0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(2):662–671

doi: 10.1210/en.2005-0829

Estrogen Up-Regulates Inducible Nitric Oxide Synthase, Nitric Oxide, and Cyclooxygenase-2 in Splenocytes Activated with T Cell Stimulants: Role of Interferon-γ

Ebru Karpuzoglu, Jillian B. Fenaux, Rebecca A. Phillips, Andrea J. Lengi, François Elvinger, and S. Ansar Ahmed

Department of Biomedical Sciences and Pathobiology (E.K., J.B.F., R.A.P., A.J.L., S.A.A.), Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061; and Department of Large Animal Clinical Sciences (F.E.), The Laboratory for Study Design and Statistical Service, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia 24060

Estrogen is implicated in many autoimmune diseases and is a robust immunomodulator. For example, it regulates interferon (IFN)-γ, a cytokine believed to up-regulate inducible nitric oxide synthase (iNOS). A notable gap in the literature is a lack of information on the regulation of nitric oxide in immune tissues by estrogen. We now show that activation of splenocytes with T cell stimulants [concanavalin-A (Con-A) or anti-CD3 antibodies] results in copious release of nitric oxide in splenocyte cultures from estrogen-treated but not placebo-treated mice. Moreover, even a low dose of T cell stimulants induced nitric oxide in splenocytes from estrogen-treated, but not placebo-treated, mice. Con-A-activated splenocytes from estrogen-treated mice also have up-regulated iNOS mRNA, iNOS protein, and cyclooxygenase-2 (a nitric oxide-regulated downstream proinflammatory protein) when compared with controls. Our studies suggest that the induction of nitric oxide by activated splenocytes from estrogen-treated mice is mediated in part by IFNγ. First, blocking costimulatory signals mediated through interactions of CD28 and B7 molecules by CTLA-4Ig markedly decreased not only IFNγ but also nitric oxide. Second, estrogen treatment of IFNγ-knockout (IFNγ^{-}/_) mice did not induce iNOS protein or nitric oxide. Finally, *in vitro* addition of recombinant IFNγ to Con-A-activated splenocytes from IFNγ($^{-}$/_) mice induced iNOS protein primarily in estrogen-treated mice. Overall, this is the first report to show that estrogen treatment up-regulates IFNγ-inducible-iNOS gene expression, iNOS protein, nitric oxide, and cyclooxygenase-2 as an indirect consequence of activation of T cells. These findings may have wide implications to immunity and inflammatory disorders including female-predominant autoimmune diseases. (*Endocrinology* 147: 662–671, 2006)

IT IS NOW well appreciated that nitric oxide has broad physiological and pathological effects on many tissues including the immune system (1, 2). Nitric oxide, a short-lived ($t_{1/2}$ sec) but highly reactive biomolecule, rapidly diffuses through membranes and readily reacts with diverse substances in a wide range of cell types. Nitric oxide is known to interact with: aqueous oxygen (to produce relatively unreactive nitrate and nitrite), transition metals (to form metal-nitric oxide adducts), superoxide (to generate highly toxic peroxynitrite), heme, Fe-S clusters, Zn-S clusters, and sulfadyryl clusters (to exert distinct biological effects) (3–6). It is therefore not surprising that nitric oxide plays a central role in the physiology and pathology of various tissues. Nitric oxide is generated by nitric oxide synthases (NOS), which catalyze the production of nitric oxide and L-citrulline from L-arginine in the presence of nicotinamide adenine dinucleotide phosphate reduced (NADPH)-derived electrons and O_2. There are three well-known isoforms of NOSs: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (2). In lymphoid tissues, iNOS is the principal isoform of iNOS. Macrophages appear to be a major source of iNOS (1, 2, 7). The iNOS differs from other two NOS isoforms (eNOS and nNOS) in at least two main aspects: 1) iNOS is not constitutively present but rather is induced by cytokines, *e.g.* interferon (IFN)-γ, TNFα, or other immunological stimuli (*e.g.* lipopolysaccharides), and 2) relatively high levels of nitric oxide (in the nanomolar range) are generated by iNOS.

The role of nitric oxide in immunity is well appreciated (2, 8). For example, the healing of excisional wounds is significantly delayed in iNOS-deficient mice when compared with wild-type mice (2). Nitric oxide-derived from iNOS is critical for controlling infections (1, 9, 10). Infected or activated macrophages produce nitric oxide derived from iNOS, which assists in killing the invading microbes by increasing double-stranded DNA (dsDNA) breaks and oxidation of lipids and DNA. Moreover, nitric oxide will interact with oxygen-free radicals such as superoxide to result in highly toxic peroxynitrite. Nitric oxide may also play a pivotal role in inflammation by increasing the production and/or increasing the enzymatic activity of cyclooxygenase-2 (COX-2) (11, 12).

First Published Online November 17, 2005

Abbreviations: Con-A, Concanavalin-A; COX-2, cycloxygenase-2; dsDNA, double-stranded DNA; eNOS, endothelial NOS; IFN, interferon; iNOS, inducible nitric oxide synthase; IRF, IFN regulatory factor-1; JAK, Janus kinase; nNOS, neuronal NOS; NOS, nitric oxide synthase; rIFNγ, recombinant IFNγ; STAT, signal transducer and activator of transcription.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

COX-2 is inducible directly or indirectly by nitric oxide. It is expressed in many cells, especially in macrophages and monocytes (13–17).

Whereas an appropriate increase in nitric oxide is critical for effective immunity, aberrant levels of nitric oxide have been associated with many pathological conditions such as neurodegenerative disorders, apoptosis, hypertension, hypercholestrolemia, diabetes, heart failure, infections, autoimmune diseases, asthma, and septic shock (1, 18–20). Autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and glomerulonephritis associated with increased IFNγ levels demonstrate high expression of iNOS and nitric oxide (1, 19). This implies that the level of nitric oxide and the duration of its presence must be regulated to maintain health. One of the important regulators of NOS is estrogen. In several nonimmune tissues, estrogen has been shown to be a powerful regulator of nitric oxide that is presumably generated by nNOS and eNOS (21, 22). Whereas estrogen has been shown to regulate nitric oxide in nonimmune (reproductive and nervous) tissues, there is little information on the regulation of nitric oxide by estrogen in immune tissues, particularly in relation to T-macrophage interactions. Therefore, this study addresses this important aspect. It is especially appropriate, considering that estrogen is implicated in many inflammatory and autoimmune diseases (23–29) and has recently been shown by us (30, 31) and others (32) to up-regulate IFNγ in activated splenocytes. To date, there have been no studies that have addressed the relationship among IFNγ, iNOS/nitric oxide, and COX-2 in T cell-activated splenocytes in relation to estrogen treatment. This is the first study that establishes such a link.

Materials and Methods

Mice

Three- to 4-wk-old C57BL/6 wild-type male mice were obtained from Charles River Laboratories (Wilmington, MA) and housed three to five animals per cage. C57BL/6 mice with IFNγ gene deleted (IFNγ knockout mice) were obtained from Jackson Laboratories (Bar Harbor, ME) and C57BL/6 mice with interferon regulatory factor-1 (IRF)-1 gene deleted (IRF-1 knockout mice) were obtained from Amgen (a kind gift from Dr. Georgio Senaldi, Thousand Oaks, CA). Mice were housed in standard cages, fed a diet devoid of phytoestrogens and synthetic estrogens, and housed in rooms on a 14-h light, 10-h dark cycle at the Center for Molecular Medicine and Infectious Diseases animal laboratory facility. All animal procedures including housing, treatment, and termination (by cervical dislocation) were approved by Animal Care Committee of Virginia Polytechnic Institute and State University Institutional Animal Care guidelines.

Estrogen treatment

Four- to 5-wk-old mice were orchiectomized and given SILASTIC brand implants (Dow Corning Corp., Midland, MI) that were either placebo (empty implant as a control) or estrogen implants containing 17β-estradiol (Sigma-Aldrich Inc., St. Louis, MO) by standard procedures that have been extensively reported previously (30, 31, 33, 34). Mice were killed after 1–2 months of treatment.

Isolation of splenic lymphocytes

Spleens were collected under sterile conditions, and lymphocytes were isolated according to our previously published methods (30, 31, 35). Briefly, spleens were gently dissociated by teasing on a sterile 60-mesh steel screen (Sigma-Aldrich). The cell suspensions were washed in incomplete RPMI 1640 media (CellGro, Mediatech, Herndon, VA). Lymphocytes were enriched by removing red blood cells from splenocytes after treating with ACK lysis buffer [0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA (pH 7.4)] (36). Care was taken to culture cells in phenol-red-deficient media (because phenol-red is estrogenic) and charcoal-stripped fetal bovine serum (to remove estrogens and estrogen-binding proteins) to ensure that estrogen exposure was restricted to the *in vivo* period only. Cells were thus washed and suspended in phenol red-free RPMI 1640 media (Mediatech) that was supplemented with 10% charcoal-stripped, heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 200 μM L-glutamine (Mediatech), 5000 IU/ml penicillin, 5000 μg/ml streptomycin (Mediatech), and 5 ml of 100 × nonessential amino acids (Mediatech). Isolated lymphocytes were then counted, and cell viability was assessed by the trypan blue exclusion method as described in our earlier studies (35). Cell numbers were assessed with a CASY-1 cell counter and analyzer system (Scharfe System GmbH, Reutingen, Germany) and adjusted to 5×10^6 cells/ml as described in our previous studies (35). According to our experience and the manufacturer's instructions, cells ranging from 5 to 10 μm predominantly constitute the lymphocyte population in mice (35, 37). Cells beyond 10 μm are generally aggregate cells and cellular debris and were a minor subpopulation.

Culture of lymphocytes

Five hundred microliters of cells at 5×10^6 cells/ml were added to 24-well, round, flat-bottomed plates containing 500 μl/well of complete phenol red-free RPMI 1640 media with or without the T cell mitogen, concanavalin-A (Con-A, a mitogenic dose at 10 μg/ml; Sigma-Aldrich) or anti-CD3 antibodies (a mitogenic dose at 10 μg/ml; eBioscience Inc., San Diego, CA). In selected experiments, low dose of Con-A (1 μg/ml) or anti-CD3 antibodies (low doses at 0.1 or 1 μg/ml) were used. Additionally, in some cultures, cells were exposed to Con-A (10 μg/ml) in the presence or absence of CTLA-4Ig fusion protein (15 μg/ml) (Alexis Biochemicals, San Diego, CA). The CTLA-4Ig fusion protein blocks the interaction of B7.1/B7.2 molecules (on antigen-presenting cells such as macrophages) with CD28 costimulatory molecules (on T cells). To investigate the direct *in vitro* response of splenocytes from estrogen- or placebo-treated wild-type or IFNγ-deficient mice to recombinant IFNγ, cells were cultured with recombinant IFNγ (rIFNγ; 1000 pg/ml or 10 ng/ml; BD PharMingen, San Diego, CA). Cell cultures were incubated for 24 or 48 h at 37 C with a 5% CO_2 environment. Supernatants were collected in microcentrifuge tubes and centrifuged at 5000 rpm for 5 min to collect the cellular fractions and supernatants, which were immediately frozen at −70 C until use.

Determination of IFNγ protein

Levels of IFNγ in the supernatants of activated lymphocytes in culture were analyzed with an IFNγ-specific ELISA that has been extensively described previously (30, 31). Briefly, 96-well Maxisorp high binding immunoassay plates were coated overnight with anti-IFNγ antibodies (anti-IFNγ clone R4-6A2, ATCC, HB-170; BD PharMingen) in PBS and then blocked with 2% BSA for 1 h. Plates were incubated for 2 h with either supernatants (diluted as necessary) or serial dilutions of known concentrations of IFNγ to generate a standard curve. Next, biotin-conjugated anti-IFNγ antibodies (BD PharMingen) were applied to the plates for 1 h and then overlaid with an avidin-horseradish peroxidase solution (Vector Laboratories, Burlingame, CA). The color was developed using 3,3′,5,5′-tetramethylbenzidine as the substrate (KPL, Gaithersburg, MD) and 0.18 M sulfuric acid to stop the reaction. Plates were read at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). The IFNγ protein levels were calculated with the formula obtained from the standards using SoftMax Pro software (Molecular Devices).

Isolation of RNA, cDNA synthesis, and primer design

RNA was isolated from splenic lymphocytes that were cultured for 24 h in media alone (unstimulated) or stimulated with Con-A by using RNeasy minicolumns (QIAGEN Inc., Valencia, CA) per the manufacturer's instructions. After the elution of RNA with sterile water from RNeasy columns, RNA was used to synthesize cDNA using the Super-

Script first-stand synthesis system following the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Primers were designed to span a large segment of the target genes, without regard to introns, using an online program: Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primers were designed with reference to the cDNA sequences, and the intent was to amplify large fragments. Designed primers were as follows: β-actin (965 bp), forward 5′-ATTGTTACCAACTGGGACGA-3′, reverse 5′-CTGCGCAAGTTAGGTTTTGT-3′; and iNOS (854 bp), forward 5′-TGTGTTCCACCAGGAGATGT-3′, reverse 5′-AGGTGAGCTGAACGAGGAG-3′ (One Trick Pony Oligos, Ransom Hill Bioscience, Ramona, CA).

RT-PCR assay

The RT-PCR was used to detect β-actin and iNOS mRNA expression for the generation of standards. After cDNA synthesis, the PCRs were performed using Platinium PCR SuperMix (Invitrogen) per the manufacturer's instructions. The β-actin and iNOS primers were purchased from Maxim Biotech Inc. (San Francisco, CA). The PCR was performed, using an Eppendorf mastercycler gradient, under the following conditions: 35 cycles of 94 C for 1 min, annealing at 58 C for 1 min, and amplification at 72 C for 1 min. At the end of the RT-PCR, 12 μl of PCR products were run on a 2% agarose gel containing ethidium bromide (10 μl per 100 ml of gel). Photographs of the PCR products were taken and the density of bands determined by scanning densitometry and a Kodak Image Station (PerkinElmer Life Sciences Inc., Wellesley, MA). The data were normalized to β-actin, a housekeeping gene, and expressed as relative densities. To permit direct quantification, sets of primers were used to amplify target cDNA without coamplifying the potentially contaminating genomic DNA. The PCR products obtained from the large fragments were used for the generation of a standard curve for real time-PCR analysis. These products were visualized over UV light.

Extraction of target genes

The PCR products were excised from the agarose gel and isolated using a MinElute Extraction kit according to the manufacturer's instructions (QIAGEN) to be used for the generation of standards and the standard curve method. Briefly, the gel slice was weighed and 3 vol of buffer AG were added to 1 vol gel. The gel was incubated at 50 C for 10 min and vortexed every 2–3 min during the incubation. After the gel was dissolved completely, 1 vol isopropanol was added, mixed via inverting, applied to the MinElute column, and centrifuged for 1 min. After discarding the flow-through, the column was applied with buffer QG and spun for one minute. The MinElute column was washed with buffer PE and DNA was eluted using DNase-RNase-free water.

Measurement of total cDNA after gel extraction

PicoGreen (Molecular Probes Inc., Eugene, OR) is considered to be a sensitive reagent for detecting dsDNA, such as cDNA, in solution. Following the manufacturer's instructions, a 2-μg/ml stock solution of dsDNA in Tris/EDTA (TE) buffer was prepared and samples were diluted 20-fold in DNase-RNase-free Tris/EDTA buffer. One hundred microliters of sample and PicoGreen were added to a 96-well plate and incubated in the dark for 5 min. After incubation, the sample and standards were read using a fluorometer (CytoFlour, PerSeptive Biosystems Inc., Framingham, MA) (excitation ~480 nm, emission ~520 nm). dsDNA, diluted 2-fold to generate a standard curve of fluorescence *vs.* DNA concentration, was used to detect the amount of cDNA in the samples.

Real-time PCR

After cDNA synthesis, real-time PCR was performed, using the same primers as in RT-PCR, using Platinum UDG PCR SuperMix (Invitrogen) and SybrGreen I (Molecular Probes) following the manufacturer's instructions. The β-actin and iNOS primers were purchased from Maxim Biotech. Positive controls for iNOS and β-actin were used to validate the assay. The real-time PCR was performed under the following conditions: 35 cycles of 94 C for 30 sec, annealing at 58 C for 30 sec, and amplification at 72 C for 30 sec with a Bio-Rad iCycler. β-Actin mRNA encodes a cytoskeleton protein, which is expressed among almost all cell types. It is widely used to normalize results in real-time RT-PCR assays. On amplification of the target gene, integration of the dsDNA binding dye, SybrGreen I, into the amplification product results in an increase in fluorescence, which is a direct consequence of target amplification during PCR. Direct quantification of the PCR products was performed using the standard curve generated from appropriate standards that had known amounts of cDNA in femtograms. The target gene expression was normalized to β-actin.

Detection of nitric oxide

A Greiss assay was used to determine the presence of nitric oxide. In this assay, nitrite, an end product of nitric oxide oxidation, is measured in tissue culture fluid. Greiss assay reagents included 1% (wt/vol) sulfanilamide (Sigma-Aldrich) and 0.1% (wt/vol) naphthylenediamine dihydrochloride (Sigma-Aldrich). Both reagents were dissolved separately in 2.5% phosphoric acid. Immediately after collecting the tissue culture, 50 μl of both reagents were added to an equal volume of the culture supernatant in a 96-well round-bottom plate. A serial dilution of sodium nitrite beginning at 1000 μM to 0.5 μM was used as a standard. Five to ten minutes after adding the reagents, the plates were read on an optical density plate reader set at 550 nm (Molecular Devices). The levels of nitric oxide were calculated using the formula obtained from the standards using SoftMax Pro software (Molecular Devices).

Determination of iNOS and COX-2 protein

Western blot assays were used to detect the iNOS and COX-2 protein levels in cell lysates of placebo and estrogen-treated splenic lymphocytes that were cultured with Con-A (10 μg/ml) or Con-A (10 μg/ml) plus CTLA-4Ig (15 μg/ml). At the end of the incubation period, cells were harvested and resuspended in 15 μl of lysis buffer [50 mM Tris (pH 7.4), 300 mM NaCl, 2 mM EDTA (pH 8), 0.5% Triton X-100] and 30 μl of freshly added mammalian protease inhibitor cocktail (10 μg/ml, Sigma-Aldrich), vortexed, and incubated on ice for 15 min. Fifteen microliters of 2× sample buffer (Laemmli sample buffer; Sigma-Aldrich) were added to the 15 μl of lysate and heated at 95 C for 5 min. Samples were electrophoresed on a 12.5% gel at 25 mA constant current until the dye ran off the bottom. The proteins were transferred to polyvinyl difluoride (PVDF) transfer membranes (Amersham Biosciences, Piscataway, NJ) by blotting for 1 h 15 min at 240 mA constant current. After transferring the proteins to a polyvinyl difluoride membrane, it was blocked in 5% milk in TBS-T for 1 h at room temperature. The blot was incubated with anti-iNOS antibody [rabbit polyclonal IgG (sc-650), Santa Cruz Biotechnology Inc., Santa Cruz, CA] and COX-2 primary antibody [rabbit polyclonal IgG (sc-1745), Santa Cruz Biotechnology] diluted in blocking buffer (1:1000) overnight on a rocking platform. The blots were rinsed briefly in TBS-T and then washed three times for 10 min with TBS-T. The secondary horseradish peroxidase-conjugated antibody (antirabbit IgG, 1:2000, Santa Cruz Biotechnology) was applied in blocking buffer to each blot and incubated for 1 h. After blots were washed, they were labeled using an ECL protocol (Amersham Pharmacia Biotech, Piscataway, NJ), and the bands were visualized using a Kodak Image Station (PerkinElmer Life Sciences).

Statistics

Data were analyzed using SAS software (SAS Institute Inc., Cary, NC) at the Laboratory for Study Design and Statistical Service, Virginia-Maryland Regional College of Veterinary Medicine, by coauthor Dr. F. Elvinger. Normality of data was assessed using UNIVARIATE procedure of SAS, and if warranted, data were transformed logarithmically. Treatment and other effects were assessed using the MIXED procedure of SAS, by ANOVA of a split plot model including treatment, date of experiment, and their interactions tested by the main plot error term ID (treatment × date of experiment), with stimulant and stimulant × treatment interaction in the subplot tested by the overall error term. In addition, the general linear model (GLM) procedure of SAS by ANOVA of a split plot model including treatment tested by the main plot error term ID (treatment), with stimulant, time, their interaction, and interactions with treatment in the subplot tested by the overall error term was used to assess treatment and other effects. Significant interactions at $P < 0.05$ were further investigated using the SLICE option to test simple main

effects. Data are represented as geometric means with SEM. Note that graphical representation of ± SEMs of a geometrical mean is not symmetrical about the mean.

Results

Estrogen induces IFNγ

Consistent with our recently reported studies (30, 31), Con-A-activated splenocytes from estrogen-treated mice tended to have increased levels of IFNγ when compared with similar cultures from placebo-treated mice [media: not detectable; Con-A: placebo: 2591.9 (SEM +557/−458) pg/ml, estrogen: 6469.9 (SEM +1322/−362) pg/ml after 24 h of culture; n = 15 mice per treatment; $P < 0.05$]. It is likely that IFNγ-inducible parameters may also be altered in splenocytes from estrogen-treated mice. Therefore, we focused on IFNγ-inducible iNOS and its products.

Activation of splenocytes with T cell stimulation up-regulates the levels of nitric oxide in estrogen-treated mice

Activation of splenocytes with a T cell stimulant, Con-A, led to a marked increase in the levels of nitric oxide in cultures from estrogen-treated mice when compared with placebo-treated mice both at 24 h ($P < 0.05$) and 48 h ($P < 0.005$) of culture (Fig. 1). The level of nitric oxide in the supernatants of Con-A-activated splenocytes from estrogen-treated mice was higher at 48 h of culture, compared with 24 h of culture. It is noteworthy that nitric oxide was not detectable in the supernatants of unstimulated cells of even estrogen-treated mice, implying that T cell activation is necessary for nitric oxide release. Because nitric oxide is not detectable in unstimulated cultured (media only), in subsequent studies we focused on splenocytes that were activated with mitogens (Con-A or anti-CD3 antibodies).

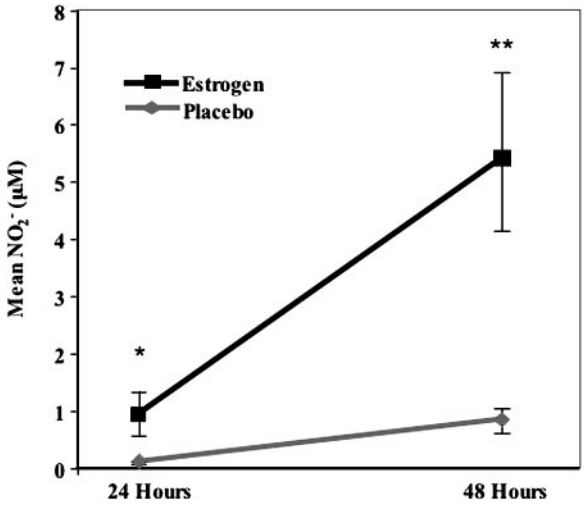

FIG. 1. Estrogen augments the levels of IFNγ-inducible nitric oxide. Splenocytes from estrogen- or placebo-treated orchiectomized mice were stimulated with Con-A (10 μg/ml) or left unstimulated in media, and the level of nitric oxide in the supernatants were determined by a Griess assay at 24 h (n = 14 mice per placebo or estrogen treatment; *, $P < 0.05$) and 48 h (n = 13 mice per placebo or estrogen-treatment; **, $P < 0.005$). In the supernatants of splenic lymphocytes left unstimulated (media), nitric oxide levels were not detectable. Data are presented as geometric means with SE values.

Anti-CD3-activated splenocytes from estrogen-treated mice also demonstrated increased levels of nitric oxide

Next, we used another T cell mitogen, anti-CD3 antibodies, to confirm that activation of splenocytes from estrogen-treated mice will induce higher levels of nitric oxide when compared with similar cultures from placebo-treated mice. As is apparent in Fig. 2, higher levels of nitric oxide were evident only in supernatants of anti-CD3 stimulated splenocytes from estrogen-treated mice, especially in cultures exposed to 1 μg/ml of anti-CD3 antibodies ($P < 0.0001$). It is also noteworthy that even 10 times lower dose of anti-CD3 antibodies (0.1 μg/ml) induced noticeable levels of nitric oxide in splenocytes cultures from estrogen-treated mice but not in similar cultures from placebo-treated mice ($P < 0.0001$). Nitric oxide was not detectable in cultures that were left unstimulated (media only) or exposed to control antibodies (data not shown), suggesting that activation is essential for the release of nitric oxide.

Estrogen treatment increases iNOS mRNA expression and iNOS protein in Con-A or anti-CD3-activated splenocytes

Because estrogen increased nitric oxide production in the supernatants of Con-A or anti-CD3-activated splenic lymphocytes from estrogen-treated mice, we next investigated whether this increase in nitric oxide is also reflected at the level of iNOS gene transcription. By real-time PCR analysis, Con-A-activated splenocytes from estrogen-treated mice demonstrated significantly increased levels of expression of the iNOS gene when compared with similar cultures from placebo-treated mice (Fig. 3A, placebo: n = 8, estrogen: n = 8; $P < 0.0001$). A representative gel of end products of the real-time PCR assay is shown at the top of this panel. Figure 3B shows a representative melt curve of iNOS expression to confirm a single amplification product in a real-time PCR. Furthermore, the PCR product determined by RT-PCR was

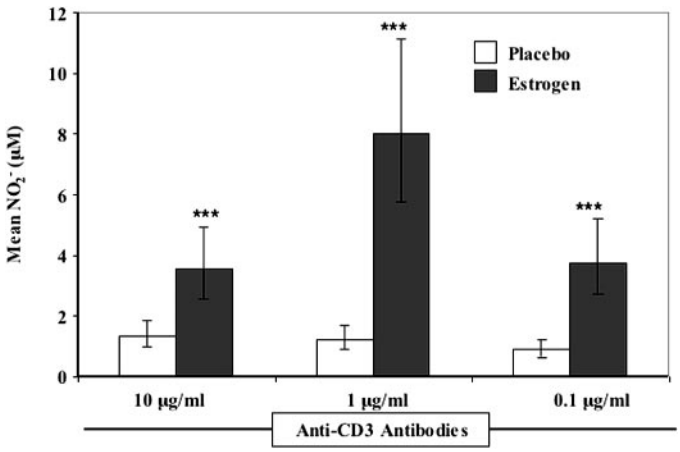

FIG. 2. Anti-CD3 activation of splenocytes from estrogen-treated mice induces increased levels of nitric oxide. Splenocytes from estrogen- and placebo-treated mice (n = 5 per treatment) were left unstimulated (media only) or stimulated with various doses of anti-CD3 antibodies (0.1, 1, or 10 μg/ml) for 48 h. Higher levels of nitric oxide were evident in the culture supernatants from estrogen-treated mice when compared with similar cultures from placebo-treated mice (***, $P < 0.0001$). Data are presented as geometric means with SEs.

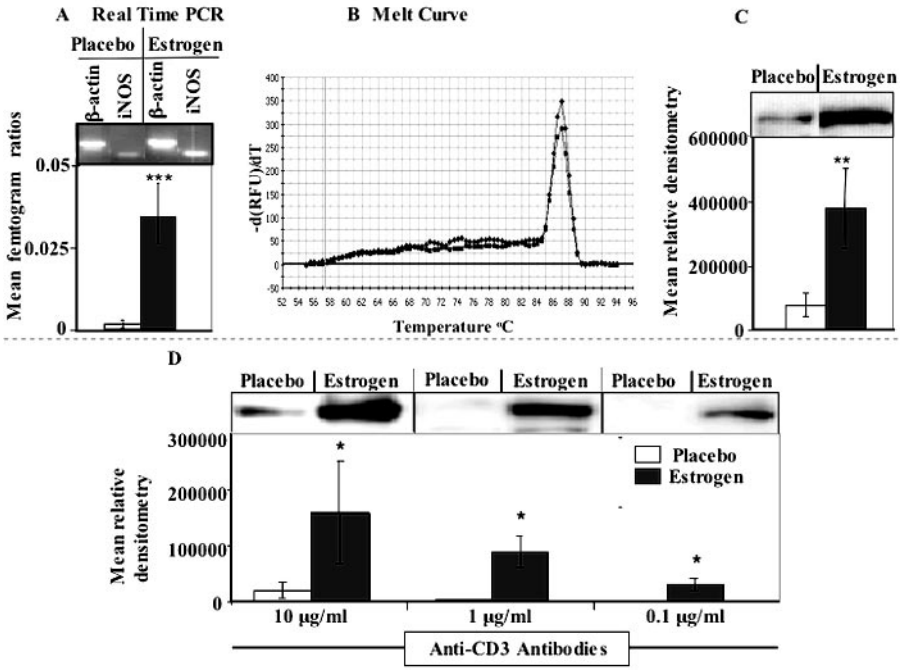

FIG. 3. Estrogen up-regulates iNOS mRNA and protein expression. Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml) for 24 h. The quantitative detection of iNOS mRNA expression was performed with a real-time PCR assay (A; placebo: n = 8 mice, estrogen: n = 8 mice; ***, $P < 0.0001$). A, Representative end products of real-time PCR. B, Representative melt curve for iNOS mRNA expression. C (*Top portion*), Representative experiment with Con-A (10 μg/ml)-stimulated cells from estrogen- or placebo-treated orchiectomized male mice. C (*Bottom portion*), Mean relative densitometry data for iNOS protein expression as detected with Western blot assay (placebo: n = 6 mice, estrogen: n = 6 mice; **, $P < 0.005$). D, Mean relative densitometry data for iNOS protein expression in splenocytes stimulated with 10 μg/ml of anti-CD3 (n = 3 mice per placebo or estrogen-treatment; *, $P < 0.05$), 1 μg/ml of anti-CD3 (n = 4 mice per placebo or estrogen treatment; *, $P<0.05$), or 0.1 μg/ml of anti-CD3 antibodies (n = 5 mice per placebo or estrogen-treatment; *, $P < 0.05$). D (*Top portion*), Representative gel. There was no detectable iNOS protein in unstimulated (media) splenocytes from estrogen or placebo-treated mice. All data are presented as geometric means with SE *bars*.

comparable with the PCR product of real-time PCR (data not shown).

Given that Con-A- or anti-CD3-activated splenocytes from estrogen-treated mice had increased levels of nitric oxide and increased expression of the iNOS gene, we next determined whether iNOS protein is also expressed at a higher level in these cells. Con-A-activated splenocytes from estrogen-treated mice had increased expression of iNOS protein, compared with similar cultures from placebo-treated mice (Fig. 3C, $P < 0.005$). Similarly, splenocytes from estrogen-treated mice, when exposed to various doses of anti-CD3 antibodies, also demonstrated increased levels of iNOS protein (Fig. 3D, $P < 0.05$). Importantly, exposure of splenocytes to a low dose of anti-CD3 (0.1 μg/ml) induced detectable expression of iNOS protein bands only in cells from estrogen-treated mice but not cells from placebo-treated mice (Fig. 3D, $P < 0.05$).

Activated splenocytes from estrogen-treated mice had increased nitric oxide-regulated COX-2 protein expression

Several studies have shown that nitric oxide up-regulates levels of COX-2 (38, 39). Given our observation of increased levels of nitric oxide in activated splenocytes from estrogen-treated mice, we then determined whether the expression of COX-2 protein levels in these cells was also altered (Fig. 4A). The relative expression of COX-2 protein was up-regulated in Con-A-activated splenocytes from estrogen-treated mice when compared with controls (Fig. 4B, $P < 0.05$). Similarly, splenocytes from estrogen-treated mice, when activated with anti-CD3, also had increased expression of COX-2, compared with controls (n = 4 per treatment, data not shown). There was no detectable COX-2 protein in unstimulated (media) splenocytes (data not shown).

Blocking CD28 and B7 molecular interactions inhibits the secretion of IFNγ and nitric oxide

The importance of molecular interactions of the costimulatory molecules CD28 on T cells with B7.1 (CD80)/B7.2 (CD86) on antigen-presenting cells in the context of IFNγ and nitric oxide was investigated. Remarkably, blocking the interaction of CD28 with B7 molecules with a CTLA-4Ig fusion protein dramatically decreased the level of IFNγ in the supernatants (Fig. 5A, $P < 0.0001$). The decrease in IFNγ was evident in both estrogen and placebo-treated samples, indicating the importance of this pathway and the interaction of T cells with antigen presenting cells. Blockade of CD28 and B7 interactions by CTLA-4Ig also significantly decreased the levels of nitric oxide in the supernatants of Con-A-activated splenocytes after 48 h of culture (Fig. 5B, $P < 0.05$). The decrease in IFNγ and nitric oxide levels due to coculturing with CTLA-4Ig was not due to cytotoxic effects of this fusion protein because the viability of cells that were cultured with and without CTLA-4Ig for 24 h of incubation were comparable (media: placebo = $9.8 \times 10^5 \pm 3.9 \times 10^5$, estrogen = $1.7 \times 10^6 \pm 9.3 \times 10^5$; Con-A: placebo = $5.9 \times 10^5 \pm 2.8 \times 10^5$,

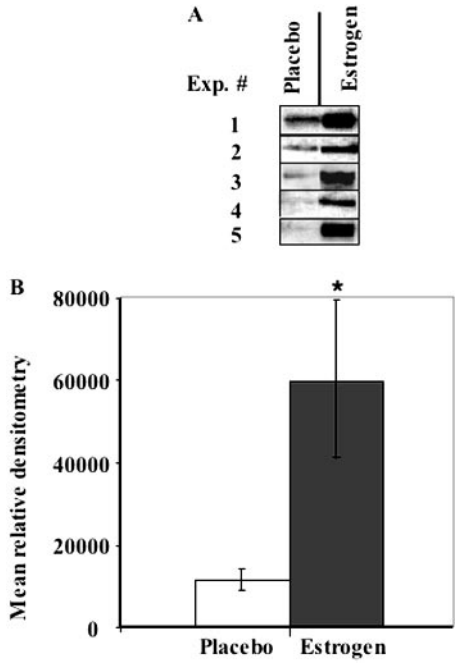

FIG. 4. COX-2 expression is increased in estrogen-treated splenic lymphocytes. Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml) and cultured for 48 h. A, COX-2 protein expression from five different experiments as determined by Western blot analysis. B, Relative densitometry data of COX-2 protein expression presented [geometric means with SE *bars* (placebo: n = 5 mice, estrogen: n = 5 mice; *, $P < 0.05$)] for Con-A-stimulated cells from estrogen- or placebo-treated mice.

estrogen = $6.2 \times 10^5 \pm 2.2 \times 10^5$; Con-A and CTLA-4Ig: placebo = $8.4 \times 10^5 \pm 1.9 \times 10^5$, estrogen = $6.9 \times 10^5 \pm 2.4 \times 10^5$). Stimulation of enriched T cells only or enriched macrophages only with Con-A did not induce detectable levels of nitric oxide, thereby indicating that physical interactions of T cells with macrophages are necessary for nitric oxide induction (data not shown).

Blockade of CD28 and B7 interactions also decreases COX-2 levels

Splenocytes from estrogen- and placebo-treated mice were cultured with Con-A in the presence or absence of CTLA-4Ig to determine the level of COX-2. As expected, Con-A activation of splenocytes from estrogen-treated mice, but not from placebo-treated mice, resulted in increased expression of COX-2 protein (Fig. 5C). Coculturing with CTLA-4Ig tended to decrease the level of expression of COX-2 (Fig. 5C; $P<0.05$).

The estrogen-induced increase in nitric oxide: role of IFNγ

To demonstrate the importance of IFNγ in the induction of iNOS and nitric oxide, IFN$\gamma^-/_$ mice were used. In none of the five IFN$\gamma^-/_$ mice that were treated with estrogen (n = 5) or placebo (n = 5), iNOS mRNA was detectable by real-time PCR in Con-A-activated splenocytes (data not shown). Furthermore, there were detectable levels of neither iNOS protein in Con-A-activated splenocytes from estrogen or placebo-treated IFN$\gamma^-/_$ mice (Fig. 6) nor nitric oxide in

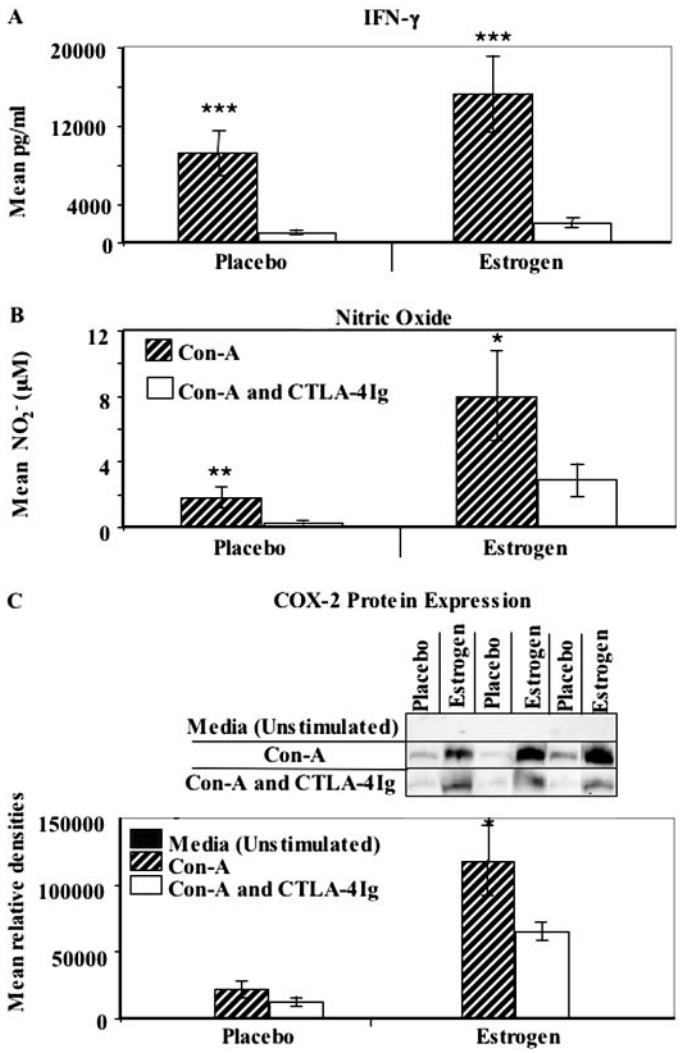

FIG. 5. Blocking CD28-B7 molecular interactions inhibits IFNγ protein, IFNγ-inducible nitric oxide, and COX-2 protein expression. Splenic lymphocytes isolated from estrogen- or placebo-treated gonadectomized male mice were cultured with Con-A (10 μg/ml) in the presence or absence of CTLA-4Ig fusion protein (15 μg/ml) for 24 or 48 h. The levels of IFNγ in the supernatants of Con-A-stimulated splenic lymphocytes were significantly decreased in cultures with CTLA-4Ig after 24 h of incubation. This was particularly evident in cultures from estrogen-treated mice (A; Con-A: placebo n = 15 mice, estrogen n = 20 mice; Con-A and CTLA-4Ig: placebo n = 15 mice, estrogen n = 15 mice; ***, $P < 0.0001$). Similarly, blocking CD28 and B7 interactions with CTLA-4Ig also abrogated nitric oxide release (B; Con-A: placebo n = 15 mice, estrogen n = 20 mice; Con-A and CTLA-4Ig: placebo n = 15 mice, estrogen n = 15 mice; *, $P < 0.05$, **, $P < 0.005$). IFNγ and nitric oxide were not detected in supernatants from unstimulated (media) cells. C, COX-2 expression is decreased by CTLA-4Ig inhibition. The *top portion* shows three representative experiments of COX-2 protein expression in cells stimulated with Con-A or Con-A and CTLA-4Ig or left unstimulated in media for 48 h. The *bottom portion* shows mean relative densitometry of COX-2 protein expression (placebo: n = 3 mice, estrogen: n = 3 mice; *, $P < 0.05$). All data are presented as geometric means with SE *bars*.

the supernatants of these cultures. Estrogen treatment by itself did not induce iNOS protein expression in unstimulated or Con-A-activated splenocytes. The importance of IFNγ in the conditional expression of iNOS was also con-

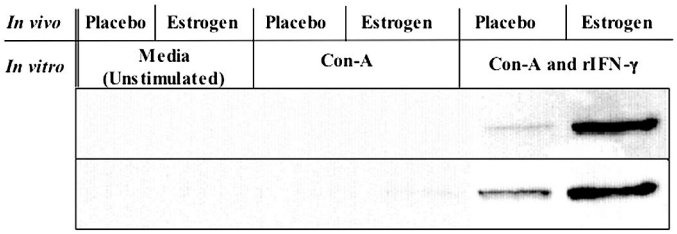

FIG. 6. Deliberate addition of IFNγ to Con-A-activated splenocytes increases iNOS protein expression in IFNγ ($^{-}/_{-}$) estrogen-treated mice. Splenic lymphocytes from estrogen- or placebo-treated IFNγ knockout mice on a C57BL/6 background were cultured with Con-A (10 μg/ml), Con-A (10 μg/ml) and rIFNγ (1000 pg/ml), or left unstimulated (media) for 24 h. The iNOS protein expression from estrogen or placebo-treated IFNγ knockout mice were detected with a Western blot assay. Two representative individual experiments are shown (n = 5 mice per placebo or estrogen treatment).

firmed after addition of recombinant IFNγ to Con-A-activated splenocytes. Con-A-activated splenocytes from estrogen-treated IFNγ$^{-/-}$ mice, when exposed to rIFNγ, demonstrated higher levels of iNOS, compared with similar culture from placebo-treated mice (Fig. 6). These data demonstrated that IFNγ promotes iNOS protein, and estrogen does not directly up-regulate iNOS in the absence of IFNγ.

Moreover, we also used estrogen (n = 10) and placebo (n = 10) treatment to IRF-1-deficient mice. The transcription factor, IRF-1, has been shown to be important for optimal secretion of IFNγ (40). These mice had very low levels of IFNγ (media: not detectable; Con-A: placebo: 210.97 ± 42.2 pg/ml, estrogen: 413.33 ± 108.3 pg/ml after 24 h of culture; media: not detectable; Con-A: placebo: 670.02 ± 116.6 pg/ml, estrogen: 1432.8 ± 240.5 pg/ml after 48 h of culture; n = 10 per treatment) and undetectable levels of nitric oxide or iNOS. Because these mice are deficient of IRF-1, it is not surprising that these mice had low levels of IFNγ. Estrogen tended to increase IFNγ in IRF-1 knockouts, although this trend was markedly weaker than that observed in wild-type mice. Overall, these data imply that estrogen-induced expression of iNOS is inducible by IFNγ. Because the above data implied the importance of IFNγ in induction of iNOS/nitric oxide (rather than direct effect of estrogen induction of iNOS/nitric oxide), we next used wild-type estrogen-treated mice, and splenocytes from these mice were stimulated with a lower dose of Con-A (1 μg/ml) in the presence or absence of rIFNγ (10 ng/ml). Interestingly, stimulation of splenocytes with a lower dose of Con-A (1 μg/ml) induced detectable levels of nitric oxide in the supernatants of estrogen-treated wild-type mice, when compared with placebo-treated mice (Fig. 7; $P < 0.05$). No nitric oxide was detectable in unstimulated (media) cultures from estrogen or placebo-treated mice. Next, deliberate addition of rIFNγ to aliquots of these cultures induced nitric oxide (Fig. 7), indicating that nitric oxide generation is responsive to and dependent on IFNγ.

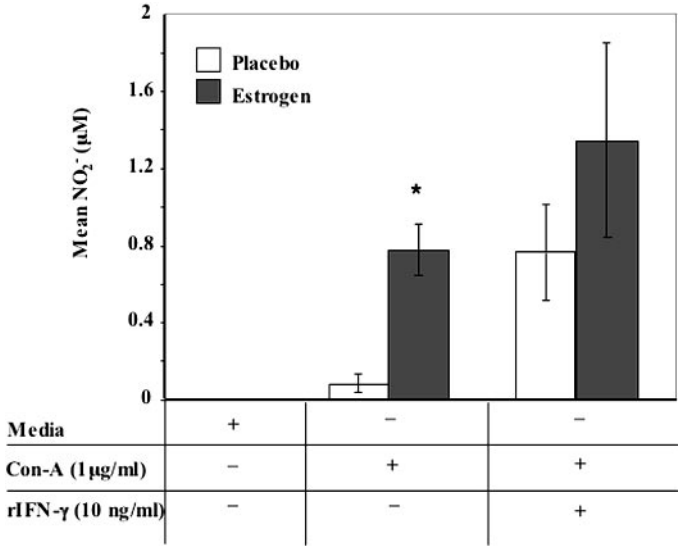

FIG. 7. Low doses of Con-A and rIFNγ increase nitric oxide. Splenic lymphocytes from estrogen- or placebo-treated mice were stimulated with low dose of Con-A (1 μg/ml) with or without rIFNγ (10 ng/ml) for 24 h. Levels of nitric oxide in the supernatants of Con-A and rIFNγ-stimulated splenic lymphocytes were significantly increased (placebo: n = 8 mice, estrogen: n = 8 mice; *, $P < 0.05$). Data are presented as geometric means with SE *bars*.

Discussion

It is now recognized that human exposure to estrogens occurs through multiple means including endogenous, pharmaceutical, and environmental sources (24, 41). Many studies have clearly shown that the immune system is highly responsive to estrogens (24, 25, 42–45). All the major cells of the immune system (T, B, macrophages, and dendritic cells) are potential targets for estrogens. Furthermore, estrogens have been implicated in many inflammatory and autoimmune conditions (24, 25, 29, 43). Therefore, it is not surprising that studies aimed at understanding the effects of estrogen on the immune system are recognized as being of increasing biomedical importance.

A significant novel observation in this study is that splenocytes from estrogen-treated mice, when exposed to T cell stimulants, release abundant amounts of nitric oxide in supernatants. The levels of nitric oxide in the supernatants of Con-A-activated splenocytes from estrogen-treated mice were particularly high after 48 h of culture. Estrogen appears to sensitize splenocytes to release nitric oxide because splenocytes from estrogen-treated mice, but not from placebo-treated mice, when exposed to even low doses of anti-CD3 antibodies (0.1 μg/ml) or Con-A (1 μg/ml), induced detectable levels of nitric oxide. The increase in nitric oxide in Con-A or anti-CD3-activated splenocytes from estrogen-treated mice is due to estrogen-induced up-regulation of iNOS mRNA (as demonstrated by real-time PCR) and a concomitant increase in iNOS protein (as demonstrated by Western blot assays). Our studies show that one indirect consequence of T cell activation is the release of nitric oxide.

Recent studies have also shown that nitric oxide can induce the expression of COX-2 or prostaglandin H synthase, an enzyme that catalyzes arachadonic acid for the formation of prostaglandin in tissues (46–48). For example, studies in mouse skin cells have shown that the suppression of iNOS by specific inhibitors also inhibited COX-2, whereas the addition of nitric oxide donors induced the expression of COX-2 (46). Similarly, studies in three colon cancer cell lines have shown that nitric oxide also up-regulated the expression of COX-2 (47). Furthermore, nitric oxide is necessary for main-

taining prolonged COX-2 mRNA expression in IFNγ and lipopolysaccharide-stimulated macrophage cell lines (15, 38) as well as peritoneal macrophages (16), and iNOS and nitric oxide up-regulated COX-2 in myocardial cells (48). To our knowledge, the relationship of nitric oxide and COX-2 in immune cells such as splenocytes has thus far not been investigated. Our studies clearly show that Con-A activation of splenocytes up-regulated not only nitric oxide but also COX-2 expression.

It is noteworthy that T cell stimulation was necessary to induce the release of nitric oxide because nitric oxide was not released in unstimulated cultures. It is also of note that interactions of activated T cells with macrophages are essential for enhanced release of nitric oxide. Blocking the molecular interactions of CD28 and B7 molecules with a CTLA-4Ig fusion protein markedly decreased not only IFNγ secretion but also iNOS mRNA and nitric oxide. Blocking CD28 and B7 interactions also significantly decreased COX-2 levels. We further show that the up-regulation of iNOS/nitric oxide by estrogen is mediated by IFNγ. This cytokine has been known to be up-regulated by estrogen (30–32, 49). The dependence of nitric oxide on IFNγ is also evident by the observations that estrogen administration to either IFNγ-deficient mice or IRF-1-deficient mice (which have very low levels of IFNγ) did not up-regulate the levels of nitric oxide or iNOS mRNA. Moreover, direct addition of recombinant IFNγ to Con-A-activated splenocytes from wild-type or IFNγ-deficient mice tended to up-regulate nitric oxide or iNOS protein, respectively.

These data show estrogen priming is necessary for IFNγ induction of iNOS protein (Fig. 6). Furthermore, the kinetics of induction of IFNγ and nitric oxide also suggests that estrogen induces IFNγ first, which is then followed by nitric oxide. For example, intracellular IFNγ is readily measured in freshly isolated splenocytes from estrogen-treated mice, and detectable levels of IFNγ in the supernatants of Con-A-activated splenocytes from estrogen-treated mice become evident as early as 6 h of culture (30). High levels of IFNγ in cultures of Con-A-activated splenocytes from estrogen-treated mice are evident at 24 h of culture (which continue to rise at 48 h of culture). IFNγ is thought to act on macrophages to up-regulate iNOS gene expression via Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Fig. 8). Nitric oxide or iNOS was not evident in freshly isolated splenocytes. At 24 h of culture, the levels of nitric oxide are detectable and high levels become evident only after 48 h of culture. The enhanced expression of iNOS mRNA and protein in the activated splenocytes does not appear to be due to increased levels of phosphorylated STAT-1. In a separate series of studies, we found that phos-

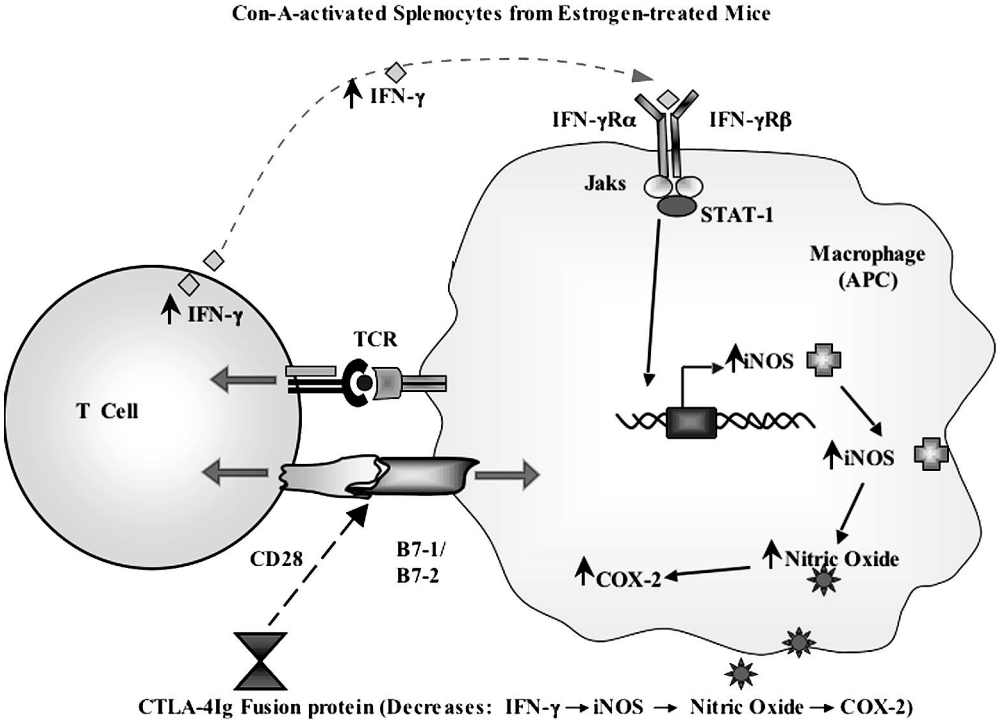

FIG. 8. The effect of estrogen-activated splenocytes IFNγ, IFNγ-inducible iNOS, nitric oxide, and nitric oxide-stimulated COX-2. Signaling through T cell receptor (TCR) and costimulatory molecules such as CD28 and B7.1/B7.2 is critical to the activation of lymphocytes. Con-A, a strong pan-T cell stimulant, results in increased levels of IFNγ protein in the supernatants of splenic lymphocytes from estrogen-treated mice. IFNγ has been shown to activate macrophages, increase iNOS activity, induce COX-2 via IFNγ-inducible nitric oxide, and confer immunity against intracellular pathogens. Macrophages are important target cells for IFNγ. IFNγ binds to specific IFNγ receptors, which often exist as heterodimers of IFNγ-Rα and IFNγ-Rβ chains. The binding of IFNγ to its receptors results in the activation of members of the Janus kinase family, Jak-1 by IFNγ-Rα and Jak-2 by IFNγ-Rβ, which in turn leads to activation of the STAT-1 pathway. Activation of cells by IFNγ results in activation of IFNγ-inducible genes, such as iNOS. Under the influence of iNOS, there is conversion of L-arginine to L-citrulline, resulting in the generation of nitric oxide. The increase in nitric oxide could also result in increased COX-2 protein expression. Blocking CD28-B7.1/B7.2 interactions of Con-A activated splenocytes with CTLA-4Ig results in decreased IFNγ secretion, which in turn decreases the activation of iNOS genes and nitric oxide release. Diminished nitric oxide levels also decrease COX-2 protein. APC, Antigen presenting cells.

phorylated STAT-1 (pSTAT-1) levels (as demonstrated with cytometric bead array and tyrosine or serine phosphorylation of STAT-1) in activated splenocytes from estrogen-treated mice were comparable with placebo-treated controls (Lengi, A. J., E. Karpuzoglu, R. A. Phillips, and S. Ansar Ahmed, submitted for publication).

Several *in vitro* studies have also shown the effect of estrogen on iNOS or nitric oxide in nonimmune cells such as isolated rat aortic rings, ovine coronary artery cells, rat myocytes, rat uterus and endothelial cells, and macrophage-like cell lines (J774, RAW-264.7) and peritoneal macrophages (50–55). Depending on the cell type and stimulus, direct exposure of these cells to estrogen either increased or suppressed iNOS or nitric oxide. For example, estrogen increased iNOS expression in RAW-267.7 cells and peritoneal macrophages (50, 55) or suppressed IL-1-induced iNOS expression in isolated rat aortic cells (52). Our studies differed from these studies in several important respects. First, to date no studies have been conducted on the *in vivo* effects of estrogen on IFNγ-inducible iNOS, nitric oxide, and COX-2 by immune cells. Second, our studies examined the consequence of T cell activation in splenocytes by studying the interactions of T cells and macrophages. The use of splenocytes simulates as natural a state as possible, in which lymphocytes (T, B) and antigen-presenting cells (*e.g.* macrophages, dendritic cells) physically coexist and interact to influence one another. This last point is highlighted by the fact that blocking the interactions of T and antigen-presenting cells through CTLA-4Ig markedly decreased IFNγ and nitric oxide. Therefore, the costimulation that occurs between antigen-presenting cells and other cell populations in whole cell culture is critical to production of detectable levels of nitric oxide and IFNγ. In this experimental setting, *in vivo* estrogen treatment did not directly induce iNOS or nitric oxide; rather these effects were mediated through induction of cytokines such as IFNγ as demonstrated using IFNγ knockout mice.

These studies provide a new understanding of estrogen modulation of the immune system via release of nitric oxide, especially considering that nitric oxide has potent effects on the immune system including defense against microbial agents, cellular proliferation, apoptosis, and cell survival (2, 56, 57). Furthermore, one of the downstream nitric oxide-induced events is the induction of COX-2 (17, 47, 48), which suggests that estrogen also modulates eicosanoid production that is involved in inflammation. Thus, estrogen promotion of inflammation and certain autoimmune diseases may be mediated by proinflammatory IFNγ-mediated events such as induction of iNOS and nitric oxide. Nitric oxide in turn can induce COX-2 (Fig. 8), which is largely responsible for prostaglandin E2 secretion (46). These are new observations that may provide insights into mechanisms of estrogen-induced promotion of inflammatory diseases and aid design of new therapeutic interventions to manipulate levels of nitric oxide (nonsteroidal antiinflammatory drugs).

Acknowledgments

We are grateful to Amgen (Thousand Oaks, CA; Dr. G. Sinaldi) for supplying the IRF-1($^{-/-}$) mice; Mary Nickle for animal care and maintenance of animals; Virginia-Maryland Regional College of Veterinary Medicine for the graduate stipend awarded to Ms. E. Karpuzoglu (Karpuzoglu-Sahin); and Ms. Carrie Hardy for her excellent technical assistance.

Received July 5, 2005. Accepted November 3, 2005.

Address all correspondence and requests for reprints to: Dr. S. Ansar Ahmed, Center for Molecular Medicine and Infectious Diseases, 1410 Prices Fork Road, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia 24060-0342. E-mail: ansrahmd@vt.edu.

This work was supported by grants from the National Institutes of Health (5RO1 AI51880-03), the U.S. Department of Agriculture (USDA)-Hatch, and the USDA-Animal Health and Disease programs (to S.A.A.).

References

1. **Bogdan C** 2001 Nitric oxide and the immune response. Nat Immunol 2:907–916
2. **MacMicking J, Xie QW, Nathan C** 1997 Nitric oxide and macrophage function. Annu Rev Immunol 15:323–350
3. **Wink DA, Hanbauer I, Grisham MB, Laval F, Nims RW, Laval J, Cook J, Pacelli R, Liebmann J, Krishna M, Ford PC, Mitchell JB** 1996 Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. Curr Top Cell Regul 34:159–187
4. **Lipton SA** 1996 Distinctive chemistries of no-related species. Neurochem Int 29:111–114
5. **Kolb H, Kolb-Bachofen V** 1998 Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? Immunol Today 19:556–561
6. **Ludwig ML, Marletta MA** 1999 A new decoration for nitric oxide synthase—a Zn(Cys)4 site. Structure Fold Des 7:R73–R79
7. **Liew FY, Cox FE** 1991 Nonspecific defence mechanism: the role of nitric oxide. Immunol Today 12:A17–A21
8. **Sicher SC, Vazquez MA, Lu CY** 1994 Inhibition of macrophage Ia expression by nitric oxide. J Immunol 153:1293–1300
9. **Bogdan C** 1997 Of microbes, macrophages and nitric oxide. Behring Inst Mitt:58–72
10. **Bogdan C, Rollinghoff M, Diefenbach A** 2000 The role of nitric oxide in innate immunity. Immunol Rev 173:17–26
11. **Fu JY, Masferrer JL, Seibert K, Raz A, Needleman P** 1990 The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. J Biol Chem 265:16737–16740
12. **Xie WL, Chipman JG, Robertson DL, Erikson RL, Simmons DL** 1991 Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA 88:2692–2696
13. **Hinz B, Brune K, Pahl A** 2000 Prostaglandin E(2) upregulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. Biochem Biophys Res Commun 272:744–748
14. **Hinz B, Brune K, Pahl A** 2000 Cyclooxygenase-2 expression in lipopolysaccharide-stimulated human monocytes is modulated by cyclic AMP, prostaglandin E(2), and nonsteroidal anti-inflammatory drugs. Biochem Biophys Res Commun 278:790–796
15. **Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P** 1993 Nitric oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci USA 90:7240–7244
16. **Habib A, Bernard C, Lebret M, Creminon C, Esposito B, Tedgui A, Maclouf J** 1997 Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. J Immunol 158:3845–3851
17. **Chun KS, Cha HH, Shin JW, Na HK, Park KK, Chung WY, Surh YJ** 2004 Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-κB. Carcinogenesis 25:445–454
18. **Vladutiu AO** 1995 Role of nitric oxide in autoimmunity. Clin Immunol Immunopathol 76:1–11
19. **Singh VK, Mehrotra S, Narayan P, Pandey CM, Agarwal SS** 2000 Modulation of autoimmune diseases by nitric oxide. Immunol Res 22:1–19
20. **Kroncke KD, Fehsel K, Kolb-Bachofen V** 1998 Inducible nitric oxide synthase in human diseases. Clin Exp Immunol 113:147–156
21. **Rosenfeld CR, Chen C, Roy T, Liu X** 2003 Estrogen selectively up-regulates eNOS and nNOS in reproductive arteries by transcriptional mechanisms. J Soc Gynecol Investig 10:205–215
22. **Grohe C, Kann S, Fink L, Djoufack PC, Paehr M, van Eickels M, Vetter H, Meyer R, Fink KB** 2004 17β-Estradiol regulates nNOS and eNOS activity in the hippocampus. Neuroreport 15:89–93
23. **Olsen NJ, Kovacs WJ** 1996 Gonadal steroids and immunity. Endocr Rev 17:369–384
24. **Ansar Ahmed S, Hissong BD, Verthelyi D, Donner K, Becker K, Karpuzoglu-Sahin E** 1999 Gender and risk of autoimmune diseases: possible role of estrogenic compounds. Environ Health Perspect 107(Suppl 5):681–686
25. **Ansar Ahmed S, Penhale WJ, Talal N** 1985 Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. Am J Pathol 121:531–551
26. **Homo-Delarche F, Fitzpatrick F, Christeff N, Nunez EA, Bach JF, Dardenne**

M 1991 Sex steroids, glucocorticoids, stress and autoimmunity. J Steroid Biochem Mol Biol 40:619–637
27. **Cutolo M, Seriolo B, Villaggio B, Pizzorni C, Craviotto C, Sulli A** 2002 Androgens and estrogens modulate the immune and inflammatory responses in rheumatoid arthritis. Ann NY Acad Sci 966:131–142
28. **Friedman A, Waksman Y** 1997 Sex hormones and autoimmunity. Isr J Med Sci 33:254–257
29. **Lahita RG** 1999 The role of sex hormones in systemic lupus erythematosus. Curr Opin Rheumatol 11:352–356
30. **Karpuzoglu-Sahin E, Hissong BD, Ansar Ahmed S** 2001 Interferon-γ levels are upregulated by 17-β-estradiol and diethylstilbestrol. J Reprod Immunol 52:113–127
31. **Karpuzoglu-Sahin E, Zhi-Jun Y, Lengi A, Sriranganathan N, Ansar Ahmed S** 2001 Effects of long-term estrogen treatment on IFNγ, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. Cytokine 14:208–217
32. **Fox HS, Bond BL, Parslow TG** 1991 Estrogen regulates the IFNγ promoter. J Immunol 146:4362–4367
33. **Ansar Ahmed S, Dauphinee MJ, Montoya AI, Talal N** 1989 Estrogen induces normal murine CD5+ B cells to produce autoantibodies. J Immunol 142:2647–2653
34. **Ansar Ahmed S, Verthelyi D** 1993 Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but not dihydrotestosterone. J Autoimmun 6:265–279
35. **Donner KJ, Becker KM, Hissong BD, Ansar Ahmed S** 1999 Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. Cytometry 35:80–90
36. **Ansar Ahmed S, Gogal Jr RM, Walsh JE** 1994 A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J Immunol Methods 170:211–224
37. **Calemine JB, Gogal Jr RM, Lengi A, Sponenberg P, Ansar Ahmed S** 2002 Immunomodulation by diethylstilbestrol is dose and gender related: effects on thymocyte apoptosis and mitogen-induced proliferation. Toxicology 178:101–118
38. **Perkins DJ, Kniss DA** 1999 Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. J Leukoc Biol 65:792–799
39. **Ishimura N, Bronk SF, Gores GJ** 2004 Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. Am J Physiol Gastrointest Liver Physiol 287:G88–G95
40. **Taniguchi T, Ogasawara K, Takaoka A, Tanaka N** 2001 IRF family of transcription factors as regulators of host defense. Annu Rev Immunol 19:623–655
41. **Ansar Ahmed S** 2000 The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology 150:191–206
42. **Puzanov IJ, Bennett M, Kumar V** 1996 IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells. J Immunol 157:4282–4285
43. **Ansar Ahmed S, Talal N** 1989 Sex hormones and autoimmune rheumatic disorders. Scand J Rheumatol 18:69–76
44. **Castagnetta L, Granata OM, Traina A, Cocciadiferro L, Saetta A, Stefano R, Cutolo M, Carruba G** 2002 A role for sex steroids in autoimmune diseases: a working hypothesis and supporting data. Ann NY Acad Sci 966:193–203
45. **Verthelyi D** 2001 Sex hormones as immunomodulators in health and disease. Int Immunopharmacol 1:983–993
46. **Steer SA, Corbett JA** 2003 The role and regulation of COX-2 during viral infection. Viral Immunol 16:447–460
47. **Liu Q, Chan ST, Mahendran R** 2003 Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. Carcinogenesis 24:637–642
48. **Xuan YT, Guo Y, Zhu Y, Han H, Langenbach R, Dawn B, Bolli R** 2003 Mechanism of cyclooxygenase-2 upregulation in late preconditioning. J Mol Cell Cardiol 35:525–537
49. **Karpuzoglu-Sahin E, Gogal Jr RM, Hardy C, Sponenberg P, Ahmed SA** 2005 Short-term administration of 17-β estradiol to outbred male CD-1 mice induces changes in the immune system, but not in reproductive organs. Immunol Invest 34:1–26
50. **Azenabor AA, Yang S, Job G, Adedokun OO** 2004 Expression of iNOS gene in macrophages stimulated with 17β-estradiol is regulated by free intracellular Ca2+. Biochem Cell Biol 82:381–390
51. **Tamura K, Yamaguchi K, Kogo H** 2000 17β-estradiol inhibits ovariectomy-induced expression of inducible nitric oxide synthase in rat aorta *in vivo*. Life Sci 66:PL259–PL264
52. **Kauser K, Sonnenberg D, Diel P, Rubanyi GM** 1998 Effect of 17β-oestradiol on cytokine-induced nitric oxide production in rat isolated aorta. Br J Pharmacol 123:1089–1096
53. **Baker AE, Brautigam VM, Watters JJ** 2004 Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor β. Endocrinology 145:5021–5032
54. **Nuedling S, Karas RH, Mendelsohn ME, Katzenellenbogen JA, Katzenellenbogen BS, Meyer R, Vetter H, Grohe C** 2001 Activation of estrogen receptor β is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes. FEBS Lett 502:103–108
55. **Hong M, Zhu Q** 2004 Macrophages are activated by 17β-estradiol: possible permission role in endometriosis. Exp Toxicol Pathol 55:385–391
56. **Kroncke KD, Fehsel K, Suschek C, Kolb-Bachofen V** 2001 Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. Int Immunopharmacol 1:1407–1420
57. **Dimmeler S, Zeiher AM** 1997 Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. Nitric Oxide 1:275–281

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**