Suppression of Endothelial P-Selectin Expression Contributes to Reduced Cell Trafficking in Females
An Effect Independent of NO and Prostacyclin

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Objective—Sex hormones underlie the lower incidence of cardiovascular disease in premenopausal women. Vascular inflammation is involved in the pathogenesis of several cardiovascular diseases and it has been reported that sex hormones modulate inflammatory responses but mechanisms responsible for these effects are not yet fully established. Herein, we assessed whether sex differences in leukocyte recruitment might exist and investigated the underlying mechanisms involved in this response.

Methods and Results—Treatment with interleukin-1β (IL-1β) or tumor necrosis factor-α caused leukocyte rolling, adhesion, and emigration in mesenteric postcapillary venules in vivo that was substantially reduced in female mice compared with male mice; this difference was abolished by ovariectomy and partially restored by estrogen replacement. Deletion of endothelial nitric oxide (NO) synthase or cyclooxygenase-1 alone or in combination did not alter the leukocyte recruitment in IL-1β-treated females but significantly enhanced this response in male mice. Treatment of murine pulmonary endothelial cells with IL-1β increased expression of P-selectin in male but not female cells.

Conclusion—We have demonstrated a profound estrogen-dependent and NO and prostacyclin-independent suppression of leukocyte recruitment in females. (Arterioscler Thromb Vasc Biol. 2011;31:1075-1083.)

Key Words: endothelium ■ gender ■ leukocytes ■ nitric oxide ■ prostacyclin

A lower incidence of cardiovascular disease in premenopausal women compared with age-matched male counterparts and postmenopausal women1,2 suggests that female sex underlies a protective effect on the cardiovascular system. Indeed, a wealth of evidence from observational and experimental studies, in both animals and humans, supports the concept of a protective effect of ovarian hormones, predominantly estrogens.3,4 Understanding the molecular pathways that underlie the beneficial effects of estrogens may, therefore, identify novel strategies that could be taken to harness the therapeutic potential of hormone therapy safely and novel targets to treat cardiovascular disease in both sexes.

A number of targets/pathways/mediators have been proposed to play a role in mediating the beneficial effects of female sex hormones.3,5-7 Of these mechanisms, there is a substantial body of evidence implicating estrogen-induced or estrogen-enhanced activation of the endothelium.5 Endothelial dysfunction is thought to be instrumental in precipitating the vascular inflammation that is an early and crucial event in the pathogenesis of a number of cardiovascular disorders (eg, atherosclerosis, ischemia/reperfusion injury).8,9 In health, the endothelium is critical in maintaining an antiinflammatory, and thereby antiatherogenic, phenotype of the blood vessel wall.10,11 This activity has been attributed to its capacity to release factors that not only alter the tone and growth of the underlying smooth muscle but also regulate the reactivity of circulating white cells, erythrocytes, and platelets and govern vascular permeability. The prevailing wisdom currently promotes the thesis that estrogens upregulate the synthesis, release, and activity of protective endothelial factors and thereby sustain the protective phenotype conveyed by the endothelium and underpin the protection of females from cardiovascular disease.7

A significant proportion of this cytoprotective activity of the endothelium has been attributed to alterations in NO bioavailability by upregulation of synthesis, activity, or both.5,12 However, the role of other potentially beneficial endothelial mediators, including prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF), has received little attention. There is some evidence, from diverse animal models of inflammation, to suggest the existence of sex hormone–dependent male/female differences in inflam-
matory cell recruitment.\textsuperscript{13–18} However, the exact pathways (i.e., whether this regulation occurs at the level of the endo-
thelium or at the level of the circulating cell) and the mediators involved are uncertain.

In this study, we demonstrate that reduced expression of P-selectin underlies the attenuated leukocyte recruitment that occurs in female mice in response to acute inflammatory stimuli. Moreover, we demonstrate that although both NO and PGI\textsubscript{2} tonically repress leukocyte recruitment in response to inflamma-
tory stimuli in males, neither mediator has a role in females.

Materials and Methods

Animals
All experiments were conducted according to the Animals (Scientific Procedures) Act 1986 (United Kingdom). The experiments were performed on age-matched (6 to 7 weeks) female and male wild-type (WT; C57BL6, Charles River), endothelial nitric oxide (NO) synthase knockout (eNOS\textsuperscript{-/-}),\textsuperscript{19} cyclooxygenase-1 knockout (COX-1\textsuperscript{-/-}),\textsuperscript{20} and eNOS\textsuperscript{-/-}/COX-1\textsuperscript{-/-} double knockout (dKO) mice.\textsuperscript{21} All knockout mice were bred in-house. In addition female WT ovariec-
tomized (OVX) and sham-operated control animals were purchased from Charles River. Ovariectomy was performed on sexually imma-
ture mice at 4 weeks of age. In 16 OVX mice, Alzet isomotic pumps (model 1004) were implanted at the time of surgery (Charles River), containing vehicle (polyethylene glycol 400, Sigma-Aldrich) or a replacement dose of estrogen of 0.4 mg/day providing physiological levels in mice as previously demonstrated.\textsuperscript{22} Experiments were conducted on these animals 2 weeks after surgery. Mice were maintained in a 12-hour light/dark-cycle room with free access to food and water.

Determination of Plasma Estrogen Concentration
Blood was collected into heparin by cardiac puncture from mice anesthetized with isoflurane. Following centrifugation at 14,000 g for 10 minutes at 4°C plasma was collected and estrogen concentra-
tion determined according to the manufacturer’s instructions using a commercially available enzyme-linked immunoassay (extradiol EIA, Cayman Chemical Co, Inc).

Myeloperoxidase Activity
Mesenteric tissue, collected at 0 minutes, 90 minutes, or 4 hours after interleukin-1\textbeta (IL-1\textbeta; 5 ng/mouse IP) treatment, was homogenized in 1 mL of 0.5% hexadecyltrimethylammonium bromide in MOPS buffer (10 mmol/L, pH 7). After homogenization, samples were centrifuged at 4000 g for 20 minutes at 4°C, and the supernatant was collected for determination of myeloperoxidase (MPO) levels as previously described.\textsuperscript{23} MPO levels are expressed relative to protein content as determined by Bradford assay.

Hematoxylin and Eosin Staining
Whole sections of mesentery were prepared on microscope slides and dried overnight. General structures were identified by staining with hematoxylin and eosin to enable identification of polymorpho-
nuclear and mononuclear cells.

Intravital Microscopy
WT, eNOS\textsuperscript{-/-}, COX-1\textsuperscript{-/-}, and dKO (10 to 15 g) mice received either murine IL-1\textbeta (5 ng, Peprotech),\textsuperscript{24} murine tumor necrosis factor-\alpha (TNF-\alpha; 300 ng, R&D Systems),\textsuperscript{25} or saline vehicle IP. After 1.5, 4, or 24 hours, mice were anesthetized with diazepam (6 mg/kg SC) and Hypnorm (0.7 mg/kg fentanyl citrate and 20 mg/kg fluanisone IM), cautery incisions were made along the abdominal region, and the mesenteric vascular bed was exteriorized for intrav-
itral microscopy recordings (for full details, see the Supplemental Methods, available online at http://atvb.ahajournals.org). The extent of basal and cytokine-elicited leukocyte rolling was analyzed by counting the number of cells passing a fixed point per minute (cells/min). A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period of at least 30 s. Adherent cells were expressed as the number per a 100-\textmu m length of venule counted over 1 minute.

Air Pouch Model
IL-1\textbeta was used to induce cell migration (>90% neutrophils) into the mouse air pouch as previously described.\textsuperscript{26} On day 6, 20 ng of murine recombinant IL-1\textbeta was dissolved in 0.5 mL of 0.5% carboxymethyl-
 cellulose and injected into the pouches. Control mice received carboxymethylcellulose alone. In both cases, air pouches were washed 4 hours after administration of the stimulus with 2 mL of phosphate-
buffered saline containing heparin (50 U/mL) and EDTA (3 mmol/L), and the samples were collected. These were then centrifuged at 220g for 15 minutes at 4°C, and pellets were resuspended in 2 mL of phosphate-
buffered saline containing heparin and EDTA. Polymorphonuclear numbers were estimated by counting after specific staining with Turk’s solution using a Neubauer hemocytometer.

Muirne Primary Lung Endothelial Cell Culture
Endothelial cells were prepared from lungs of C57BL6 WT mice according to validated and standard techniques\textsuperscript{27,28} (see Supplemental Methods for details).

Flow Cytometry
Whole blood, air pouch supernatants, and murine endothelial cell cultures were collected and subjected to fluorescence-activated cell sorting analysis to assess both cell types and adhesion molecules expressed using a FACS Calibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson) (see Supplemental Methods for full details).

Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction
Expression of key neutrophil chemokine mRNA (CXCL1, CXCL2, CXCL5) in mouse mesenteric tissue was determined by real-time quantitative reverse transcription–polymerase chain reaction (see Supplemental Methods for details).

Statistical Analysis
All data are expressed as mean±SEM. Statistical analyses were performed using GraphPad Prism 5.0 (San Diego) and significance determined using the Student t test for differences between 2 data groups, 1-way ANOVA for more than 2 groups, and 2-way ANOVA for comparison between sexes with and without cytokine treatment fol-
lowed by Bonferroni post test where appropriate. The n values quoted similarly indicate the number of experiments and animals used.

Results

Sex Differences in Granulocyte Infiltration in Mouse Air-Pouch Model
IL-1\textbeta caused a significant increase in GR-1-positive (Figure 1A and 1B) leukocyte recruitment into air pouches of male but not female WT mice, as assessed at the 4-hour time point (Figure 1C). In contrast to intact female mice, cell recruit-
m ent was significantly raised in response to IL-1\textbeta in OVX mice (Figure 1B).

Plasma estrogen concentration was substantially lower in OVX (28.1±8.5 pg/mL, n=12) compared with sham-operated control mice (46.01±6.4 pg/mL, n=16, P<0.05). The remaining measured estrogen likely relates to extra-
gonadal generation due to the activity of aromatase.\textsuperscript{29} In addition, uterus weight was significantly decreased in OVX mice (9.4±5 mg, n=22) compared with sham-operated control mice (82.1±16.5 mg, n=21, P<0.001). There were no
differences in body weight between groups (OVX = 21.09 ± 0.8 g; sham-operated control = 20.7 ± 0.5 g). These results confirm successful surgery in OVX mice.

**Leukocyte Rolling Studies**

**Sex Differences in Leukocyte Rolling**

IL-1β (5 ng IP) treatment caused a time-dependent increase of leukocyte rolling and adhesion that was significantly greater in magnitude in male compared with female mice (2-way ANOVA, \( P < 0.05 \); Figure 2A and 2B). Histological assessment of whole mesentery demonstrated that neutrophils represented the predominant cell type recruited in these experimental conditions (Figure 2C). Accordingly, in a separate experiment, a time-dependent increase in MPO levels was evident from 0 to 4 hours following IL-1β treatment in male but not female mice (2-way ANOVA \( P < 0.05 \); Figure 2D).

**Figure 1.** Flow cytometry analysis of leukocytes infiltrated into air pouches. Six-day-old air pouches were injected with IL-1β (20 ng) at time 0, with lavage fluids being collected 4 hours later. A, Representative forward scatter (FSC)/side scatter (SSC) dot-plot of the whole cell population collected from air pouches and histogram of GR-1 positive expression (black line) versus control (grey line) of gated cells in the population R1. B, Density dot-plots of air pouch lavage fluids collected from vehicle control and IL-1β (5 ng)-treated male WT mice. C, Total number of GR-1 positive cells recovered from air pouches following vehicle or IL-1β treatment in male (n = 5), female (n = 5 control, n = 9 IL-1β), and OVX female (n = 5) mice. Data are mean ± SEM. Statistical significance was assessed using 2-way ANOVA among the 3 groups at \( P < 0.05 \) (interaction probability value was not significant) and with Bonferroni post tests shown as \( \# P < 0.05 \).

**Figure 2.** Leukocyte–endothelial cell interactions in mouse mesenteric postcapillary venules. WT female and male mice were treated with IL-1β (5 ng IP) at time 0 and then, at the reported times, instrumented for intravital microscopy analysis of the mesenteric microcirculation conducted for assessment of leukocyte rolling (A) and leukocyte adhesion (B) in female and male mice (n = 10 to 15 animals per group). C, Typical hematoxylin and eosin staining of mesenteric postcapillary venules (v) from a male WT mouse treated with IL-1β (5 ng, 1.5 hours). The arrows identify multilobed nuclei of granulocytes. Magnification \( \times 400 \). D, Time-course of the cell recruitment in response to IL-1β (5 ng) as determined by measurement of MPO in WT male (n = 12 to 15) and female (n = 8 to 15) mice. Leukocyte recruitment assessed using intravital microscopy 1.5 hours after administration of TNF-α (300 ng IP) and shown as leukocyte rolling (E) and leukocyte adhesion (F) in male (n = 8) and female (n = 10) mice. All data shown as mean ± SEM of n mice per group. Statistical significance determined using 2-way ANOVA is shown as \( * P < 0.05 \) for differences between the sexes (in all cases, interaction probability value was not significant), with \( \# P < 0.05 \) representing the Bonferroni post test (A and B), the Dunnett post test (D), and the unpaired Student test (E and F).
Because the greatest difference in cell rolling between the sexes was evident at 1.5 hours following cytokine treatment (26.9 ± 7.9 cell/min [n = 14] in males and 9.7 ± 1.3 [n = 9] cells/minute in females, Figure 2A), this time point was used to investigate the mechanisms involved in the sex differences in further experiments. This sex difference in leukocyte recruitment was also apparent in response to the distinct cytokine TNF-α (Figure 2E and 2F), where both leukocyte rolling and adhesion were significantly lower in female (n = 8) compared with male (n = 10) mice. There were no significant differences in venular hemodynamics between the sexes (Table 1).

**Role of Sex Hormones in Sex Differences on Leukocyte Rolling**

Although ovariectomy had no effect on basal leukocyte rolling compared with sham-operated animals, IL-1β-induced leukocyte rolling was significantly increased in OVX mice (2-way ANOVA, P < 0.01; Figure 3A). This effect of ovariectomy on the response to IL-1β was in part reversed by estrogen replacement (Figure 3B, n = 8 for both groups, P < 0.05).

**IL-1β Elevates Chemokine Expression in Both Sexes**

Quantitative polymerase chain reaction of mesenteric tissue of IL-1β-treated WT animals revealed increases in mRNA expression, above that measured in saline-treated controls (Figure 4), of all 3 of the neutrophil-specific chemokines measured. However, this IL-1β-induced chemokine elevation was similar in both sexes (2-way ANOVA: not significant; Figure 4).

**Effect of IL-1β on Circulating Blood Cells**

IL-1β (5 ng IP) provoked a significant rise in the number of circulating granulocytes in male compared with female WT mice. No significant differences in circulating monocyte or lymphocyte numbers were evident between the sexes (Table 2). Fluorescence-activated cell sorting analysis demonstrated P-selectin glycoprotein ligand-1 on the surface of all cell types measured in both sexes. In addition, t-selectin expression was significantly elevated by cytokine treatment on granulocytes in both sexes. Interestingly, however, the percentage of granulocytes expressing t-selectin under basal conditions was higher in male compared with female mice (Table 3).

**Sex Differences in P-Selectin Expression on**  
**Primary Murine Endothelial Cells**

Treatment with IL-1β (20 ng/mL; 1.5 hours of treatment) did not stimulate P-selectin expression (Figure 5A and 5B) in cultures of female primary endothelial cells (94.1 ± 0.5% purity as identified by positive intercellular adhesion molecule-2 expression; Figure 5C and 5D); in contrast, P-selectin expression was significantly elevated in corresponding male endothelial cells (2-way ANOVA, P < 0.05; Figure 5B).

**Sex Differences in Basal and IL-1β-Induced**  
**Leukocyte Rolling: Role of Endothelial NOS and COX-1 Enzymes**

Basal leukocyte rolling was significantly greater in male compared with female eNOS-/- mice (2-way ANOVA, P < 0.001; Figure 6A), and although IL-1β treatment appeared to cause a minor elevation of leukocyte rolling in both sexes, this did not reach significance (Figure 6A). Basal leukocyte rolling in female COX-1-/- mice was similar to male COX-1-/- mice (Figure 6B); however, whereas IL-1β caused a profound increase in rolling in males, no such effect was evident in age-matched female COX-1-/- animals (2-way ANOVA, P < 0.001; Figure 6B). These effects were, again, unrelated to differences in venular hemodynamics, which did not vary between the groups (Table 1).
Sex Differences in IL-1β-Induced Leukocyte Rolling of dKO Mice

IL-1β treatment significantly increased leukocyte rolling in male dKO mice, whereas in female dKO mice, rolling remained at basal levels up to 4 hours after cytokine treatment (Figure 6C). Venular hemodynamics were not altered in either genotype or sex in comparison with WT controls (Table 1).

Discussion

Female sex exerts a permissive influence over inflammatory responses, a phenomenon thought to be driven principally by the activity of female sex hormones and believed to play an important role in underlying the cardioprotection evident in premenopausal females.3,4 However, the exact mechanisms involved in this effect are unclear. In this report, we have demonstrated the existence of a sex difference in leukocyte recruitment under inflammatory conditions and implicated female sex hormones in mediating this effect. In addition, we have demonstrated that neither NO or PGI2 is involved in this effect. Female sex hormones play a role in mediating this protective phenotype. In addition, our findings also suggest that female sex hormones play a role in mediating this apparent reduced sensitivity to cytokine-induced cell recruitment because it has recently been demonstrated that, in rats, female sex worsens leukocyte recruitment in response to an ischemia/reperfusion insult in the hepatic microcirculation.31

Table 2. Total Number of Leukocytes in Whole Blood of Female and Male WT Mice Under Basal Conditions and After IL-1β Stimulation

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Female Control</th>
<th>Male Control</th>
<th>Female + IL-1β</th>
<th>Male + IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td>1.95±0.8</td>
<td>1.2±0.4</td>
<td>2.3±1.0</td>
<td>3.79±1.4*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>7.04±0.75</td>
<td>6.55±0.91</td>
<td>6.46±0.76</td>
<td>6.56±1.17</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1.96±0.52</td>
<td>1.63±0.74</td>
<td>2.98±0.22</td>
<td>2.87±0.58</td>
</tr>
</tbody>
</table>

Mice were left untreated or received IL-1β (5 ng IP, 1.5 hours). Data (No. of cells×10^6/ml) shown are mean±SEM for 5 animals per group. Statistical analysis using 1-way ANOVA followed by Bonferroni post tests shown.

Table 3. L-Selectin and PSGL-1 Expression in Circulating Leukocytes in Basal Conditions and After IL-1β Stimulation

<table>
<thead>
<tr>
<th>Cell Type (Adhesion Molecule)</th>
<th>Female Control</th>
<th>Male Control</th>
<th>Female + IL-1β</th>
<th>Male + IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes (L-selectin)</td>
<td>26.8±1.7</td>
<td>39.4±3.6</td>
<td>51.8±6.7</td>
<td>54.0±5.5</td>
</tr>
<tr>
<td>Granulocytes (PSGL-1)</td>
<td>99.9±0.1</td>
<td>99.6±0.1</td>
<td>99.6±0.07</td>
<td>99.8±0.07</td>
</tr>
<tr>
<td>Lymphocytes (L-selectin)</td>
<td>46.6±4.1</td>
<td>44.9±5.9</td>
<td>51.6±7.5</td>
<td>50.8±9.2</td>
</tr>
<tr>
<td>Lymphocytes (PSGL-1)</td>
<td>98.8±0.1</td>
<td>97.1±1.9</td>
<td>99.9±0.03</td>
<td>99.8±0.05</td>
</tr>
<tr>
<td>Monocytes (L-selectin)</td>
<td>31.9±10.6</td>
<td>20.9±1.2</td>
<td>45.8±7.7</td>
<td>42.1±6.4</td>
</tr>
<tr>
<td>Monocytes (PSGL-1)</td>
<td>98.4±0.3</td>
<td>97.3±1.0</td>
<td>99.1±0.2</td>
<td>99.0±0.2</td>
</tr>
</tbody>
</table>

Values report the percentage of L-selectin-positive and PSGL-1-positive events for each respective leukocyte population. Mice were left untreated or received IL-1β (5 ng IP, 1.5 hours). Data are shown as mean±SEM for 3 to 5 animals per group.
similar in magnitude to the response evident in males. Previous characterization of this model of inflammation indicates that the cellular infiltrate in response to IL-1β at this time point (4 hours) is predominantly neutrophilic, a view in part supported in the present study by fluorescence-activated cell sorting analysis indicating that the cells recruited were granulocytes. Interestingly, although substantial changes in cell number were evident in response to IL-1β, there were no significant differences in the number of cells recruited in response to the vehicle control, suggesting that sex hormones play an important role in controlling leukocyte recruitment principally under inflammatory conditions. There is considerable evidence suggesting that the beneficial effects of female sex hormones relate predominantly to the activity of estrogen, and to determine whether this hormone may underlie the effects of female sex in the present study, we investigated the effects of estrogen replacement in OVX females. Our data suggest that the sex differences are, in part, likely to be related to a suppressive effect of estrogen, because the effects of ovariectomy were partially inhibited by estrogen replacement. The dose of estrogen used in our studies to replace the levels of this hormone in female mice has previously been shown to restore physiological levels and to exert antiinflammatory effects in mouse models. The incomplete reversal of the effect of the cytokine may indicate that other nonestrogenic influences/factors, such as progesterone, also play a role.

Leukocyte recruitment to sites of tissue injury is a dynamic, multistep process involving leukocyte rolling, adhesion, and emigration. To investigate whether the altered cell recruitment evident in the air-pouch studies might be due specifically to a sex-dependent suppression of a specific step in the recruitment paradigm, we used the technique of intravital microscopy. Our findings demonstrate that enhanced leukocyte rolling and adhesion induced by IL-1β are suppressed in female WT mice compared with male mice, an effect that was not due to inherent differences in venular hemodynamics. Histological analysis of the mesenteric vasculature confirmed that the cellular target for this effect was the neutrophil, a fact substantiated by measurement of MPO in whole mesentry homogenates.

Our data support the thesis that estrogens exert tight control on the early process of cell recruitment (leukocyte rolling phenomenon), rather than having a generalized effect on all steps of the cell recruitment paradigm, ie, rolling, adhesion, and emigration. This is supported by the fact that although IL-1β treatment resulted in an increase in the levels of neutrophil chemokines that we have previously shown to be pivotal in IL-1β-induced cell adhesion (namely CXCL1,

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Flow cytometry analysis of murine primary endothelial cells. A, Representative histogram of P-selectin expression in the presence of IL-1β (20 ng/mL, 1.5 hours) of isotype (black), male (light grey), and female (dark grey) positive cells. B, Median fluorescence intensity (MFI) of P-selectin expression in the absence and presence of IL-1β (20 ng/mL, 1.5 hours). C, Representative forward scatter (FSC)/side scatter (SSC) dot-plot of isolated cell population (R1). D, Representative histogram of intercellular adhesion molecule-2 (ICAM-2) expression of positive cells (black) and isotype control (grey) in R1. Data shown are mean±SEM for 7 primary cultures of WT cells, each prepared from 3 animals. Statistical significance was determined using 2-way ANOVA of *P*=0.05 between the sexes (interaction probability value was not significant), with Bonferroni post test #P*=0.01 (C) (in all cases, interaction probability value was not significant), with Bonferroni post tests shown as #P*=0.001 (A) and **P*=0.001 (B) and **P*<0.01 (C) (in all cases, interaction probability value was not significant), with Bonferroni post tests shown as #P*=0.05.

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Basal and IL-1β-stimulated leukocyte rolling in mouse mesenteric postcapillary venules. Mice were left untreated or received IL-1β (5 ng IP), and the extent of cell rolling was determined 1.5 hours later in male (n=6 to 8) and female (n=6 to 9) eNOS+/+ mice (A), 1.5 hours later in male (n=6 to 7) and female (n=6 to 13) COX-1−/− mice (B), and 0 to 4 hours later in eNOS−/− /COX-1−/− (dKO, n=5 to 18) mice (C). Data are mean±SEM. Statistical significance determined using 2-way ANOVA demonstrated a significant difference between the sexes of **P*<0.001 (A and B) and **P*<0.01 (C) (in all cases, interaction probability value was not significant), with Bonferroni post tests shown as #P*<0.05.
CXCL2, and CXCL5), no difference between the levels of expression were evident between the sexes. These findings are congruent with observations in healthy volunteers demonstrating no difference in serum levels of CXCL1 or other CXC chemokines.

Our findings and proposed model are at odds with studies suggesting higher levels of certain CXCL chemokines in the sera of healthy women versus age-matched men at baseline or following an inflammatory stimulus of lipopolysaccharide, and studies demonstrating estrogen-induced suppression of rat vascular CXCL chemokine expression in response to the inflammatory stress of vascular balloon injury in vivo or expression by cultured smooth muscle cells in response to TNF-α. The reason for this discrepancy is uncertain but may reflect differences in the inflammatory stimulus or the stage at which the inflammatory response was sampled following initiation of the response.

We considered whether the differences in cell recruitment might simply reflect differences in circulating cell numbers. Indeed, our studies demonstrated that although under control conditions there were no significant differences in the circulating numbers of granulocytes, monocytes, or lymphocytes between sexes, following IL-1β treatment granulocyte numbers rose significantly in male but not female mice. Supporting these findings are observations that the risk of coronary artery disease is correlated to increasing circulating granulocyte numbers and particularly MPO levels (implicating neutrophils) in men, an effect that is not evident in women. It is unlikely that the differences in the extent of circulating cells might relate to specific sex differences in the levels of leukocyte adhesion molecule expression because no differences in either L-selectin or PSGL-1, the key leukocyte adhesion molecules involved in neutrophil rolling, were found in our study. Thus, it is likely that the lower numbers of circulating cells, in part, underlie the lower numbers recruited in response to IL-1β. However, sex differences in the numbers of cells recruited (air-pouch experiments) in response to IL-1β persists even after normalization for circulating cell numbers giving values of near 1×10^5 cells for males and 8×10^4 cells for females. This observation suggests that sex differences in cell recruitment pathways likely play a role.

Several studies have demonstrated that the beneficial effects of female sex hormones within the circulation relate to upregulation of pathways at the endothelial cell that are critical in maintaining an antiinflammatory phenotype of the blood vessel wall. Evidence supports the view that estrogens upregulate the synthesis, release, and activity of protective endothelial factors and suppress the expression of pathogenic mediators by the endothelium. NO and PGI2 have been identified as important targets for estrogen activity; however, our investigations using mice with targeted disruption of eNOS, COX-1, or both (the principal enzymes involved in endothelial generation of NO and PGI2, respectively) demonstrated that neither of these endothelial mediators played a role in mediating the protection against inflammatory cell recruitment in females. In contrast, the loss of either or both of these proteins resulted in substantial increases in leukocyte rolling under basal conditions and following cytokine treatment in male mice. These findings suggest that although both NO and PGI2 play important roles in sustaining the antiinflammatory phenotype of endothelial cells in male mice, they are redundant in female animals. This profile of reactivity with respect to inflammatory cell recruitment mimics our findings with respect to other differences evident in the vasoprotective effects of endothelial mediators between the sexes. Recently, we proposed that although NO and PGI2 play essential roles in maintaining a vasodilated microvasculature of male mice, it is predominantly EDHF that subserves this role in female mice. Moreover, that this provision of EDHF is the predominant endothelial-derived vasorelaxant factor that confers protection against hypertension in females. It is possible, therefore, that the differences between the sexes demonstrated in the present study reflect a difference in EDHF bioactivity and support the view that the remit of EDHF extends beyond vasodilatation.

The identity of EDHF remains a hotly disputed issue, with a number of distinct candidates having been proposed since the term EDHF was first coined in 1988. One of the putative EDHF candidates, at least in certain vascular beds, is C-type natriuretic peptide (CNP). Our previous work has demonstrated that exogenous CNP prevents both leukocyte recruitment and platelet aggregation. Thus, it is tempting to speculate that CNP, as an EDHF, may underpin the antileukocyte, antiatherogenic phenotype of the endothelium of females. Further investigation of the role of CNP in leukocyte recruitment and any sex differences that may be apparent is clearly warranted.

Early leukocyte rolling in vivo is mediated by the interaction of endothelial (particularly P-selectin) and leukocyte adhesion molecules (particularly PSGL-1). Our evidence suggests that although alterations in leukocyte adhesion molecules are unlikely to mediate the comparatively lower cell recruitment in females, changes in endothelial adhesion molecule expression might explain this phenomenon. Using primary endothelial cells isolated and cultured from male and female WT mice, we demonstrated that whereas P-selectin expression is increased significantly in response to IL-1β treatment of endothelial cells of male mice, expression of this adhesion molecule was largely unchanged following treatment of endothelial cells from female mice. These results support the view that female sex hormones exert protection, at least in part, by modulating expression of P-selectin on endothelial cells during leukocyte rolling. Previous work from our laboratory has demonstrated that the reduction of leukocyte rolling exerted by CNP in mesenteric postcapillary venules is due to a suppression of P-selectin expression, an observation furthering a role for EDHF in the cytoprotective effects of female sex on leukocyte recruitment. A limitation of our findings, however, is that lung endothelial cells rather than cells of mesenteric origin were used to assess the P-selectin response to the cytokine. These cells were used because of the difficulty in obtaining sufficient purity and numbers of cells from the venular side of the mesenteric circulation.

In summary, the present study demonstrates that leukocyte rolling is modulated by female sex hormones in the present of an inflammatory stimulus and that this effect is independent
of both NO and PGI₂, and possibly due to EDHF activity. In turn, we propose that our evidence supports modulation of the expression of P-selectin on endothelial cells by sex hormones to prevent leukocyte rolling, which has downstream consequences for the extent of cell adhesion and emigration. A growing body of evidence suggests that leukocyte recruitment is likely to be pathogenic in atherosclerotic disease. Indeed, circulating neutrophil numbers and levels of neutrophil chemokines⁵⁸ are correlated with severity in acute coronary syndromes, as well as being evident in thrombi and at sites of plaque and rupture in patients.⁵⁹–⁶¹ In addition, leukocytes have been identified at lesional sites in the early stages of plaque formation, and neutrophil depletion is associated with decreased atherosclerotic load in mouse models of disease.⁵²–⁵⁵ The role of P-selectin in these phenomena is uncertain, especially because genetic deletion of this adhesion molecule appeared to have no impact on the beneficial effects of estradiol treatment on atherosclerosis in a mouse model of disease,⁶⁶ whereas vascular cell adhesion molecule-1 has been implicated.¹⁶,⁶⁶ However, the possibility that other adhesion molecules might have been upregulated and, therefore, compensated for the absence of P-selectin was not investigated. We suggest that because leukocyte recruitment has been implicated in cardiovascular disease and our findings suggest that leukocyte recruitment is specifically attenuated in inflammation in females, these effects of sex on leukocyte recruitment may play a role in mediating the cardioprotection evident in premenopausal females.

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Disclosures
None.

References


Suppression of Endothelial P-Selectin Expression Contributes to Reduced Cell Trafficking in Females: An Effect Independent of NO and Prostacyclin

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Suppression of endothelial P-selectin underlies reduced cell trafficking in females: an effect independent of NO and PGI2

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Mesenteries were superfused with bicarbonate buffer at 37°C [in g/liter: NaCl, 7.71; KCl, 0.25; MgSO4, 0.14; NaHCO3, 1.51; and CaCl2, 0.22 (pH 7.4); gassed with 5% CO2 and 95% N2] at a rate of 2 ml/min. The temperature of the stage was maintained at 37°C. Red blood cell (rbc) velocity was measured in venules by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station). Venular blood flow was calculated from the product of mean rbc velocity ($V_{\text{mean}} = $ centerline velocity/1.6) and microvascular cross-sectional area, assuming a cylindrical geometry. Wall shear rate was calculated by the Newtonian definition: shear rate = 8,000 × ($V_{\text{mean}}$/diameter). Three to five randomly selected post-capillary venules (diameter 20-40µm, length >100µm) were observed for each mouse; measurements were taken 5–10min after exposure of the chosen vessels.

Murine Primary Lung endothelial cell culture

For each experiment lungs from three mice were collected, placed in ice-cold Dulbecco’s modified Eagle’s medium (DMEM) and the tissue minced and then digested for 1 h at 37°C in 0.1% collagenase-A (Sigma, UK). The cellular digest was passed through a blunt 14-gauge needle followed by filtration through a 70-µm nylon mesh, then centrifuged at 800 g for 10 min at room temperature. The pellet was resuspended in DMEM/F12 medium (37°C) [containing 20% FBS, 50 µg/ml endothelial mitogen (Biomedical Technologies, UK), 100 µg/ml heparin, and penicillin-streptomycin] and plated in 0.1% gelatin-coated T75 flasks. Cells were washed after 24 h and cultured for 2–4 days. Magnetic beads (5 µg/4x10⁶ beads, Dynabeads, Invitrogen, UK) were coated with anti-mouse CD102 (clone 3C4; Pharmingen, UK) antibody. Per flask, 4x10⁶ beads were added and incubated for 1 h at 37°C. Cells were trypsinized and selected in a magnetic field for 10 min, washed, and then plated in 0.1% gelatin-coated 6-well plates. Experiments were performed on cultures that were 80-90% confluent (~100,000/well).

Flow Cytometry

Whole blood and murine air pouch supernatants

Blood (50 µl) collected by cardiac puncture from WT mice or samples from air pouch experiments (200 µl) were incubated with PBS containing 0.1% sodium azide, 10% rat serum and the blocking antibody FcgIIIR mAb (CD16/CD32) for 30 min at 4°C. Cells were then labelled with FITC-conjugated
CD62L (2.5 µg/mL eBioscience, UK) for L-selectin or RPE-conjugated CD162 (0.5 µg/mL ebioscience, UK) for PSGL-1 combined with one of the following PE or FITC-conjugated mAb for 30 min at 4°C: anti-GR-1 for granulocytes (Clone RB6-8C5; ebioscience, UK), anti-CD3e for lymphocytes (Clone 145-2C11; ebioscience, UK) or anti-F4/80 for monocytes and macrophages (Clone BM8; ebioscience, UK). After labelling, erythrocytes were lysed (blood samples only) with (500 µl) lysing reagent for 1-2 min and 125 µl of fixative (Beckman Coulter, UK), then all cells were washed, centrifuged, and suspended in PBS containing 0.1% sodium azide for analysis with FACS calibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuestTM software (Becton Dickinson).

**Murine primary endothelial cells.** Endothelial cells were harvested in trypsin/EDTA and subcultured in six-well plates (Falcon, UK). Cells were then treated with murine IL-1β (20 ng/ml) for 90 min. Cells were then washed in 2 ml DMEM/F12 for 1 min. Rat anti-mouse monoclonal antibody against P-selectin [FITC-conjugated CD62P (clone RB40.34, BD Pharmingen, UK) was diluted with PBS containing 0.1% sodium azide and incubated in the dark at 37°C for 30 min with cells. After this time endothelial cells were collected using trypsin/EDTA (0.025% trypsin 0.01% EDTA Invitrogen; 500 µl per well) at 37°C for 2 min and then trypsin activity blocked by the addition of 500 µl of Trypsin Neutralizing Solution (Invitrogen). Following this FACS analysis was performed.

In all cases, forward- and side-scatters were set to exclude erythrocytes and/or dead cells. Positive and negative populations were identified based on single or dual-colour staining performed with a PE- and FITC-conjugated IgG isotype (ebioscience, UK). The cell type and expression pattern of PSGL-1, L-selectin and P-selectin were determined by measuring the PE and FITC mean fluorescence intensity (MFI) of the specific antibody and the percentage of positive cells.

**Real-time quantitative RT-PCR**

Expression of key neutrophil chemokine mRNA was determined by real-time quantitative RT-PCR. Briefly, mesenteric vascular beds were removed from control or IL-1β-treated (5ng, 90 min) mice as described above, snap frozen, and stored at -80°C until use. Samples were homogenized using ceramic beads (Precellys 24, Bertin Technologies, UK) and total RNA isolated using a NucleoSpin RNA II purification kit (Macherey-Nagel, UK). cDNA was synthesized from 1 µg of total RNA with Moloney
Murine Leukemia virus reverse transcriptase (Promega, UK) using oligo(dT) nucleotides. The following primers were used:

**CXCL1 (KC):**
5'-TGAGCTGCGCTGTCAGTGCCT-3'
5'-AGAAGCCAGCGTTACCAGA-3';

**CXCL2 (MIP-2):**
5'-GAGTTTGAGTGTGACGCCCCCAGG-3'
5'-GTAGCCTTGCCTTTGTTCAGTATC-3'

**CXCL5 (LIX):**
5'-GCATTCTGTTGCTGTCAGCT-3'
5'-CCTCCTTTCTGTTTTTCAGTTAGC-3';

**18S:**
5'-AGCCTGCGGCTAATTTGAC-3'
5'-CAACTAAGAACGGCCATGCA-3'.

Standard curves for were generated to determine the amplification efficiencies of target and reference genes. Quantitative PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, UK) utilizing SYBR green (ABgene, UK) with 100nM primers and 20ng of cDNA. Chemokine expression was normalized to 18S and expressed as a relative value using the comparative threshold cycle (Ct) method ($2^{-\Delta \Delta Ct}$). The levels of mRNA expression of genes of interest were normalized to male control.

Reference List