreticulin may form a complex that, when altered by deregulation of calreticulin, leads to the recruitment and binding of other proteins to form an antigenic complex. These possibilities are currently under investigation. Taken together, our results suggest that estradiol tightly regulates calreticulin expression in normal human T cells. Deregulation of calreticulin, in addition to other estrogen-responsive genes in the calcium signaling pathways including, calcineurin and CD154 could account for abnormal activation responses in female SLE T cells and contribute to the strong gender bias in this autoimmune disorder.

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a

Activated T Cell Extract

Estradiol Concentration (mol/L)

b

Figure 1.

Calreticulin expression increases in response to physiological concentrations of estradiol in activated T cells. T cells were isolated from healthy volunteers and cultured for 24 h without (-E) or with three physiological concentrations (10^{-9} to 10^{-7} M estradiol). The T cells were activated during the last 4 h of culture. RNA and protein was sequentially separated as detailed in the text. (A) Calreticulin mRNA was measured using real-time PCR from samples in triplicate. Calreticulin increased at the three doses tested with maximum expression at 10^{-8} M estradiol. Data are mean values $\pm$ SD. (B) Calreticulin protein expression increased in response to estradiol at the three physiological concentrations tested. The amount of proteins was greatest (1.9-fold increase) at 10^{-8} M estradiol stimulation. Data shown are representative of two independent experiments.

a

Activated T Cell Extract

b

Figure 2.

Calreticulin expression increases temporally in response to estradiol in activated T cells. T cells were isolated from healthy volunteers and cultured in estradiol (10^{-7} M) containing medium for the indicated times. The T cells were activated during the last 4 h of culture. RNA and protein was sequentially separated as detailed in the text. (A) Calreticulin mRNA was measured using real-time PCR from samples in triplicate. Data are mean values $\pm$ SD. (B) Calreticulin protein increased in response to estradiol as assessed by Western blotting. The amount of protein increased approximately 1.7-fold at 6 h after stimulation and remained increased (1.8-fold) at 24 h. Data shown are representative of two independent experiments.

Figure 3.

Calreticulin expression increased ($p = 0.028$) in response to estradiol in activated control T cell samples. T cell samples obtained from normal volunteers were cultured for 18 h without and with estradiol. The samples were activated for 4 h as described in the text and the amount of calreticulin in the same samples without or with estradiol was compared using real time PCR. Data are mean values $\pm$ SD from samples in triplicate. Black bars are data from T cells cultured with estradiol and white bars are data from T cells cultured without estradiol.

Figure 4.

Calreticulin expression does not change ($p = 0.87$) in response to estradiol in activated SLE T cell samples. T cell samples obtained from SLE patients were cultured for 18 h without and with estradiol. The samples were activated for 4 h and the amount of calreticulin in the same samples without or with estradiol was compared using real time PCR. Data are mean values $\pm$ SD from samples in triplicate. Black bars are data from T cells cultured with estradiol and white bars are data from T cells cultured without estradiol.

a

Resting T Cell Extract

Time in Culture (10^{-7} M Estradiol)

b

Figure 5.

Calreticulin expression decreases at 24 h in resting T cells. T cells were isolated from healthy volunteers and cultured in medium containing estradiol (10^{-7} M) for the indicated times. RNA and protein was sequentially separated as detailed in the text. (A) Calreticulin mRNA was measured using real-time PCR. Data are mean values $\pm$ SD of samples in triplicate. (B) Calreticulin protein increased in response to estradiol at 6 and 12 h post stimulation as assessed by Western blotting. The protein declined at 24h post stimulation to levels similar to that of T cells cultured without estradiol. Data shown are representative of two independent experiments.

Figure 6.

Calreticulin expression is downregulated ($p = 0.044$) by estradiol in resting control T cells. T cell samples obtained from normal volunteers were cultured for 18 h without and with estradiol. The amount of calreticulin in the same samples without or with estradiol was compared using real time PCR. Data are mean values $\pm$ SD from samples in triplicate. Black bars are data from T cells cultured with estradiol and white bars are data from T cells cultured without estradiol.

Figure 7.

Calreticulin expression does not change ($p = 0.77$) in response to estradiol in resting SLE T cell samples. T cell samples were obtained from SLE patients and cultured without and with estradiol. The amount of calreticulin in the same samples without or with estradiol was compared using real time PCR. Data are mean values $\pm$ SD from samples in triplicate. Black bars are data from T cells cultured with estradiol and white bars are data from T cells cultured without estradiol.

a

kDa

b

C

Figure 8.

Calreticulin and estrogen receptor- α associate in T47D breast cancer cells and human T cells. (A) Separation of nuclear and cytosolic fractions from T47D breast cancer cells shows enrichment of histone deacetylase (HDAC) in the nuclear fraction. T47D breast cancer cells were homogenized and cytoplasmic and nuclear fractions were separated. The extracts were size fractionated by SDS-PAGE and the proteins were transferred to nitrocellulose by standard methods. The membrane was reacted with an antibody to detect HDAC which migrated with an apparent molecular mass of 62 kDa. Arrows indicate the position of molecular size standards. (B) Calreticulin and estrogen receptor- α associate in T47D breast cancer cells. T47D breast cancer cells were homogenized and nuclear and cytoplasmic fractions were separated. Calreticulin was immunoprecipitated and the immunoprecipitates were size fractionated by SDS-PAGE. Proteins in the immunoprecipitates were transferred to nitrocellulose and the membrane was reacted with an estrogen receptor- α antibody which detected a protein at 68 kDa. Lane 1, molecular size standards; Lane 2, no extract, negative control; Lane 3, total T47D extract (10 μ g); Lane 4, T47D cytosolic extract; Lane 5, T47D nuclear extract. Data shown are representative of three independent experiments. (C)

Estrogen receptor- α and calreticulin associate in T47D breast cancer cells. T47D breast cancer cells were homogenized and nuclear and cytoplasmic fractions were separated. Estrogen receptor- α was immunoprecipitated and the immunoprecipitates were size fractionated by SDS-PAGE. Proteins in the immunoprecipitates were transferred to nitrocellulose and the membrane was reacted with a calreticulin antibody which detected a protein at 58 kDa. Lane 1, total T47D extract, positive control (10 μ g); Lane 2, T47D cytosolic extract; Lane 3, T47D nuclear extract. Arrows indicated the position of the molecular size standards. Data shown are representative of two independent experiments. (D) Calreticulin and estrogen receptor- α associate in normal resting T cells. T cells were homogenized and nuclear and cytoplasmic fractions were separated. Calreticulin was immunoprecipitated and the immunoprecipitates were size fractionated by SDS-PAGE. Proteins in the immunoprecipitates were transferred to nitrocellulose and the membrane was reacted with an estrogen receptor- α antibody. Lane 1, T47D nuclear extract, positive control, 10 μ g); Lane 2, T cell cytosolic extract; Lane 3, T cell nuclear extract. Arrows indicate positions of molecular size standards. The size of the estrogen receptor- α on the immunoblots was approximately 68 kDa. Data shown are representative of two independent T cell samples.

Table 1

Characteristics of SLE patients enrolled in the study

Patient	SLEDAI	Estradiol (pg/ml)	SSA/Ro	Calreticulin (pg/ml)
SLE 1	10	nd	0.18	11.98
SLE 2	2	79.7	1.56*	5.36
SLE 3	2	157.2	0.15	4.63
SLE 4	4	97.2	0.28	5.56
SLE 5	2	188.8	0.30	4.87
SLE 6	12	120.7	0.08	4.97
SLE 7	5	123.7	0.11	6.20
SLE 8	4	98.6	0.26	4.91
SLE 9	13	nd	nd	nd
SLE 10	11	95.6	0.12	5.26
SLE 11	4	nd	0.07	20.03
SLE 12	16	nd	0.1	6.48

* Positive for SSA/Ro autoantibody

Table 2

Characteristics of Healthy Control Females Enrolled in the Study

Sample	Estradiol (pg/ml)	SSA/Ro	Calreticulin (pg/ml)
CTRL 1	171.3	0.19	5.57
CTRL 2	108.58	0.14	11.35
CTRL 3	nd	0.21	5.7
CTRL 4	108.2	0.06	7.21
CTRL 5	116.8	0.18	6.91
CTRL 6	nd	0.14	4.56
CTRL 7	175.18	0.09	8.03
CTRL 8	114.78	0.08	7.87
CTRL 9	139.2	0.07	6.41