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Estradiol Coupling to Human Monocyte Nitric Oxide Release Is Dependent on Intracellular Calcium Transients: Evidence for an Estrogen Surface Receptor

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We tested the hypothesis that estrogen acutely stimulates constitutive NO synthase (cNOS) activity in human peripheral monocytes by acting on an estrogen surface receptor. NO release was measured in real time with an amperometric probe. 17β-estradiol exposure to monocytes stimulated NO release within seconds in a concentration-dependent manner, whereas 17α-estradiol had no effect. 17β-estradiol conjugated to BSA (E₂-BSA) also stimulated NO release, suggesting mediation by a membrane surface receptor. Tamoxifen, an estrogen receptor inhibitor, antagonized the action of both 17β-estradiol and E₂-BSA, whereas ICI 182,780, a selective inhibitor of the nuclear estrogen receptor, had no effect. We further showed, using a dual emission microfluorometry in a calcium-free medium, that the 17β-estradiol-stimulated release of monocyte NO was dependent on the initial stimulation of intracellular calcium transients in a tamoxifen-sensitive process. Leeching out the intracellular calcium stores abolished the effect of 17β-estradiol on NO release. RT-PCR analysis of RNA obtained from the cells revealed a strong estrogen receptor-α amplification signal and a weak β signal. Taken together, a physiological dose of estrogen acutely stimulates NO release from human monocytes via the activation of an estrogen surface receptor that is coupled to increases in intracellular calcium. The Journal of Immunology, 1999, 163: 3758–3763.

In examining the estrogen-immunocyte literature, it is becoming apparent that this hormonal signaling molecule exerts a cellular immunosuppressive action (1). Functionally, estrogen exerts a suppressive effect on neutrophil granulocytes and monocyte-directed migration in response, in a tamoxifen-sensitive manner, to various chemotactic agents (1–10). Estrogen also diminishes immunocyte phagocytosis in a tamoxifen-sensitive manner (11–13). As expected, this action also can be initiated by inhibiting the adhesion potential of the immunocytes and endothelial lining of the vasculature (3, 14–19). The finding of estrogen receptors on immunocytes complements this literature. Peripheral blood monocytes from patients with systemic lupus erythematosus and healthy controls were found to express estrogen receptor mRNA as well as estrogen receptor binding sites (20–22). This estrogen binding site has also been found by others to be present on monocytes (23–25).

Given these recent findings, we examined human peripheral monocytes to determine whether they exhibited an estrogen surface receptor (ESR)3 that when stimulated released constitutive NO synthase (cNOS)-derived NO in a calcium- and tamoxifen-sensitive manner. We demonstrate for the first time that human peripheral monocytes exhibit an ESR whose NO release is calcium-dependent, tamoxifen-sensitive, and ICI 182,780-insensitive. Thus, the NO produced as a result of estrogen stimulation may, in part, be the process whereby this hormone causes cellular immunosuppression as well as other immune actions.

Materials and Methods

Direct measurement of NO release

Human peripheral monocytes were obtained from the Long Island Blood Services (Melville, NY). The cells were isolated via the Accurate (Westbury, NY) monocyte kit and washed as previously described in great detail (26–28).

NO release from the incubated monocytes (10⁷ cells/chamber) was measured directly using an NO-specific amperometric probe (World Precision Instruments, Sarasota, FL) as described by Stefano and colleagues (26, 29). Briefly, the cells were placed in a superfusion chamber in 2 ml PBS. A micromanipulator (Zeiss-Eppendorff, Oberkochen, Germany) attached to the stage of an inverted microscope (Nikon Diaphot, Melville, NY) was employed to position the amperometric probe 15 μm above the cell surface. The system was calibrated daily using different concentrations of the nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (Sigma, St. Louis, MO). S-acetyl-ret-penicillamine (SAP) was used as a negative control to generate a standard curve. Baseline levels of NO release were determined by evaluation of NO release in PBS. Cells were stimulated with the respective ligand, and the concentration of NO gas in solution was measured.

3 Abbreviations used in this paper: ESR, estrogen surface receptor; cNOS, constitutive NO synthase; eNOS, endothelial cNOS; L-NAME, Nω-nitro-l-arginine methyl ester; E₂-BSA, 17β-estradiol conjugated to BSA; ER, estrogen receptor.
in real time with the DUO 18 computer data acquisition system (World Precision Instruments). The amperometric probe was allowed to equilibrate for at least 12 h in PBS before being transferred to the superfusion chamber containing the cells, and manipulation of the cells was performed only with glass instruments. Each experiment was repeated four times. Each experiment was simultaneously performed with a control from the same tissue source (vehicle alone) to exclude experimental drift in NO release unrelated to the study drugs.

To evaluate NO release, the cells were exposed to a concentration gradient of various ligands. If an antagonist or a NOS inhibitor was used, it was administered 2 min before that of the various estrogen ligands. The NOS inhibitor, N-nitro-l-arginyl methyl ester (l-NAME) was used in these studies.

Data were evaluated by Student’s t test. Data acquisition was by the computer-interfaced DUO-18 software (World Precision Instruments). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, San Rafael, CA) for graphic representation and evaluation. Data gatherers were unaware of the experimental treatments.

Ligands
The monocytes were stimulated with various concentrations of 17β-estradiol (10^{-11} \text{ to } 10^{-7} \text{ M}) or 17β-estradiol conjugated to BSA (E2-BSA) (10^{-13} \text{ to } 10^{-7} \text{ M} of 17β-estradiol). They were also stimulated with 17α-estradiol (10^{-8} \text{ M}) (n = 4), tamoxifen (10^{-8} \text{ M}), or ICI 182,780 (10^{-9} \text{ to } 10^{-5} \text{ M}), estrogen receptor antagonists (n = 4), or tamoxifen (10^{-9} \text{ M}) plus 17β-estradiol (10^{-5} \text{ M}) (n = 4), or tamoxifen (10^{-9} \text{ M}) plus E2-BSA (10^{-13} \text{ to } 10^{-7} \text{ M} of 17β-estradiol), E2-BSA (10^{-9} \text{ M}) (n = 4) or ICI 182,780 (10^{-9} \text{ M}) plus E2-BSA (10^{-9} \text{ M}) (n = 4). Tamoxifen and ICI 182,780 were added to the milieu 2 min before 17β-estradiol or E2-BSA. To determine that there was no dissociation between 17β-estradiol and BSA, an RIA kit optimized for the direct quantitative determination of very low concentrations of 17β-estradiol (ICN Pharmaceuticals, Costa Mesa, CA) was used. 17β-estradiol was measured in the cytosolic fraction of monocytes (10^7 cells/ml) treated with 10^{-9} \text{ to } 10^{-5} \text{ M} E2-BSA. After washing, the cells were put through a freeze-thawing cycle (three times), cellular debris was pelleted (12,000 rpm 15 min), and the cytosolic material evaluated for free estradiol. Estradiol was not detected in the cytosol. The assay sensitivity was 0.2 pg/tube.

Intracellular calcium levels monitored by calcium imaging
Monocytes were allowed to adhere in chamber slides (Nunc, Naperville, IL) using PBS (30) supplemented with 10% FCS at 37°C in a 5% CO_2 atmosphere (31). To promote rapid adherence, the chambers were rinsed with 1% BSA. The cells were left under these conditions for 2 h before experimentation commenced. We estimate that at the end of this period we lost ~50% of the cells due to the introduction of DMSO, which causes the cells to break their adherence. The cells were diluted, equaling ~100 monocytes per chamber slide chamber. Intracellular calcium levels were measured by dual emission microfluorometry using the fluorescent dye fura 2-AM. Images were acquired in real time with the DUO 18 computer data acquisition system (World Precision Instruments). The amperometric probe was allowed to equilibrate in PBS without calcium/magnesium. When the wavelength used in the denominator of the ratio. Images were acquired every 0.4 s with an image-processing system COMPIX C-640 SIMCA (Compix, Mars, PA) and an inverted Nikon microscope. Experiments were conducted at room temperature in PBS without calcium/magnesium. When the respective receptor antagonists were used, they were administered 2 min before the respective agonist. The antagonists did not stimulate [Ca^{2+}]_i at the test concentrations. Furthermore, under control conditions, the cells exhibited a low level [Ca^{2+}]_i in the 0.2–2.1 nM range.

A two-way ANOVA was used for statistical analysis on the peak [Ca]_i time, 7 s after agonist exposure to the cells. Each experiment was simultaneously performed with up to eight cells. The mean value was combined

with the mean value taken from four other replicates. The SEM represents the variation of the mean of the means.

All drugs were purchased from Sigma, except ICI 182,780 that was kindly provided by Zeneca Pharmaceuticals (Costa Mesa, CA).

RT-PCR analysis
Human monocytes were obtained from the Pasteur Institute (Lille, France). The cells were isolated using Magnetic Cell Sorting MicroBeads (MACS) as described by the manufacturer (Miltenyi Biotec, Heidelberg, Germany). CD14 MicroBeads were used to enrich monocytes/macrophages from peripheral blood. After MACS separation, monocytes purity was >99%.

Total RNA from monocytes was extracted using Trizol (Life Technologies/BRL, Strasbourg, France). A total of 3 μg RNA was reverse transcribed into cDNA using random hexamers and Moloney murine leukemia virus RT (Life Technologies/BRL), as previously described (34). One-sixth of the first strand synthesis reaction was amplified for 40 cycles using 1U Taq polymerase and 100 pmol of each forward and reverse primer. The cycling parameters were 94°C for 90 s, 65°C for 90 s, and 72°C for 120 s. Negative control RT-PCR reactions were performed by omitting reverse transcriptase and RNA from the reaction mixture. In both pairs, the priming sites were separated by an intron, thus preventing amplification of any contaminating genomic DNA (data not shown). For the ER α amplification, the primer pair (25 mer) was designed to amplify a 281-bp cDNA fragment (residues 83–177, according to Ref. (35)). For the ER β amplification, the primer pair (25 mer) generated a 265-bp cDNA product (residues 381–469, according to Ref. (36)). As an internal control, GAPDH mRNA was also amplified using a primer pair (37) design to amplify a 470-bp cDNA (residues 36–192, according to Ref. (38). The PCR products were cloned-into TA cloning vector systems (Stratagene, La Jolla, CA) and sequenced to verify the specificity of the amplification.

Results
Direct evaluation of NO release
NO release was measured in real time using a NO-specific amperometric probe following stimulation of the monocytes either with 17β-estradiol or E2-BSA (Fig. 1, inset). Normally, monocytes release low levels of cNOS-derived NO (0–1 nM range) (26).

17β-estradiol stimulates NO release. In real time, 17β-estradiol (10^{-9} \text{ M}) stimulated the release of NO (13 nM peak value) over a 10-min time period (Fig. 1 and inset). This 17β-estradiol-stimulated process can be inhibited by first exposing the tissue to tamoxifen, an estrogen receptor blocker (Fig. 2). Increasing concentrations of 17β-estradiol (10^{-11} \text{ to } 10^{-7} \text{ M}) resulted in a dose-dependent increase in NO release with a maximal effect observed at 10^{-9} \text{ M} (Fig. 1). This increase peaked before the 2-min

![FIGURE 1. Dose-dependent release of NO after in vitro stimulation of monocytes (10^7 cells/ml) by 17β-estradiol and E2-BSA. The graphed values represent peak values obtained 2 min post drug exposure. The cells are exposed to the agents for the entire observation period. Each experiment was repeated four times and the resulting mean value (=SEM) graphed. Inset, Real time representation of 17β-estradiol (10^{-9} \text{ M})-stimulated NO from peripheral monocytes.](http://www.jimmunol.org)
observation period upon 17β-estradiol exposure to the monocytes (Figs. 1 and 2). Addition of 10^{-11} M 17β-estradiol failed to stimulate a significant increase in NO release.

**The action of estradiol is steroid-specific.** 17α-estradiol (10^{-9} M) did not stimulate any release from the monocytes (data not shown). Tamoxifen (10^{-9} M), an estradiol receptor inhibitor, significantly diminished (p < 0.005) 17β-estradiol-stimulated endothelial NO release (Fig. 2).

**17β-estradiol acts as a surface receptor.** 17β-estradiol appears to stimulate NO release by acting on the membrane surface, not on an intracellular receptor. E2-BSA (10^{-9} M), which does not penetrate the cellular membrane due to its size, also stimulates monocyte NO release within 2 min of its application in a tamoxifen-sensitive process (Figs. 1 and 2). As with 17β-estradiol, the E2-BSA-stimulated NO release is dose-dependent (Fig. 1). Stimulation of either tissue with 10^{-11} M E2-BSA failed to stimulate a significant increase in NO release. The median effective concentration (EC_{50}) for E2-BSA-stimulated NO release is ~5 x 10^{-10} M. It appears in these cells that E2-BSA is as potent as 17β-estradiol in stimulating NO release (Figs. 1 and 2). In this regard, it is important to note that testosterone and progesterone were without effect (Fig. 1).

To further establish that this indeed is the case, l-NAME (100 μM), a NOS inhibitor, blocked the NO-stimulating activities of 17β-estradiol (Table I). To further establish the specificity of this phenomenon, we attempted to inhibit the estrogen-stimulated NO release using ICI 182,780, a nuclear estrogen receptor antagonist.

**Table I. l-NAME inhibits estrogen-stimulated NO release from monocytes**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Estrogen Agonist (10^{-9} M)</th>
<th>l-NAME (μM)</th>
<th>NO Level (nM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td>17β-estradiol</td>
<td>NO</td>
<td>11.3 ± 1.1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>17β-estradiol</td>
<td>100</td>
<td>1.8 ± 0.6</td>
</tr>
</tbody>
</table>

*Each experiment was replicated four times. p < 0.01 in comparing NO release in presence of l-NAME to that in the appropriate tissues without it.*

**Direct evaluation of intracellular calcium release**

In a few recent reports from our laboratory, we demonstrated that morphine, anandamide, and estrogen stimulated cNOS-derived NO release from endothelial cells, which was dependent on intracellular calcium transients. In this regard, we performed the same experiments with the monocytes in a calcium-free medium. In real time, 17β-estradiol (10^{-9} M) stimulated a rapid intracellular calcium transient within 6 s of its exposure to these cells (Fig. 4). This event could be blocked by prior tamoxifen (10^{-9} M) exposure but not by ICI 182,780 (Fig. 4). The EC_{50} value for 17β-estradiol is 6 x 10^{-10} M, and the IC_{50} value for tamoxifen is 9 x 10^{-10} M.

In comparing the sequence of events concerning 17β-estradiol’s action in stimulating both [Ca^2+] and NO production in the monocytes, we found that the first event precedes the second by 40 s (from four experiments; Fig. 5). Given the fact that cNOS requires Ca^{2+}, we determined whether these events were linked. Over a 3-h period, we changed the Ca^{2+}-free incubation medium of the cells five times in an attempt to leach out the intracellular calcium stores (31). After the 3-h incubation, 17β-estradiol (10^{-9} M) increased [Ca], to 2.2 ± 0.7 nM ± SEM (Fig. 4f). This level is substantially lower than those previously described under non-leaching conditions (Fig. 4a). Furthermore, NO release was barely above background in the Ca^{2+}-leached monocytes following 17β-estradiol (NO 2.3 ± 0.8 nM, compared with a peak value of 13.6 ± 2.4) exposure, strongly suggesting that intracellular Ca^{2+} levels regulate cNOS activity and that Ca^{2+} originates from the estrogen receptor’s coupling to [Ca].

**Estrogen receptor α and β gene expression in human monocytes**

To determine which estrogen receptor genes were expressed in monocytes, we performed RT-PCR analysis of RNA extracted from three independent blood samples. The presence of GAPDH transcripts was also assessed as a control. As shown in Fig. 6, single bands of 281 bp (ER α), 265 bp (ER β), and 470 bp...
ifen was administered 2 min before 17\( \beta \)-estradiol, which was identical and were identified as the sequence of ER human breast cell lines (MCF7 and MDA MB231) and monocytes. The nature of the PCR products was further assessed after subcloning and both genes could be expressed within the same cell type. The natrium of the calcium transients and NO release from human monocytes. E2-BSA applied. 2 min; vertical line in horizontal bar indicates point at which the drug is maintained in a calcium-free medium. Vertical bar, 26 nM; horizontal bar, 2 min; vertical line in horizontal bar indicates point at which the drug is applied.

FIGURE 4. Real time representation of 17\( \beta \)-estradiol (10\(^{-9}\) M)-stimulated [Ca\(^{2+}\)] from cultured peripheral monocytes. a, 17\( \beta \)-estradiol-stimulated [Ca\(^{2+}\)]; b, Lack of ICI-182,780 (10\(^{-8}\) M) antagonism of 17\( \beta \)-estradiol-stimulated [Ca\(^{2+}\)]; ICI-182,780 was administered 2 min before 17\( \beta \)-estradiol. c, Tamoxifen (10\(^{-7}\) M) antagonizes 17\( \beta \)-estradiol-stimulated [Ca\(^{2+}\)]; Tamoxifen was administered 2 min before 17\( \beta \)-estradiol. d, Progesterone (10\(^{-9}\) M) and testosterone (e) (10\(^{-9}\) M) do not stimulate [Ca\(^{2+}\)]; f, 17\( \beta \)-estradiol (10\(^{-9}\) M) does not stimulate [Ca\(^{2+}\)] from cultured peripheral monocytes maintained in a calcium-free medium. Vertical bar, 26 nM; horizontal bar, 2 min; vertical line in horizontal bar indicates point at which the drug is applied.

(GAPDH) were detected. The sizes of the PCR amplification products corresponded to the sizes predicted from the genomic sequences. ER\( \alpha \) amplification signal was observed, as was ER\( \beta \) in monocytes; however, the ER\( \alpha \) material exhibited a higher density reading (+69% over ER\( \beta \), determined by Gel Pro Density Analysis (Media Cybernetics, Silver Spring, MD)). It was apparent that both genes could be expressed within the same cell type. The nature of the PCR products was further assessed after subcloning and sequencing of the specific bands. cDNA sequences obtained for human breast cell lines (MCF7 and MDA MB231) and monocytes were identical and were identified as the sequence of ER\( \alpha \) and ER\( \beta \) receptor.

Discussion

The present study demonstrates that at physiological concentrations, 17\( \beta \)-estradiol rapidly stimulates NO release from human monocytes. This process appears to be mediated by a specific estradiol receptor, as noted by its antagonism by tamoxifen and the lack of 17\( \alpha \)-estradiol action. The fact that E\(_2\)-BSA, an impermeable cell membrane estradiol analogue, also stimulates NO release and ICI 182,780 cannot block its action, indicates that this receptor is located on the surface of the cNOS-containing cells. Furthermore, 17\( \beta \)-estradiol and E\(_2\)-BSA-stimulated NO release is inhibited by l-NAME, a NOS inhibitor, indicating that the effect of the agonists on NO release is mediated by coupling the membrane estrogen receptor to cNOS. Additionally, the estrogen-stimulated release of monocyte cNOS-derived NO is dependent on the initial stimulation of intracellular calcium transients, supporting the cNOS activation by estrogen, since it is calcium-dependent. Taken together, the study not only demonstrates a monocyte ER but its ability to couple the estrogen receptor to cNOS activation via the stimulation of intracellular calcium transient.

FIGURE 5. Sequence of events regarding the real-time E\(_2\)-BSA-stimulated calcium transients and NO release from human monocytes. E\(_2\)-BSA (10\(^{-9}\) M) addition to the medium results in immediate calcium transients (application at base of the steep increase) that is then followed by a progressive decrease lasting about 2 min. Approximately 40 s later, an increase in NO release (peak level 13 nM for E\(_2\)-BSA) occurs, which lasts for 10 min. The raw data were graphed and connected with spline curves so that the precise times could be better visualized.

FIGURE 6. Estrogen receptor gene expression in human monocytes. RT-PCR was performed using either no RNA (negative control, lane 1) or 3 \( \mu \)g of human breast cancer cell lines (positive control, lane 2) monocytes (lane 3). MCF7 and MDA MB231 cell lines were respectively used for ER\( \alpha \) and \( \beta \) amplification control. PCR amplification was also performed using a primer pair specific for human GAPDH as a cDNA control. The PCR products (one-fifth of the ER\( \alpha \) and \( \beta \) reaction, one-tenth for GAPDH amplification) were separated by a 2% agarose gel electrophoresis and revealed by ethidium bromide and photographed under UV light. DNA markers (1 kb ladder) were run in parallel. The sizes of the amplified products are indicated in bp on the right.
nM range. Therefore, we surmise that the use of micromolar concentrations of ICI-182,780 is abnormally high for the receptor $K_3$ and may have nonspecific actions, whereas at $10^{-5}$ M, tamoxifen exerts its action and is in the $K_3$ ER receptor range. Supporting this hypothesis are unpublished data from our laboratory, demonstrating that the use of $10^{-5}$ M tamoxifen or $10^{-5}$ M ICI-182,780 reduced by 23% and 20%, respectively, morphine-stimulated NO release from human endothelial cells (29). This indicates that at high doses these drugs become less selective. Taking this into account, ER $\alpha$ and ER $\beta$ cDNA-expressing cells presented both membrane and nuclear estrogen receptors (49). Here, we also note via RT-PCR that monocytes express both ER $\alpha$ and $\beta$ materials with an apparent higher level of the former. However, human granulocytes only express ER $\alpha$ and exhibit both NO release and calcium transients in response to 17$\beta$-estradiol and E$_2$-BSA in a tamoxifen-sensitive and ICI-182,780-insensitive process (G. B. Stefano, unpublished observations), suggesting that ER $\alpha$ is the ESR mediating these phenomena in monocytes.

Furthermore, tamoxifen, although it is often considered as an antagonist of the nuclear estrogen receptor, is also able to antagonize the effect of estradiol on its membrane receptor as shown by our group (50) and by others (51, 52). In this regard, Benten et al. (53) found in splenic T cells isolated from mice that 17$\beta$-estradiol stimulated $[Ca^{2+}]$, in a tamoxifen-insensitive manner. This finding supports our present observations regarding an estrogen surface receptor, since the authors reached the same conclusion using E2-BSA as well. The lack of tamoxifen-sensitivity in their studies and its efficacy in ours may simply be due to species and/or cell variations.

The structure of the membrane ERs is unknown, but since single cDNA and RNA are capable of producing both membrane and nuclear receptors, the membrane ER must be very similar to the classical nuclear ER (49). Posttranslational modification of some ER protein must occur to ensure targeting to the membrane, a phenomenon that may also explain the ICI 182,780 insensitivity at low concentrations. Interestingly, Razandi et al. (49) have shown that membrane ER were G protein linked. Thus, one possible mechanism for the acute estradiol-induced NO release in monocytes could be that 17$\beta$-estradiol activates the G protein pathway leading to intracellular calcium stores mobilization and then to cNOS activation and NO release.

In another recent study, we demonstrated that 17$\beta$-estradiol stimulates NO release from human internal thoracic artery fragments and from cultured arterial endothelial cells by acting on an ESR, given that E$_2$-BSA was as potent as 17$\beta$-estradiol in stimulating NO release by both types of endothelial cells (41). In this study, ICI-182,780 did not block the cNOS stimulatory action of estradiol as did tamoxifen. Estradiol short-term stimulating action, i.e., NO release, via a specific ESR on arterial endothelial cells and monocytes is supported by other recent studies that demonstrate a vasodilatory role for estrogen involving NO occurs quickly (42, 54–56). Beside its short-term stimulating action, estradiol can have a long-term action via a nuclear receptor on NO release from endothelia (39, 57). Estradiol can indeed increase ecNOS expression within 8 h after its application on human vein endothelial cells, via a nuclear receptor-mediated system, and this action can be inhibited by the selective nuclear estrogen receptor antagonist, ICI-182,780 (15). The presence of two left-half palindromic sites of an estrogen receptor-binding element on the human ecNOS gene supports a potential receptor-mediated effect of estrogen on gene expression (57).

In regard to monocytes, estrogen down-regulates immunocyte functions, i.e., chemotaxis and phagocytosis (1–13). This action can also be initiated by inhibiting the adhesion potential of the immunocytes and endothelial lining of the vasculature (3, 14–16). Furthermore, in accordance with our present observations, monocytes express estrogen receptor mRNA as well as an estrogen receptor binding site (20–25).

Taken together, estrogen’s ability to stimulate cNOS-derived NO is significant since NO is also considered as an important inhibitory agent that diminishes immunocyte adhesion and the vascular endothelium’s capability to adhere immunocytes as well as down-regulating various immunocytes both before and after proinflammatory events (58, 59). In this regard, estrogen is acting in parallel with endogenous morphine and the endocannabinoid anandamide (58, 59).

At first glance, it may appear that we have a redundant immunovascular down-regulating process. However, we believe that each signaling system performs this common function, i.e., cNOS-derived NO release, under different circumstances. Morphine, given its long latency before increases in its levels are detected, arises after trauma/inflammation to down-regulate these processes in neural and immune tissues (59–61). Anandamide, by being part of the always present arachidonate and eicosanoid signaling processes, serves to maintain tonal NO in vascular tissues (62). We surmise that estrogen, since testosterone or progesterone don’t exert this NO generating action, provides an extra-degree of immunocyte and vascular down-regulation in females. This is most probably due to both the immune and vascular trauma associated with cyclic reproduction activities, i.e., endometrial buildup, when a high degree of vascular and immune activities are occurring. Given the high degree of proliferative growth capacity during estrogen peak levels in this cycle, NO may function to enhance down-regulation of the immune system to allow for these changes. In this regard, it is not difficult to understand the reports documenting various cancers with blocking estrogen actions and, conversely, reports documenting its anti-cancer protective actions (63).

Our work establishes that a physiological dose of estrogen acutely stimulates NO release from human monocytes via the activation of an ESR and increases intracellular $Ca^{2+}$ transients. This finding promises to open up new areas of investigation concerning estrogen-associated biomedical phenomena.

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