Activation of the Annexin A1 Pathway Underlies the Protective Effects Exerted by Estrogen in Polymorphonuclear Leukocytes

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Objective—The anti-inflammatory properties of the female sex hormone estrogen have been linked to a reduced incidence of cardiovascular disease. In the present study, we addressed whether estrogen could activate vasculoprotective mechanisms via annexin A1 (AnxA1) mobilization in human polymorphonuclear cells (PMNs).

Methods and Results—Using whole-blood flow cytometry, we demonstrated that premenopausal women expressed higher levels of surface AnxA1 on circulating PMNs compared with males. This correlated with high plasma estrogen during the menstrual cycle. The addition of estrogen in vitro to male PMNs induced rapid mobilization of AnxA1, optimal at 5 ng/mL and a 30-minute incubation period; this effect was abolished in the presence of the estrogen receptor antagonist ICI182780. Estrogen addition to human PMNs induced a distinct AnxA1hi CD62Llo CD11blo phenotype, and this was associated with lower cell activation as measured by microparticle formation. Treatment of human PMNs with E2 inhibited cell adhesion to an endothelial cell monolayer under shear, which was absent when endogenous AnxA1 was neutralized. Of interest, addition of estrogen to PMNs flowed over the endothelial monolayer amplified its upregulation of AnxA1 localization on the cell surface. Finally, in a model of intravital microscopy, estrogen inhibition of white blood cell adhesion to the postcapillary venule was absent in mice nullified for AnxA1.

Conclusion—We unveil a novel AnxA1-dependent mechanism behind the inhibitory properties of estrogen on PMN activation, describing a novel phenotype with a conceivable impact on the vasculoprotective effects of this hormone. (Arterioscler Thromb Vasc Biol. 2011;31:2749-2759.)

Key Words: immune system ■ immunologic techniques ■ leukocytes ■ microcirculation ■ pharmacology

The effects that sex hormones have on the processes regulating vascular inflammation have recently been reevaluated, identifying novel cell targets and pathways. These efforts are prompted by epidemiological evidence that gender affects the incidence of cardiovascular and inflammatory diseases. Compared with age-matched males, premenopausal, but not postmenopausal, females are protected against cardiovascular disease, implicating a protective role for female sex hormones. Indeed, there is unequivocal evidence for vasoprotective and anti-inflammatory properties of estrogen (17-β estradiol [E2]), raising the question of its cellular targets and molecular mechanisms. In the vasculature, estrogen has a direct positive effect on vascular function, improving blood flow, and inhibiting vascular injury through regulation of endothelial-derived nitric oxide. It is interesting to note that the timing of estrogen administration is a key factor in determining beneficial outcomes: when estrogen is given prophylactically, the extent of disease, its incidence, or both is reduced, but this protection is lost if administered following disease onset.

In the context of acute inflammation, estrogen has been shown to be anti-inflammatory, suppressing the nuclear factor-κB pathway, as well as downregulating specific adhesion molecules on the vascular endothelium, including the recently reported modulation of CD62P expression in the vessel wall. Regulation of adhesion molecules by estrogen would lead to modulation of the infiltration of leukocytes into the vascular tissue. Furthermore, estrogen reduces generation of the polymorphonuclear cells (PMN) chemoattractant cytokine-induced neutrophil chemoattractant-2β (CXCL3, GRO3), thereby inhibiting PMN migration. It is accepted that estrogen modulation of cytokines and chemokines might underlie its regulatory properties in vascular inflammation, providing a potential explanation to clinical observations.

Annexin A1 (AnxA1) is a 37-kDa protein with anti-inflammatory properties in the context of experimental inflammation. In human resting PMNs, AnxA1 is abundant in the cytoplasm, with only a small proportion present on PMN surface. On PMN activation, AnxA1 is rapidly mobilized to the cell surface, where it binds to its receptor, the G-protein coupled receptor formyl-peptide receptor 2/ lipoxin A4 receptor (FPR2/ALX). Following receptor ligation, AnxA1 is able to evoke a number of anti-inflammatory mechanisms,
both in an autocrine and paracrine fashion, inhibiting distinct stages of the leukocyte transmigration cascade. AnxA1 is also endowed with proresolving properties including promotion of efferocytosis of apoptotic PMNs. On activation, PMNs are also capable of releasing microparticles (also known as ectosomes). These are small (<1 μm) membrane-bound structures that can be released from most cell types and express surface molecules derived from their parent cell. Recent evidence suggests that microparticles can induce cellular cross-talk and inhibit inflammatory responses. Of interest to us, PMN-derived microparticles expressing AnxA1 are capable of evoking anti-inflammatory actions.

Currently, there is little evidence indicating that estrogen affects PMN reactivity, and most studies have focused on the endothelial cell as the primary target to evoke vasculoprotective properties. Here, we started with the observation that premenopausal women express higher levels of AnxA1 on the PMN surface as compared with age-matched males, and this correlates with estrogen levels during the menstrual cycle. On these bases, we have studied the association between estrogen engagement of the AnxA1 pathway in human PMNs and cell activation in vitro and in vivo. We propose that this novel mechanism might at least contribute to the protective effects of estrogen in vascular inflammation.

**Materials and Methods**

Detailed information on protocols and ethics are in the Supplemental Methods, available online at http://atvb.ahajournals.org.

**Cell Culture and Reagents**

Unless otherwise stated, all cell culture reagents were obtained from Sigma-Aldrich (Poole, Dorset, United Kingdom). Human PMNs were isolated via density gradient as described. PMNs (106 cells per test) or whole-blood aliquots (50 μL) were incubated with E2 or vehicle, E2 or E2 plus monoclonal antibody 1B for 30 minutes at 37°C before flow over HUVEC monolayer at a rate of 1 dyn/cm2 for 8 minutes, as previously described. PMN/HUVEC interaction in the flow chamber was monitored on 6 random fields recorded for 10 seconds. Analysis of total cell capture and rolling and firmly adherent PMNs was carried out off-line by manual quantification. In some cases, cells flowed over the HUVEC monolayer were collected and compared with resting (preflowed) or adherent PMNs for the extent of cell-surface AnxA1, CD62L, and CD11b expression using the flow cytometric protocol described above.

**Flow Cytometric Detection of Surface AnxA1 on Human PMNs**

Cells or whole blood were incubated for 1 hour at 4°C with mouse anti-human AnxA1 monoclonal antibody 1B, produced in-house or anti-human FPR2/ALX (Genovac, Freiburg, Germany), using a 3-step protocol (Supplemental Methods). Briefly, after the first 1-hour incubation, cells were washed and incubated with a rabbit anti-mouse IgG fluorescein isothiocyanate–conjugated secondary antibody to CD16 (PE, clone eBioCB16), to L-selectin (PE-Cy5, clone DREG-56), or, in some samples, to CD11b (APC, clone ICRF44) was run.

For detection of plasma microparticles, after incubation with E2 in the presence or absence of rIL-1β, platelet-free plasma was double stained for CD66b (PE, clone G10F5) (or IgM isotype control) to identify PMN-derived plasma microparticles and costained for AnxA1, FPR2/ALX, or CD62L. Beads (1 μm each; Becton Dickinson, San Jose, CA) were also run, in selected samples, to control for microparticles size.

In all cases, 20,000 events were acquired by using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo analysis software (version 9.2, Treestar Inc, Stanford, CA).

**Western Blot Analysis**

PMNs were separated into cytosolic and membrane fractions as reported, subjected to standard SDS–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (Millipore, Watford, United Kingdom). These were incubated with monoclonal antibody 1B (1 μg/mL) and horseradish peroxidase–conjugated goat anti-mouse IgG (Dako, Cambridge, United Kingdom). Proteins were detected using an enhanced chemiluminescence detection kit and visualized on Hyperfilm (GE Healthcare).

**Determination of Plasma of E2, Progesterone, and Cortisol Concentrations**

Blood was taken from females who were not on any oral contraceptives (for demographics, see Supplemental Table I) at days 2, 12, 19 and 26 of individual menstrual cycles. All blood samples were taken between 10 and 11 AM. Plasma prepared from blood of female volunteers was tested for E2 and progesterone concentrations using specific enzyme immunoassays (estradiol EIA kit, Invitrogen; progesterone and cortisol EIA, Cayman Chemical Co Inc, Ann Arbor, MI).

**Flow Chamber Assay**

Human umbilical vein endothelial cells (HUVECs) were cultured until they reached confluence and stimulated with 10 ng/mL tumor necrosis factor-α for 4 hours (Sigma-Aldrich). Isolated PMNs were incubated with vehicle, E2, or E2 plus monoclonal antibody 1B for 30 minutes at 37°C before flow over HUVEC monolayer at a rate of 1 dyn/cm2 for 8 minutes, as previously described. PMN/HUVEC interaction in the flow chamber was monitored on 6 random fields recorded for 10 seconds. Analysis of total cell capture and rolling and firmly adherent PMNs was carried out off-line by manual quantification. In some cases, cells flowed over the HUVEC monolayer were collected and compared with resting (preflowed) or adherent PMNs for the extent of cell-surface AnxA1, CD62L, and CD11b expression using the flow cytometric protocol described above.

**Intravital Microscopy of Leukocyte Recruitment on Mesenteric Postcapillary Venules**

Male C57BL6 mice (AnxA1+/− and AnxA1−/−; 4 weeks old) were injected with either PBS or estradiol (100 ng/mouse, IP) 30 minutes before administration of IL-1β (10 ng/mouse, IP). After 2 hours, animals were anesthetized, and the mesenteric microvascular bed was exposed for analysis. Intravital microscopy was performed as previously reported. A cautery incision was made along the abdominal region and the mesenteric vascular bed was exteriorized, placed on a viewing Plexiglas stage, and mounted on a Zeiss Axioskop FS with a water-immersion objective lens (magnification ×40, Carl Zeiss, Welwyn Garden City, United Kingdom) and an eyepiece (magnification ×10, Carl Zeiss). Mesenteries were superfused with thermostated (37°C) bicarbonate-buffered solution at a rate of 2 mL/min. When a suitable postcapillary venule was selected (diameter 20 to 40 μm; straight vessel length 100 μm), recording was started and kept up for a 3-minute period, during which adherent and emigrated leukocytes were counted.

**Statistical Analyses**

Data are expressed as mean±SEM. The Student t test was used to compare 2 groups with parametric data distribution. For multiple comparison analyses, 1- and 2-way ANOVAs were carried out, with appropriate Dunnett, Tukey, or Bonferroni postcorrection tests. Probability values <0.05 were considered to be significant.

**Results**

E2 Modulates AnxA1 Expression in Human PMNs

Analyses of AnxA1 expression in circulating cells of healthy volunteers revealed a consistent and significant increment in the fraction of AnxA1+/− PMNs in female donors (Figure
1A). Of 6 donors per gender, there was almost a doubling in cell surface AnxA1 on female PMNs (P<0.01). This difference was not seen in the expression of the AnxA1 receptor FPR2/ALX (Figure 1A). Analysis at the single cell level revealed a significant increase in AnxA1 extent of expression on PMNs taken from female donors (2-fold increase in mean fluorescence intensity units), whereas no differences between gender could be measured for FPR2/ALX or CD62L (Table). These discrete, reproducible changes observed in PMNs purified from females, including the higher degree of AnxA1 surface expression at the cellular level, prompted us to investigate E2 effects on human PMNs in vitro.

Male donors were used for these experiments. Concentration-response and time-course analyses revealed that 5 ng/mL E2 (18 nmol/L) for 30 minutes yielded optimal mobilization of AnxA1 on the PMN surface (P<0.001; Figure 1B), and this concentration was selected for subsequent experiments. To validate fluorescence-activated cell sorting analysis data of in vitro mobilization of AnxA1, Western blot analysis was also carried out. Figure 1C shows that presence of E2 mobilized AnxA1 to the membrane fraction. Such an effect was due to engagement of the ER, because pretreatment with the antagonist ICI182780, significantly reduced the ability of E2 to mobilize AnxA1 on PMN surface: 30% of PMNs were AnxA1+ve cells in the presence of E2, whereas, only 11% of PMNs were AnxA1+ve in the presence of the ER antagonist (10 to 100 μmol/L ICI182780; P<0.001) (Figure 1D).

### Table. Expression of Annexin A1 (AnxA1), FPR2/ALX, CD62L, and CD11b on Male and Female Polymorphonuclear Cells (PMNs)

<table>
<thead>
<tr>
<th>Cell Surface Antigen</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnxA1</td>
<td>12.3±1.9</td>
<td>22.9±3.9</td>
<td>1.9</td>
<td>3.9*</td>
</tr>
<tr>
<td>FPR2/ALX</td>
<td>12.9±1.5</td>
<td>14.9±0.3</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>CD62L</td>
<td>188±19</td>
<td>165±16</td>
<td>8.7</td>
<td>9.0</td>
</tr>
<tr>
<td>CD11b</td>
<td>41±8.7</td>
<td>55±9.0</td>
<td></td>
<td></td>
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</tbody>
</table>

Values show mean fluorescence intensity units for AnxA1, formyl-peptide receptor 2/lipoxin A4 receptor (FPR2/ALX), CD62L, and CD11b on PMNs from age-matched healthy male and females. Data are expressed as mean±SEM from 6 subjects per group.

*P<0.01 compared to males.
cycle (325 versus 91 pg/mL at day 2), whereas progesterone peaked at day 26 (12.3 ng/mL). This peak in progesterone levels coincided with a resurgence in E2 plasma levels at day 26 (150 pg/mL) (Figure 2B). Plasma cortisol levels did not change throughout the time-course of the menstrual cycle.

We obtained a good degree of correlation between PMN surface AnxA1 expression and plasma E2 concentrations, with an \( r^2 \) value of 0.5628 (Figure 2C, left panel). To test whether the correlation was affected by the presence of progesterone, day 26 values (peak for progesterone) were removed from the correlation graph, yielding an even higher \( r^2 \) value of 0.7655 (Figure 2C, right panel). To assess the potential effect of progesterone on the ability of E2 to mobilize AnxA1, we incubated whole blood from male donors with progesterone (10 ng/mL; menstrual cycle levels) and E2 and observed a reduced ability to provoke AnxA1 mobilization by E2 \( (P<0.05) \) (Figure 2D).

**Profile of E2-AnxA1 Pathway in PMN Activation In Vitro**

The results so far have shown the effects of E2 on AnxA1 mobilization in the absence of cell activation. To investigate...
the potential effects of E2 associated AnxA1 externalization, we carried out a series of in vitro experiments in which whole blood was either pretreated with fMLF (10 nmol/L, 20 minutes) followed by E2 treatment (5 ng/mL, 30 minutes), or pretreated with E2 followed by fMLF (in comparison with vehicle and single-treatment controls).

Figure 3A shows box-and-whisker plots of this set of experiments. As previously observed, E2 treatment significantly increased the expression of AnxA1 and FPR2/ALX on the PMN surface. The data are mean ± SEM of 6 experiments conducted with different blood preparations. *P < 0.001 compared with vehicle control (1-way ANOVA with the Dunnett postcorrection); **P < 0.001. B, Representative histograms depicting the mean fluorescence intensity units for AnxA1, CD66b, and CD62L from the experiments presented in A. Gray histograms indicate isotype controls (IgG1 for AnxA1 and CD62L, and IgM for CD66b).

Figure 3. Estrogen confers an anti-inflammatory phenotype to human polymorphonuclear cells (PMNs). A, Male whole blood was treated with either formyl-Met-Leu-Phe (fMLF) (10 nmol/L) for 20 minutes, E2 (5 ng/mL) for 30 minutes, or a combination of treatments consisting of fMLF followed by E2 or pretreatment with E2 followed by fMLF. All blood aliquots were kept at 37°C for a total of 50 minutes of incubation time. Flow cytometry allowed quantification of the degree of expression of annexin A1 (AnxA1), formyl-peptide receptor 2/lipoxin A4 receptor (FPR2/ALX), CD62L, CD11b, and CD66b on the PMN surface. Data are mean ± SEM of 6 experiments conducted with different blood preparations. *P < 0.001 compared with vehicle control (1-way ANOVA with the Dunnett postcorrection); **P < 0.001. B, Representative histograms depicting the mean fluorescence intensity units for AnxA1, CD66b, and CD62L from the experiments presented in A. Gray histograms indicate isotype controls (IgG1 for AnxA1 and CD62L, and IgM for CD66b).
sently mobilized AnxA1 to PMN surface compared with vehicle control \((P<0.001)\). AnxA1 externalization was not observed in fMLF treatment alone or when PMNs were pretreated with fMLF before E2 (Figure 3A). In contrast, pretreatment of PMNs with E2 before fMLF resulted in significant mobilization of AnxA1 similar to levels seen in E2 treatment alone. Analysis of FPR2/ALX expression indicated lack of externalization on E2 treatment but an upregulation post-fMLF, irrespective of the presence of E2 \((P<0.001\) for all 3 treatment groups).

Figure 3B reports representative histograms from these experiments, showing AnxA1 expression alongside that of CD66b and CD62L. Analysis of CD66b expression revealed a significant augmentation in PMN surface expression, when cells were treated with fMLF alone or given as pretreatment. This was not observed, however, with vehicle or E2 (alone or as pretreatment). Vehicle and E2 alone had no effect on CD62L shedding, whereas fMLF treatment caused significant CD62L-shedding, which was unaffected by with E2 addition. Interestingly, pretreatment of PMNs with E2 (5 ng/mL) did not rescue the cells from CD62L shedding but did maintain low expression of the \(\beta_{2}\)-integrin CD11b when compared with vehicle and E2 treatment alone (Figure 3A and 3B). Therefore, E2 can selectively modulate PMN stimulation induced by fMLF.

Next, we measured neutrophil-derived microparticles as a test for E2 to inhibit downstream PMN activation, and we used the plasma from blood treated as in Figure 3. Microparticles were identified according to their size (and using 1-\(\mu\)m beads; see Supplemental Figure I). Treatment of PMNs with fMLF promoted a significant release of CD66b-positive microparticles compared with vehicle control \((P<0.001)\). Addition of E2 prevented microparticle generation elicited by fMLF, and this time, the hormone was active even when added after the stimulus (Figure 4A). These responses are shown in representative histograms (Figure 4B). Microparticles generated in these experimental conditions were characterized further quantifying specific antigens on a per-microparticle basis, reporting it as mean fluorescence intensity units.
Treatment of fMLF alone led to a 2-fold increase in the expression of AnxA1 on CD66b +ve microparticles compared with vehicle ($P<0.001$). This response was insensitive to cell exposure to $E_2$ (5 ng/mL) following fMLF, whereas estrogen was ineffective when given alone (Figure 4C). In contrast, and in line with the global data on microparticle formation, pretreatment with $E_2$ abrogated fMLF-induced generation of AnxA1/CD66b double positive microparticles (Figure 4C). The extent of expression of FPR2/ALX on PMN-derived microparticles was marked even in vehicle-treated cells and only marginally modulated by the treatments. In contrast, CD62L expression (mean fluorescence intensity units) was reduced by $E_2$ only when added before fMLF (Figure 4C).

Collectively, these data indicated that $E_2$ can modulate PMN activation recorded via microparticle generation and can modify, at least in part, some of the proteins displayed on these microstructures.

**$E_2$ Modulates PMN Reactivity Under Flow via Endogenous AnxA1**

In the next series of experiments, we wanted to determine whether $E_2$ could modulate PMN activation, and hence inflammatory responses, in an AnxA1-dependent fashion. To model the impact of $E_2$-treated PMNs in vascular inflammation, we used the flow chamber assay. Following HUVEC activation by tumor necrosis factor-α, PMNs from healthy males treated with vehicle, $E_2$, or $E_2$ plus an anti-AnxA1 neutralizing antibody were flowed over the monolayer.

Cell incubation with $E_2$ did not affect the ability of the PMNs to come in contact with the monolayer and hence did not affect cell capture (the total number of interacting cells; Figure 5, top). However, a selective interference with the extent of PMN adhesion resulted in ~3-fold decrease (Figure 5, middle; $P<0.001$). This effect was significantly lost when PMNs were coadministered with the AnxA1 neutralizing antibody and $E_2$. There were no significant differences in the capture or rolling of PMNs between the 3 treatments (Figure 5A, bottom). Figure 5B displays representative images captured from these experiments.

The experiments of flow chamber assay allowed us to investigate the phenotype of the $E_2$-treated PMNs that had encountered the inflamed endothelium in the flow chamber but did not adhere, as well as cells that had adhered to the endothelium. Addition of $E_2$ (5 ng/mL; 30 minutes) to PMNs that had flowed over the HUVEC monolayer resulted in higher levels of AnxA1 on their surface (Figure 6B) compared with hormone addition to resting PMNs (preflow in Figure 6A; $P<0.05$). These changes in AnxA1 were not reflected by any detectable change in CD62L (Figure 6A and 6B). In contrast, although postflow PMNs displayed higher CD11b levels to static (preflow) PMNs, the addition of $E_2$ (5 ng/mL; 30 minutes) significantly attenuated this cellular response (Figure 6A and 6B, bottom).

We also recovered postadherent PMNs (dislocating cells by increasing shear) and detected much higher AnxA1 levels in vehicle-treated cells (reminiscent of our old observations$^{16}$), and this could be partially modulated by $E_2$ (5 ng/mL; 30 minutes) (Figure 6C). CD11b was higher in postadherent PMNs and was no longer susceptible to the addition of the hormone.

**$E_2$ Modulates In Vivo PMN Reactivity via Endogenous AnxA1**

The effect of $E_2$ on PMN recruitment under flow was also assessed in vivo using the inflamed microvasculature of the mesentery. This model was chosen because of the tonic buffering role exerted by endogenous AnxA1.$^{25}$

Treatment of wild-type mice with interleukin (IL)-1β alone caused a significant increase in cell adhesion and emigration compared with vehicle control, as assessed at 2 hours postcytokine (Figure 5A, $P<0.001$). Pretreatment of the animals with $E_2$ (100 ng IP; ~30 minutes) significantly attenuated the effect of IL-1β in terms of both cell adhesion and emigration ($P<0.001$). Figure 5B shows representative images of the mesenteric microcirculation of wild-type mice treated with IL-1β in the presence or absence of $E_2$. Importantly, modulation of this inflammatory event by $E_2$ was dependent on endogenous AnxA1 because this hormone was ineffective when tested in AnxA1 −/− male mice (Supplemental Figure II). Hemodynamic parameters can be seen in Supplemental Table II.
Discussion

In the present study, we provide evidence that the female sex hormone estrogen exerts inhibitory effects on human PMNs, which translate to anti-inflammatory properties in vascular inflammation and provide a strong indication for a functional association with endogenous AnxA1. Together, these results readdress the target for the anti-inflammatory mechanisms of estrogen, indicating that, besides the vascular wall, this hormone can have marked effects on the blood-borne leukocyte.

The anti-inflammatory nature of female sex hormones has long been noted, and it is well established that, for instance,
inflammatory and cardiovascular diseases are exacerbated in postmenopausal women. More specifically, recent studies have shown postmenopausal women present increased levels of adipokines (eg, serum amyloid protein A^{27}), as well as proinflammatory cytokines (eg, IL-8,28) in plasma, all of which increase the risk of cardiovascular diseases. The present study was prompted by the observation that a higher degree of cell surface AnxA1 could be measured on circulating PMNs of premenopausal female volunteers compared with age-matched males.

A previous study reported the detection of the cell surface AnxA1 in blood leukocytes, specifically PMNs, which correlated with plasma cortisol. These latter results presented a functional, well-characterized association between glucocorticoids and cellular expression of the anti-inflammatory protein, AnxA1.^{30} Here our unexpected observation of sexual dimorphism, specifically for the blood PMNs, prompted us to perform more detailed analyses during the course of the menstrual cycle. These new data indicate that cell surface AnxA1 in circulating PMNs is selectively modulated by levels of circulating hormones, with a significant and robust correlation to plasma estrogen levels but neither progesterone nor cortisol. Such an association was not consequent to a global, nonselective change in PMN phenotype, because AnxA1 cell surface expression was discretely augmented in correlation with peak of estrogen level without changes in cell surface FPR2/ALX or CD66L.

Estrogen is known to modulate a variety of responses in target cells that could have an impact on the intensity of an experimental inflammatory response. For example, estrogen regulates PMN recruitment by inhibiting the chemokine cytokine-induced neutrophil chemoattractant-2B^{13} and other cytokines, likely consequent to a suppression the nuclear factor-κB pathway. Such a complex network of effects is not surprising in view of the pleiotropic action of this hormone. In the context of vascular inflammation, the majority of studies have focused on the endothelium. Estrogen maintains vascular tone by mediating key endothelial-derived factors. For example, in vitro treatment on endothelial cells with estrogen augments the production of the anti-inflammatory autacoids nitric oxide, prostacyclin, and endothelial-derived hyperpolarizing factor. The dichotomy between genders made the PMNs the focus of the present study.

We found that there was a distinct and reproducible mobilization of AnxA1 in human PMNs on incubation with estrogen; the effect was nongenomic, optimal at 30 minutes, and mediated by classical ERs as demonstrated by the use of the nonselective ER antagonist ICI182780. The data were reassuring in view of the well-documented presence of ER-α and ER-β in human PMNs. Moreover, a microarray study in the rat has reported a directly associated ER binding with gene regulation of AnxA1 in blood leukocytes. Together with our new data, we can propose that this hormone produces nongenomic and genomic regulation of this mediator of endogenous anti-inflammation.

Our ex vivo measurements showing that high AnxA1 correlate with high E_2 (but not cortisol) during the menstrual cycle added support to our in vitro findings. In the time-course analyses, we chose day 12, as E_2 is the only hormone that is high during this period of the menstrual cycle. However, E_2 levels remain high for a few days during the menstrual cycle. It is tempting to propose that the anti-inflammatory effects of E_2 (via AnxA1) could be explained by the fact that at days 13 and 14, when E_2 levels remain high, follicle-stimulating hormone and luteinizing hormone are also high. Because both follicle-stimulating hormone and luteinizing hormone have been positively correlated with AnxA1, E_2 could act in concert with follicle-stimulating hormone and luteinizing hormone and exert their anti-inflammatory actions by promoting AnxA1 mobilization. Of course, this hypothesis would need to be substantiated by future investigations.

In the human PMN, AnxA1 represents a substantial amount of the intracellular proteins, being calculated to be between 2% and 4%. We have gathered evidence for the existence of at least 2 pools of the protein in resting PMNs, one cytosolic and the other associated with subcellular organelles, such as gelatinase and azurophilic granules. More recent studies indicate that these distinct pools of intracellular AnxA1 can be mobilized in a stimulus dependent fashion; thus, cell activation consequent to outside-in signaling (eg, after cell adhesion) exports the granule/vesicular pool of the protein. In contrast, pharmacological treatment with dexamethasone, cromones, or other soluble compounds appears to engage the cytosolic pool of the protein. The latter process seems relevant to the effects of estrogen on the PMN treatment, because analyses of specific markers for intracellular granules (eg, CD66b or CD11b) failed to detect modulation of cell surface expression at concentration, and incubation time, optimal for E_2 promotion of AnxA1 mobilization. Of interest, recent work has demonstrated existence of a partial similarity between glucocorticoids and estrogens with respect to gene modulation in the human PMN, observing also how an ER antagonist can impact on the biological properties of glucocorticoids in vitro and in vivo. The present study extends the potential for this crosstalk, or at least shared properties, between these 2 classes of hormones to rapid nongenomic modulation of AnxA1 expression.

The functional relevance of estrogen-induced AnxA1 externalization was initially investigated in vitro, monitoring classical PMN activation markers. Preincubation of human PMNs with estrogen inhibited cell activation by formylated peptides, in a selective fashion, because significant inhibition was quantified against fMLF-induced CD11b and CD66b upregulation but not CD62L shedding. Chronologically, the mechanism activated by estrogens must precede the cell simulation with fMLF because the hormone was inactive when added after the PMN activator. Indeed, a distinct PMN phenotype (AnxA1^{18} CD62L^{26} CD11b^{30}) is apparent in estrogen-pretreated PMNs, as seen in several settings (see below), prompting us to suggest the existence of an E_2-generated anti-inflammatory PMN phenotype that may have an impact on further cell recruitment to sites of inflammation. The fact that estrogen could alter specific pathways in the human PMN can also be deduced from the lack of modulation of fMLF-induced FPR2/ALX upregulation. This receptor is
known to be expressed in a variety of granules and to be upregulated on cell trafficking; such a pattern is grossly similar to what described for AnxA1, yet it was not modulated by estrogen. This apparent lack of modulation of cell surface FPR2/ALX expression was also evident in circulating cells taken from healthy subjects.

Our analyses of PMN activation were concluded by quantifying the production of PMN-derived microparticles, a response that is gaining momentum both with respect to biological functions and suitability as disease biomarker. In line with the results obtained with CD11b and CD66b, addition of estrogen before fMLF reduced the production of PMN-derived microparticles, quantified as CD66b+ve microstructures. Collectively, these results indicate that estrogen application to human PMNs—before stimulation—significantly attenuates cell activation. We propose that modulation of PMN responsiveness by estrogen could be at least contributory, in association with the reported effects on vascular wall reactivity, to gender specificity with respect to gender-specific disease penetrance observed for cardiovascular pathologies. This proposition, and its link to endogenous AnxA1, is corroborated by the experiments of flow chamber where the specific effect of E2-treated PMNs on inflamed endothelium was tested. The antiadhesive effect of AnxA1 on PMNs under flow has been well documented by our group.17,38 We found that in comparison with vehicle control, there was a significantly lower degree of PMN adhesion to the inflamed endothelium with E2-treated PMNs—an effect that was lost when AnxA1 was neutralized. These data were supported by in vivo experiments where the effect of estrogen on the inflamed microcirculation was tested. A real-time protocol of intravital microscopy was applied whereby the mesenteric vasculature was inflamed by administration of IL-1β. Intraportal treatment of male mice with estrogen produced a marked attenuation of the extent of leukocyte adhesion and emigration. This finding is aligned with the antimigratory and anti-inflammatory properties of this hormone as demonstrated in ovariectomized animals, where leukocyte recruitment was significantly increased.12 These experiments provide a crucial functional link between the variety of analyses performed with human subjects and cells and the potential pathophysiological relevance, noting also that the significant attenuation of white blood cell recruitment onto the postcapillary venule vessel wall, resulting from estrogen administration, was absent in AnxA1−/− mice.

Finally, we wanted to determine whether E2-treated PMNs that had encountered the inflamed endothelium under flow but did not adhere had a phenotype similar to our in vitro experiments. Analysis of AnxA1 on postflow PMNs suggests that we might have underestimated the effect of E2 on AnxA1 mobilization: there was a 3-fold increase in AnxA1 expression on E2-treated postflow PMNs compared with the preflow PMNs (static incubation). This is a novel observation, and it stresses the importance of testing modulation of the AnxA1 pathway in the presence of shear. It is therefore plausible that the externalization of AnxA1 induced by the classes of drugs listed above (eg, glucocorticoids, cromones, and more) might be much more pronounced in intravascular settings, where the PMN is subjected to shear stress.

In line with our in vitro results in static settings, there was a significant attenuation of CD11b expression on postflow E2-treated PMNs, compared with their adherent counterparts. A similar effect has been observed in eosinophils, where glucocorticoid-induced AnxA1 mobilization resulted in inhibition of β2 integrin-intercellular adhesion molecule-1 interactions in eosinophils and to a lesser degree PMNs.48 Taken together, these results suggest that E2 has 2 distinct effects on the PMN: under noninflamed “normal” conditions, E2 induces a modest increase of surface AnxA1 with no modulation of CD11b, which could be thought of as priming mechanism. However, under inflamed, nonstatic conditions, E2 is capable of mounting a pronounced protective anti-inflammatory response by augmenting AnxA1 surface expression and modulating CD11b expression.

In conclusion, we unveil a novel mechanism whereby AnxA1 externalization can drive estrogen-regulated anti-inflammatory processes in PMN biology. It remains to be seen whether such an association could also be extended to other properties recently ascribed to AnxA1, such as modulation of the life span of migrated neutrophils and of their removal by phagocytes.

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Disclosures
None.

References


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Suchita Nadkarni, Dianne Cooper, Vincenzo Brancaleone, Stefania Bena and Mauro Perretti

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Supplemental Material

Human cells were prepared according to a protocol approved by the East London & The City Local Research Ethics Committee (Rec Ref. 05/Q0603/34 ELCHA, London, United Kingdom). Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986) and was approved by the Queen Mary University of London Ethics Committee (London, United Kingdom).

Cell culture and reagents

Unless otherwise stated, all cell culture reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK). Human PMN were isolated via density gradient as described before. All PMN cultures (10^6 cells per test) were carried out in phenol red-free and serum-free RPMI-1640 (Lonza, Slough, UK), supplemented with L-glutamine and penicillin/streptomycin, with the indicated incubation periods at 37°C. Typically, estrogen (E2) was added to cells for 30 minutes, using a concentration range of 0.5-10ng/ml, prior to stopping reactions on ice and determining AnxA1 and FPR2/ALX expression by flow cytometry (see below). In selected experiments, the estrogen receptor antagonist ICI182780 (10^-100 µM) was pre-incubated for 10 min prior to addition of 5 ng/ml E2.

In selected experiments a whole blood protocol was applied, affording minimal manipulation of cells. Similar to isolated PMN cultures, E2 was added at 5ng/ml concentration and incubated for 30 min prior to further stimulation with formyl-Met-Leu-Phe (fMLF; 10nM). Flow cytometry was then performed as described below.

Flow cytometric detection of surface AnxA1 on human PMN

All conjugated flow cytometry antibodies were obtained from eBioscience (Hatfield, UK), unless otherwise stated. Briefly, cells or whole blood aliquots (50µl) were subjected to a three-step staining protocol, starting with an incubation for 1 h at 4°C with mouse anti-human AnxA1 (monoclonal 1B, produced in-house or anti-human FPR2/ALX (Genovac, Freiburg, Germany); both at a final concentration of 20µg/ml plus IgG1k isotype control. Blood was then washed twice in PBS containing 10mM CaCl2 and 1.5% BSA, followed by 30 min incubation at 4°C with a rabbit anti-mouse IgG FITC-conjugated secondary Ab (AbD Serotec, Oxford, UK). Blood was then washed as above and incubated for a further 30 min with conjugated antibodies to CD16 (PE, clone eBioCB16) and L-selectin (PE-Cy5,clone DREG-56) or, in some samples, with CD11b (APC, clone ICRF44). Following two washes, blood
was lysed using a whole blood lysis kit (as per manufacturer’s protocol, Beckman Coulter, High Wycombe, UK). For the experiments with isolated PMNs a similar protocol was applied except for the lysis step and the use of human IgG (final concentration 8 mg/ml).

For detection of plasma microparticles, after incubation with E2 in the presence or absence of fMLF (see above), blood aliquots were centrifuged at 1200xg to remove platelets; platelet-free plasma was then double stained for CD66b (PE, clone G10F5) (or IgM isotype control) (Biolegend, Cambridge, UK) to identify PMN-derived plasma microparticles and co-stained for either AnxA1, FPR2/ALX or CD62L. Beads (1-µm each; Becton Dickinson, San Jose, CA) were also run, in selected samples, to control for microparticles size.

In all cases, 20,000 events were acquired by using a FACSCalibur flow cytometer (Becton Dickinson), and analysed using FlowJo analysis software (Version 9.2, TreeStar Inc, Stanford, CA).

**Western blot analysis**

Following treatments, PMN were separated into cytosolic and membrane fractions as previously reported\(^1,3\). Protein content of lysates was determined via a Bradford protein assay (BioRad, Hertfordshire, UK). PMN cytosol and membrane fractions (20µg total proteins) were subjected to standard SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF membranes (Millipore, Watford, United Kingdom). These were incubated with mAb 1B (1 µg/ml) and HRP-conjugated goat anti–mouse IgG (Dako, Cambridge, United Kingdom). Proteins were detected using enhanced chemiluminescence (ECL) detection kit and visualized on Hyperfilm (GE Healthcare, Little Chalfont, United Kingdom).

**Intravital microscopy of leukocyte recruitment on mesenteric post-capillary venules**

4-week old male C57Black6 mice (AnxA1\(^{+/+}\) and AnxA1\(^{-/-}\))\(^4\) were injected with either PBS or estradiol (100ng/mouse, i.p.) 30 min before or 1 h after administration of IL-1β (10ng/mouse, i.p.). After 2 hours, animals were anesthetized and the mesenteric microvascular bed was exposed for analysis. Intravital microscopy was performed as previously reported\(^5,6\). Briefly, after the treatment, mice were anesthetized and the left jugular vein was cannulated with polyethylene tubing (PE 10, internal diameter 0.28mm). A cautery incision was made along the abdominal region and the mesenteric vascular bed was exteriorized, placed on a viewing Plexiglas stage, and mounted on a Zeiss Axioskop “FS” with a water-immersion objective.
lens (magnification 40; Carl Zeiss, Welwyn Garden City, United Kingdom) and an eyepiece (magnification x10; Carl Zeiss). Tissue preparations were transilluminated with a 12 V, 100W halogen light source. A Hitachi charge-coupled device color camera (model KPC571; Tokyo, Japan) acquired images that were displayed onto a Sony Trinitron color video monitor (model PVM 1440QM; Tokyo, Japan) and recorded on a Sony super-VHS videocassette recorder (model SVO-9500 MDP) for subsequent offline analysis. A video time-date generator (FOR.A video timer, model JVC VTG-33, Tokyo, Japan) projected the time, date, and stopwatch function onto the monitor. Mesenteries were superfused with thermostated (37°C) bicarbonate-buffered solution (g/L: NaCl, 7.71; KCl, 0.25; MgSO4, 0.14; NaHCO3, 1.51; and CaCl2, 0.22, pH 7.4, gassed with 5% CO2/95%N2) at a rate of 2 mL/min. When a suitable post-capillary venule was selected (diameter 20 - 40 µm; 100 µm straight vessel length), recording was started and kept for a 3-minute period, where adherent and emigrated leukocytes were counted.

**Statistical analyses**

*Ex vivo* experiments in volunteers result from 6 donors; *in vitro* experiments with human cells and blood aliquots are from 5-6 individual experiments performed in duplicate with cells prepared from different donors; *in vivo* experiments of intravital microscopy result from 6 mice of each group. Data are expressed as mean ± SEM. Student’s *t*-test was used to compare two groups with parametric data distribution. For multiple comparison analyses, one- and two-way ANOVAs were carried out, with appropriate Dunnet’s, Tukey’s or Bonferroni post correction tests. *P*-values <0.05 were considered to be significant.

**References:**


Supplemental Table I

Demographic data of female subjects used for assessment of AnxA1 expression during the menstrual cycle.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Cycle length (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28.5 ±18</td>
<td>52.5 ±3.7</td>
<td>29.5 ± 0.55</td>
</tr>
</tbody>
</table>

Values represent age (years), weight (kg) and length of menstrual cycle (days). Values are expressed as mean ±SEM of 6 subjects.
Supplemental Figure I. Determination of plasma microparticles. Microparticles from plasma were assessed according to their forward/side scatter profile and against 1-\(\mu\)m beads.
Supplemental Figure II. The anti-inflammatory effect of estrogen is reliant on endogenous AnxA1.

A) Wild type (AnxA1\textsuperscript{+/+}) or AnxA1\textsuperscript{−/−} male mice were injected with E\textsubscript{2} (100ng, intraperitoneal, i.p.) 30 min before the i.p. injection of IL-1β (10 ng). The mesenteric microcirculation was exposed 2 h later, to monitor the degree of leukocyte adhesion and emigration with the vasculature by intravital microscopy. Data from 6 mice per group, with 2-3 vessel been analyzed per animal. **P<0.001 vs. vehicle; ## P<0.001 vs. respective inflamed control (without E\textsubscript{2}) (one-way ANOVA with Dunnett’s post correction).

B) Representative images of the microvasculature of wild type (AnxA1\textsuperscript{+/+}) mice showing white blood cells interacting with the vessel wall or emigrated into the sub-endothelial tissue (arrows). Note the lower degree of adherent and emigrated leukocytes in the IL-1β + E2 picture. Adipocytes are also visible (dashed arrows). Bar, 25 µm.
### Supplemental Table II

Hemodynamic parameters in AnxA1 WT and KO mice mesenteric microcirculation

<table>
<thead>
<tr>
<th></th>
<th>Diameters (µm)</th>
<th>Cell flux (no.cells/min)</th>
<th>Rolling velocity (µm/sec)</th>
<th>Wall shear rate (8,000xVmean/1.6/Dv)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham WT</strong></td>
<td>27.2±2.5</td>
<td>8.8±1.8</td>
<td>29.3±2.1</td>
<td>396.3±12.5</td>
</tr>
<tr>
<td><strong>IL1β WT</strong></td>
<td>29.3±1.5</td>
<td>18.1±2.3</td>
<td>18.1±3.1*</td>
<td>365.3±35.3</td>
</tr>
<tr>
<td><strong>IL1β+E2 WT</strong></td>
<td>28.5±1.3</td>
<td>11.0±6.2</td>
<td>26.3±1.9§</td>
<td>382.7±25.3</td>
</tr>
<tr>
<td><strong>Sham KO</strong></td>
<td>25.3±2.4</td>
<td>17.0±6.1</td>
<td>25.6±3.6</td>
<td>366.2±21.0</td>
</tr>
<tr>
<td><strong>IL1β KO</strong></td>
<td>26.5±2.1</td>
<td>19.8±1.7</td>
<td>15.4±2.4*</td>
<td>355.6±24.3</td>
</tr>
<tr>
<td><strong>IL1β+E2 KO</strong></td>
<td>26.7±2.1</td>
<td>18.7±5.2</td>
<td>18.3±2.6*</td>
<td>341.3±26.9</td>
</tr>
</tbody>
</table>

Diameter of the mesenteric vessels analyzed are here reported. Similarly, values for wall shear rate and cell flux are shown. Mice were treated with IL1β (10ng) after administration of vehicle or E2 along the procedure described in the Methods. Data are mean ± SEM of 8 animals per group. * vs Sham, § vs IL1β.