Estrogen Stimulates Neuronal Nitric Oxide Synthase Protein Expression in Human Neutrophils


Abstract—Recent studies have postulated the contribution of nitric oxide (NO) released by the endothelium to the beneficial effects of estrogen. Despite a neuronal-type NO synthase (nNOS) described in neutrophils, less is known about the effect of estrogen in these cells. The aim of the present study was to analyze the expression of nNOS protein in human neutrophils under different estrogenic conditions. We first analyzed nNOS expression in neutrophils obtained from premenopausal women. During the first 2 days of the follicular phase (low circulating estrogen concentrations), nNOS expression in neutrophils was reduced with respect to that found in neutrophils obtained from the same donors during the ovulatory phase (high circulating estrogen concentrations). Moreover, the expression of nNOS protein in neutrophils obtained from postmenopausal women after transdermal estrogen therapy was markedly enhanced. Thus, with respect to that observed before the treatment. In vitro incubation of neutrophils derived from men for 6 hours with 17β-estradiol (10⁻¹⁰ to 10⁻⁸ mol/L) upregulated the expression of nNOS protein. The 17β-estradiol receptor antagonists, tamoxifen (10⁻⁸ mol/L) and ICI 182780 (10⁻⁸ mol/L), inhibited the upregulation of nNOS protein induced by 17β-estradiol. The putative functional implication was denoted by a reduced expression of the CD18 antigen on the surface of 17β-estradiol-incubated neutrophils, which was accompanied by a decreased adhesive capacity. Both effects were prevented by an NO antagonist. In conclusion, the in vivo levels of circulating estrogen concentrations seem to be associated with the level of nNOS protein expression in neutrophils from women. Moreover, low doses of 17β-estradiol upregulated nNOS protein expression in neutrophils from men. The increased ability of 17β-estradiol–incubated neutrophils derived from men to produce NO reduced their adhesive properties. (Circ Res. 1999;85:1020-1026.)

Key Words: estrogen ■ leukocyte ■ nitric oxide synthase ■ premenopause ■ postmenopause

Death from cardiovascular disease is relatively rare in premenopausal women compared with age-matched men.¹ However, after menopause the risk of coronary heart disease increases dramatically.²,³ Several data from ongoing clinical studies have shown that hormone replacement therapy reduces cardiovascular disease in postmenopausal women, but the final causative mechanism remains unclear.⁴–⁶ The benefits of estrogen have been attributed to its effects on lipids and lipoproteins. However, the change in the lipid profile reportedly accounts for only 25% to 50% of the protective effect of estrogen against cardiovascular disease,⁷ and new data suggest that estrogen may also exert its beneficial effects through a direct action on the vessel wall.⁸,⁹

Nitric oxide (NO) is generated by the metabolic conversion of L-arginine to L-citrulline by the activity of the enzyme NO synthase (NOS). Recent molecular cloning studies have reported that human DNA encodes 2 distinct constitutive NOS isoforms, one from neurons (nNOS) and the other from endothelial cells (eNOS), as well as an inducible NOS (iNOS) isoform that requires cytokines or endotoxin activation for its expression.¹⁰,¹¹

Although the nNOS and eNOS isoforms were initially described as constitutive, in recent years the inducible character of both constitutive isoforms has been shown. In this regard, increasing evidence indicates that nNOS expression can be regulated by various physiological and pathophysiological conditions, including sympathetic activity and acute heat stress.¹²,¹³ Moreover, tumor necrosis factor-α downregulates eNOS expression,¹⁴ which could be upregulated by several factors, including estrogen.¹⁵

Similarly to endothelial cells, neutrophils produce NO by the activity of a constitutive NOS isoform. Recently, Wallerath et al.¹⁶ have identified the constitutively expressed NOS isoform in neutrophils as neuronal type. The NO released by neutrophils has been demonstrated to prevent neutrophil adhesion to vascular endothelium, to control ag-

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gregation of neighboring platelets and, in the absence of endothelium, to produce vasodilatory effects.\textsuperscript{11,17,18}

Estrogen causes short-term coronary vasodilatory effects in humans mediated by an increase in NO production.\textsuperscript{19} Moreover, long-term exposure to estrogen increases acetylcholine-mediated coronary vasodilation in nonhuman primates, male-to-female transsexuals, and postmenopausal women.\textsuperscript{20--22} These effects have been attributed to an increase in Norris expression by the endothelium.\textsuperscript{9,23} However, despite the growing interest in the action of estrogen on endothelial cells, less is known about the effect of estrogen on neutrophils. In this regard, several data support the involvement of estrogen in the modification of neutrophil activity during the menstrual cycles and pregnancy.\textsuperscript{24,25} Binding studies performed by Klebanoff\textsuperscript{26} demonstrated the presence of estrogen receptors (ERs) on neutrophils. However, at present there are no studies about the effect of estrogen on the NO-generating system in human neutrophils. Therefore, the aim of the present study was to evaluate the effect of estrogen on nNOS expression in human neutrophils. For this purpose, we analyzed the expression of nNOS protein in neutrophils obtained from premenopausal and postmenopausal women under different situations of circulating estrogen concentrations. Moreover, in in vitro experiments the effect of estrogen on the expression of nNOS protein in neutrophils obtained from men was also determined.

\textbf{Materials and Methods}

\textbf{Neutrophil Isolation}

Neutrophils were isolated as previously described\textsuperscript{18,27} from peripheral blood from the following groups: (1) 10 healthy premenopausal women (25 to 40 years of age) during different stages of the menstrual cycle, (2) 10 postmenopausal women (45 to 65 years of age) before and after 4 months of hormone replacement therapy, and (3) from 10 healthy men (25 to 40 years of age) for in vitro estrogen incubations. The protocols were approved by the Institutional Ethics Committee.

The nNOS protein determination was performed by Western blot as reported.\textsuperscript{14,28}

\textbf{Neutrophils Obtained From Premenopausal Women}

Neutrophils were obtained from the same donor at the following 2 different points: (1) the ovulatory phase, determined by the urinary peak of luteinizing hormone (LH) (by disposable test sticks; Donnate Wyntek Diagnostic), and (2) within the first 2 days of the follicular phase (menstruation). Serum estradiol concentrations were determined with an ELISA kit (Oxford Biomedical Research, Inc.).

\textbf{In Vivo Estradiol Treatment in Postmenopausal Women}

All women had not had a menstrual period for at least 4 years and had not received any hormone replacement treatment within this period of time. Neutrophils were obtained before and after 4 months of hormone replacement therapy (50 \textmu g/day estradiol transdermal patches; Estraderm Matrix 50, Novartis Pharma SA). Medroxyprogesterone acetate (Provera, Upjohn) was administered orally for 10 days of each cycle (10 mg/day). Blood samples were obtained 2 days before women received the progesterin.

\textbf{In Vitro Incubation of Neutrophils From Healthy Men With Estrogen}

Neutrophils ($5 \times 10^6$ cells/tube) from men were incubated with increasing concentrations of 17\textbeta-estradiol ($10^{-10}$ to $10^{-5}$ mol/L) or the solvent of 17\textbeta-estradiol (ethanol at a final concentration <0.01%) for 6 hours in an incubator at 37°C with 5% CO\textsubscript{2}. ER protein expression in male neutrophils was determined by Western blot as already described for the nNOS protein using the polyclonal antibody MC-20 (Santa Cruz Biotechnology Inc). The antisera may recognize a common sequence of \alpha and \beta receptor subtypes.\textsuperscript{29}

\textbf{Determination of NOS Activity}

The NOS activity was determined as the conversion of [\textsuperscript{3}H]-arginine into [\textsuperscript{3}H]-citrulline as previously described.\textsuperscript{18,27} Neutrophils from men were incubated with 17\textbeta-estradiol ($10^{-8}$ mol/L) or with the solvent (ethanol at final concentration <0.01%), for 6 hours at 37°C. During the last 45 minutes, neutrophils were loaded with [\textsuperscript{3}H]-arginine (1 \muCi/mL) followed by a 20-minute incubation at 37°C.

\textbf{CD18 Antigen Expression and Adhesion Assays}

The presence of the CD18 antigen in the surface of neutrophils was determined by flow cytometry as described.\textsuperscript{30} Male neutrophils were incubated with $10^{-8}$ mol/L 17\textbeta-estradiol or the solvent ethanol (final concentration, <0.01%) for 6 hours at 37°C. For adhesion experiments, 2 \muCi/mL \textsuperscript{51}Cr (Amersham Life Science, Ltd) was added to the incubation medium in the last 45 minutes to label neutrophils. \textsuperscript{51}Cr-labeled neutrophils (0.5 mL) were added on a plastic surface for 30 minutes at 37°C under static conditions as described.\textsuperscript{30}

\textbf{Statistical Methods}

Results are expressed as mean±SEM. Unless otherwise stated, each value corresponds to a minimum of 6 different experiments. The statistical significance was determined by ANOVA with the Bonferroni correction for multiple comparisons or a Student $t$ test, paired or unpaired. A $P$ value <0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

\textbf{Results}

\textbf{nNOS Expression in Neutrophils From Premenopausal and Postmenopausal Women}

We first determined the expression of nNOS protein by Western blot in neutrophils obtained from premenopausal women at 2 different phases of the menstrual cycle, one in which circulating concentrations of estrogen are elevated (ovulatory phase) and the other within the first 2 days of the follicular phase (menstruation), when circulating estrogen concentrations fall.

Neutrophils obtained from premenopausal women expressed a 155-kDa nNOS-like protein (Figure 1A). As shown in Figure 1A and 1B, during the ovulatory phase (positive urinary detection of LH), the level of nNOS expression in neutrophils was markedly enhanced with respect to that found in the neutrophils obtained from the same donor during the first 2 days of the follicular phase. At ovulation, serum estradiol levels were 520±30 pg/mL (1.9×10\textsuperscript{-9} mol/L). In the first 2 days of the follicular phase, serum estradiol concentration fell to 160±10 pg/mL (5.9×10\textsuperscript{-10} mol/L).

We then determined the effect of estrogen therapy on nNOS expression in neutrophils obtained from postmenopausal women before and after transdermal estradiol treatment. In all cases, each individual was also used as her own control before and after the treatment. In these experiments, neutrophils obtained after estrogen replacement therapy showed a significant increase in nNOS protein expression (Figure 1A and 1B), which was accompanied by a higher ability to produce NO ([\textsuperscript{3}H]-citrulline accumulation, pre-
Serum estradiol levels after estradiol treatment were 125.466.2 pg/mL, which on a molar basis corresponds approximately to 5.3x10^-11 mol/L of estradiol. Before treatment, estradiol serum concentration was 15.362.1 pg/mL (5.6x10^-11 mol/L).

The monoclonal antibody used did not cross-react with the eNOS or iNOS isoform, because an nNOS protein band was undetectable in homogenates of human endothelial cells and lipopolysaccharide (LPS)–treated rat macrophages (Figure 2). The monoclonal antibody used in our experiments specifically recognized the nNOS isoform (155 kDa) obtained from homogenates of rat pituitary (Figure 2).

**nNOS Protein Expression in 17β-Estradiol–Incubated Neutrophils From Men**

Under basal conditions, the expression of nNOS-like protein (155 kDa) was detected in neutrophils from men (Figure 3A and 3B). Although incubation with 17β-estradiol (10^-8 mol/L) did not significantly changes nNOS protein expression in the first 4 hours, nNOS protein was markedly enhanced 6 hours after the addition of 10^-8 mol/L 17β-estradiol and on the following times (Figure 3A and 3B). No effect of ethanol (final ethanol concentration, <0.01%) on nNOS expression by neutrophils from men was detected in the different periods of incubation (data not shown).

Incubation of neutrophils derived from men with 17β-estradiol (10^-10 to 10^-8 mol/L) enhanced the expression of nNOS protein in a dose-dependent fashion (Figure 4A and 4B). The maximal increase in nNOS protein expression was reached with 10^-8 mol/L 17β-estradiol (Figure 4A and 4B).

Conversely, higher doses of 17β-estradiol (10^-7 to 10^-5 mol/L) did not significantly changes nNOS protein expression in the first 4 hours, nNOS protein was markedly enhanced 6 hours after the addition of 10^-8 mol/L 17β-estradiol and on the following times (Figure 3A and 3B). No effect of ethanol (final ethanol concentration, <0.01%) on nNOS expression by neutrophils from men was detected in the different periods of incubation (data not shown).

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estradiol (10⁻⁸ mol/L) tended to reduce nNOS protein expression to the basal levels (Figure 4A and 4B).

The above-described effect of estradiol on nNOS protein expression was stereospecific, because 10⁻⁸ mol/L 17β-estradiol but not 10⁻⁸ mol/L 17α-estradiol increased nNOS protein expression in neutrophils from men (Figure 5A and 5B).

We further analyzed the accumulation of [³H]-L-citrulline in [³H]-L-arginine–loaded neutrophils as a measure of NO generation. After 6 hours of incubation with 17β-estradiol (10⁻⁶ mol/L), neutrophils from men showed a significant increase in the conversion of [³H]-L-arginine to [³H]-L-citrulline ([³H]-citrulline, basal, 36±5%; 17β-estradiol treatment, 59±4%; n=6, P<0.05). This effect was reversed by the presence of an NO antagonist, N⁵-monomethyl-L-arginine (L-NMMA) (10⁻⁴ mol/L) (inhibition 93±4%, n=6, P<0.01), demonstrating the specificity of the reaction.

We also determined whether the increased NO production by 17β-estradiol–stimulated neutrophils could occur through the expression of the iNOS isoform. Neutrophils from men failed to express the iNOS isoform under basal conditions or after exposure to 17β-estradiol (10⁻⁸ mol/L), suggesting that the effects of 17β-estradiol on the ability of neutrophils from men to produce NO occurs via the constitutive isoform.

Role of ERs

To determine the role of ER in the response to 17β-estradiol by neutrophils derived from men, nNOS protein expression was tested in the neutrophils under basal conditions and after exposure to 10⁻⁸ mol/L 17β-estradiol in the presence and in the absence of 2 different ER antagonists, tamoxifen (Tx, 10⁻⁸ mol/L) and ICI 182780 (ICI, 10⁻⁶ mol/L). B, Bar graph showing the densitometric scanning of the Western blot. Data are mean±SEM of 6 different experiments. *P<0.05 with respect to neutrophils incubated with the solvent of 17β-estradiol, ethanol (final ethanol concentration, <0.01%). **P<0.05 with respect to 17β-estradiol in the presence of ICI 182780.

(Figure 6). The monoclonal antibody used to detect the 130-kDa iNOS isofrom (Transduction Laboratories, 1:1.500) reacted with a positive control obtained from homogenates of LPS-treated mouse macrophages, whereas it failed to recognize a band in homogenates of human endothelial cells and rat pituitary (Figure 6).

We then determined whether ER protein is expressed in human neutrophils. Neutrophils were incubated in the presence or in the absence (control, C) of 17β-estradiol (10⁻⁸ mol/L) and 17α-estradiol (10⁻⁸ mol/L) for 6 hours. Additional experiments were performed in the presence and in the absence of the ER antagonists tamoxifen (Tx, 10⁻⁸ mol/L) and ICI 182780 (ICI, 10⁻⁶ mol/L). B, Bar graph showing the densitometric scanning of the Western blot. Results are represented as mean±SEM of 6 different experiments. *P<0.05 with respect to neutrophils incubated with the solvent of 17β-estradiol, ethanol (final ethanol concentration, <0.01%).

As mentioned above, 17β-estradiol (10⁻⁸ mol/L) caused an increase in nNOS protein expression when compared with the level found in basal conditions (Figure 7A and 7B). Although this effect was partially inhibited by 10⁻⁸ mol/L tamoxifen, it was completely reversed by 10⁻⁸ mol/L ICI 182780 (Figure 7A and 7B). In the absence of 17β-estradiol, the level of nNOS protein expression was not statistically modified by either of the ER antagonists (Figure 7A and 7B).

Both tamoxifen and ICI 182780 reduced NO generation by 10⁻⁸ mol/L 17β-estradiol–incubated neutrophils, although the level of inhibition achieved with 10⁻⁸ mol/L ICI 182780 was greater than with 10⁻⁸ mol/L tamoxifen (inhibition, tamoxifen, 40±6%; ICI 182780, 85±7%; n=6, P<0.05).

We then determined whether ER protein is expressed in neutrophils from men. Western blot analysis showed the

![Figure 6](https://example.com/figure6.png)  
Figure 6. Representative Western blot of the expression of iNOS in human neutrophils before and after exposure to 10⁻⁸ mol/L 17β-estradiol (17βE2) for 6 hours. The monoclonal antibody did not recognize the endothelial isoform in human umbilical endothelial cells and the neuronal isoform in rat pituitary, but it specifically recognized the iNOS isoform in homogenates of LPS-treated mouse macrophages. C indicates control.
presence of a 67-kDa band for ER protein (Figure 8A and 8B). Interestingly, incubation of neutrophils derived from men for 6 hours with 17β-estradiol enhanced the expression of the 67-kDa ER protein (Figure 8A and 8B). This effect was inhibited by tamoxifen and ICI 182780 (Figure 8A and 8B). As it occurred for nNOS protein expression, the reduction in ER expression achieved with 10^{-8} mol/L ICI 182780 on 10^{-8} mol/L 17β-estradiol–incubated neutrophils was of higher magnitude than that obtained with 10^{-8} mol/L tamoxifen (Figure 8A and 8B). Under basal conditions, a weak reduction in ER protein expression was observed with ICI 182780 (10^{-8} mol/L), although it did not reach statistical significance (Figure 8A and 8B). In the absence of 17β-estradiol, tamoxifen also failed to modify ER protein expression (Figure 8A and 8B).

### CD18 Antigen Expression in 17β-Estradiol–Incubated Neutrophils

Analysis by flow cytometry demonstrated that 17β-estradiol (10^{-8} mol/L)–treated neutrophils from men have a reduced expression of CD18 antigen when compared with the neutrophils incubated with the solvent of 17β-estradiol (Figure 9). The fluorescence intensity observed in the neutrophils incubated with the solvent of 17β-estradiol was 160±6, and it was significantly reduced in 10^{-8} mol/L 17β-estradiol–treated neutrophils (fluorescence intensity, 84±3; n=5, P<0.05). These results suggested that 17β-estradiol decreased the expression of CD18 antigen in neutrophils derived from men. This effect was fully prevented by the presence of the L-arginine–competitive analogue 10^{-4} mol/L L-NMMA (fluorescence intensity, 182±8). On the other hand, a decreased neutrophil adhesion to the plastic surface was observed 6 hours after incubation with 10^{-8} mol/L 17β-estradiol (neutrophils per well, control, 74±7×10^4; 17β-estradiol–treated neutrophils, 50±5×10^4; n=5, P<0.05). The antiadhesive effect of 17β-estradiol on neutrophils was completely prevented by 10^{-4} mol/L L-NMMA (17β-estradiol+L-NMMA, 80±6×10^4 neutrophils per well).

### Discussion

In the present study, we first evaluated the expression of nNOS protein in neutrophils obtained from healthy premenopausal women during situations of low and high levels of circulating estrogen. Wallerath et al^{15} have previously demonstrated that the nNOS type is constitutively expressed in neutrophils, which was recently confirmed by other authors.^{31} Our results showed that in the first 2 days of the follicular phase (low serum estrogen levels), the expression of nNOS protein in neutrophils was lower than during ovulation (high serum estrogen concentration), suggesting that there may exist an association between the level of estrogen and nNOS expression in neutrophils from premenopausal women. This was further confirmed in the experiments with neutrophils from postmenopausal women before and after transdermal estradiol application in which estrogen replacement therapy...
increased the expression of nNOS protein by the neutrophils, providing them with a higher ability to produce NO.

Few studies have evaluated the influence of estrogen on NO metabolism in both premenopausal and postmenopausal women. In this regard, Cicinelli et al. have reported that the highest plasma concentration of NO metabolites corresponded to the midcycle (the ovulatory phase). Moreover, it has been recently suggested that transdermal administration of estradiol in postmenopausal women induces a further significant increase in NO plasma levels, postulating that the source of NO was the endothelium. The present work provides further evidence that NO released from neutrophils stimulated by estrogen could contribute to total NO.

The in vitro experiments with neutrophils from healthy men tried to illustrate the gender specificity of the in vivo findings. Whereas low 17β-estradiol concentrations upregulated nNOS protein expression, concentrations above 10⁻⁷ mol/L tended to reduce nNOS expression in neutrophils obtained from men, which might suggest that the effect of estrogen on nNOS expression could be determined by the hormonal level.

Although nNOS-encoding genes were originally considered to be constitutive, it has become evident that their expression is regulated in different cells by a variety of stimuli. Moreover, estrogen has been shown to increase eNOS expression in endothelial cells; however, to our knowledge this is the first report demonstrating the upregulatory ability of estrogen on nNOS expression in neutrophils.

The mechanism by which estrogen stimulated nNOS protein expression in neutrophils remains to be determined, given that the 5'-flanking region of nNOS does not contain a canonical estrogen response element. However, many estrogen-regulated promoters do not contain perfect palindromic estrogen response element; therefore, an investigation of transcriptional transactivation of nNOS by estrogen is now indicated to further elucidate the underlying mechanisms.

In the present investigation, we also determined whether ERs are involved in the upregulatory effect of 17β-estradiol on nNOS expression and NO synthesis. Estrogen-mediated stimulation of nNOS protein expression in neutrophils derived from men occurs by a receptor-dependent mechanism, given that it was inhibited by the ER antagonists tamoxifen and ICI 182780. Interestingly, at equimolar concentrations, ICI 182780 seems to be more potent to inhibit 17β-estradiol–induced nNOS expression in human neutrophils than tamoxifen, which was confirmed in the experiments of NO production. McClelland et al. have also recently reported a greater inhibition of estrogen-induced events by ICI 182780 than by tamoxifen, which could be related to the known agonist/anagonistic effects of tamoxifen.

The presence of ER in neutrophils has been previously established by binding assays. Moreover, there is recent evidence suggesting that estrogen regulates a number of neutrophil functions by ER-dependent mechanisms that include phagocytosis and chemotaxis. We here demonstrate for the first time the expression of ER protein in neutrophils from men by Western blot analysis. Interestingly, estrogen seems to upregulate the expression of its receptors in neutrophils. Similar findings have been observed in cultures of testis of a nonmammalian vertebrate and in endothelium of ovine pulmonary arteries.

The ER subtypes expressed in neutrophils remain to be established. Both ERα and ERβ are inhibited by tamoxifen and ICI 182780. Thus, the mentioned effects of estrogen on nNOS expression in neutrophils from men could be mediated by either ERα, ERβ, both receptor subtypes, or a yet-unknown ER subtype.

Neutrophils play an important role in the development of inflammation. NO released by neutrophils has been previously demonstrated to prevent neutrophil adhesion by reducing the expression of CD11/CD18 adhesive proteins. The flow cytometric study demonstrated that 17β-estradiol caused a reduction of CD18 antigen expression in the surface of neutrophils from men. Moreover, 17β-estradiol–treated neutrophils from men showed a diminished ability to interact with a synthetic surface. The prevention of the 17β-estradiol effects on both CD18 expression and adhesion of neutrophils by the L-arginine-competitive analogue L-NMMA supported the involvement of NO and suggested the functional importance of the upregulation on nNOS expression by estrogen.

In summary, neutrophils obtained from premenopausal women, during the menstrual cycle, and from postmenopausal women, before and after transdermal estrogen therapy, showed different levels of nNOS, which were associated with changes in the circulating estrogen levels. Moreover, the results of the present study showed that the nNOS-type protein expressed in neutrophils from men is upregulated by low doses of 17β-estradiol through an ER-dependent mechanism. The increased expression of nNOS protein was associated with a decrease in the adhesion properties of the neutrophils.

The enhanced ability of neutrophils to produce NO after 17β-estradiol treatment may contribute to some of the beneficial effects of hormone replacement therapy, particularly in diseases associated with inflammation where neutrophils play a relevant role.

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MATERIALS AND METHODS

Neutrophil isolation
Neutrophils were isolated from peripheral blood. Neutrophils were isolated by Ficoll/Hypaque centrifugation as previously described \textsuperscript{18,27}. Neutrophils were obtained from three different populations: a) from pre-menopausal women during different stages of the menstrual cycle; b) from post-menopausal women before and after hormone replacement therapy and c) from men for \textit{in vitro} estrogen incubations. For the \textit{in vitro} experiments, neutrophils (95% pure, 98% viable by trypan blue exclusion) were resuspended in RPMI 1640 medium (without red phenol) supplemented with 0.25% bovine serum albumin, 5 mmol/L Gln, 2x10^{-5} \mu g/L penicillin and 2x10^{-5} \mu g/L streptomycin. The protocols were approved by the Institutional Ethics Committe.

\textbf{Determination of nNOS protein expression}

The expression of nNOS protein was analyzed by Western blot. Neutrophils were lysed and boiled in Laemmli buffer containing 2-mercaptoethanol. Equal amounts of protein (20 \mu g/lane) determined by bichinchoninic acid reagent (Pierce Rockford,IL) were loaded. To verify that equal amounts of protein had been loaded in the gel, a parallel gel with identical samples was run and stained with coomassie to compare the intensities of the protein band. Proteins were separated on denaturing SDS/10\% polyacrylamide gels and blotted onto nitrocellulose (Immobilon-P,Millipore, Bedford, MA) as reported \textsuperscript{14,28}. In brief, blots were blocked overnight at 4\textdegree C with 5\% nonfat dry milk in TBS-T (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1\% Tween 20). Western blot analysis was performed with a monoclonal antibody against nNOS (Transduction Laboratories, Lexington, Kentucky). Blots were incubated with the first antibody (1:2500) for one hour at room temperature and, after extensive washing, with the second antibody (horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody) at a dilution of 1:1500 for a further one hour. Specific nNOS protein was detected by enhanced chemiluminiscence (ECL, Amersham, Buckinghamshire, UK). Pre-stained protein markers (Biorad Hercules, CA) were used for molecular mass determinations.

To assess the specificity of the nNOS monoclonal antibody, we analyzed the cross-reactivity of the antibody against the endothelial NOS isoform in a homogenate of human umbilical endothelial cells and against the iNOS isoform expressed in homogenates of LPS-treated rat macrophages.
Furthermore, we confirmed the antibody ability to recognize the nNOS isoform expressed in homogenates of rat pituitary.

**nNOS expression in neutrophils obtained from premenopausal women**

The expression of nNOS protein was determined in neutrophils isolated from 10 healthy premenopausal women (25-40 years old). Neutrophils were obtained at two different times during the menstrual cycle, the ovulatory phase in which circulating estrogen concentrations are elevated and within the first two days of the follicular phase (menstruation) where circulating estrogen concentrations fall. Samples at the follicular and ovulatory phases were obtained from the same donor. The ovulatory phase in the menstrual cycle was determined by the urinary peak of luteinising hormone (LH). LH urinary peak was measured by disposable test sticks (Donnatest Wyntek Diagnostic, San Diego, CA). Urinary samples were obtained in the first morning micturition.

Serum estradiol concentrations were determined with the use of an ELISA kit (Oxford Biomedical Research. INC, Oxford, MI). The sensitivity of the assay was 10 pg/mL and the variation coefficient was <6% in the low range of values.

**In vivo estradiol treatment in postmenopausal women**

The expression of nNOS protein was also determined in neutrophils obtained from 15 healthy postmenopausal women (45-65 years old). All women had not had a menstrual period for at least four years and had not received any hormone replacement treatment within this period of time. Blood samples for neutrophil isolation were obtained before and after four months of hormone replacement therapy. For this purpose, postmenopausal women were treated for four months with 50 μg/day estradiol transdermal patches (Estraderm Matrix 50, Novartis Pharma SA, Rueil-Malmaison, France). To induce withdrawal bleeding after the treatment, medroxyprogesterone acetate (Provera, Upjohn, Kalamazoo, MI) was administered orally for 10 days of each cycle (10 mg/day). Blood samples were always obtained two days before the postmenopausal women received the progestin. Serum estradiol levels were determined as described above.

**In vitro incubation of neutrophils from healthy men with estrogen**

Neutrophils (5x10⁶ cells/tube) isolated from 10 healthy men (25-40 years old) were incubated
with increasing concentrations of 17β-estradiol (10^{-10} to 10^{-5} \text{ mol/L}) or medium supplemented with the solvent of 17β-estradiol, ethanol (final ethanol concentration <0.01%), for 6 hours and incubated in an incubator at 37°C with 5% CO₂.

To determine the role of ER activation in the effects of 17β-estradiol on nNOS protein expression, additional studies were performed in neutrophils treated with the ER antagonists tamoxifen (10^{-8} \text{ mol/L}) or ICI 182780 (10^{-8} \text{ mol/L}). Neutrophils (5x10^6 cells tube) were incubated with estradiol (10^{-8} \text{ mol/L}) in the presence or in the absence of the ER antagonists for 6 hours at 37°C with 5% CO₂.

The choice of neutrophils from men to test the effect of 17β-estradiol on nNOS expression was made in an attempt to reduce the influence of previous exposure of neutrophils to a high and cyclic estrogenic environment.

**Determination of estrogen receptors in neutrophils obtained from healthy men**

To determine if ER protein is expressed in neutrophils from men, Western blot analysis was performed as already described for the nNOS protein using the polyclonal antibody MC-20 (1:1000) directed against amino acids 580-599 in the estrogen-binding domain of human ERα (Santa Cruz Biotechnology INC, Santa Cruz, CA). Since the ligand-binding domains of the classical ERα isofrom and the newly described ERβ isofrom are highly homologous, the antisera may recognize either receptor subtype^{29}.

**Determination of ^{3}H-L-citrulline**

The conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline was determined as an indicator of NO generation. Neutrophils from men were incubated with 17 β-estradiol (10^{-8} \text{ mol/L}) or with the solvent of 17 β-estradiol, ethanol (final ethanol concentration <0.01%), for 6 hours at 37°C. During the last 45 min, neutrophils were loaded with [^{3}H]-L-arginine. For this purpose, 1 μCi/mL [^{3}H]-L-arginine was added to the incubation medium. Unincorporated [^{3}H]-L-arginine was washed twice with fresh RPMI-1640. To determine the ability of neutrophils to convert L-arginine into L-citrulline, neutrophils were then incubated for 20 min at 37°C. To stop the reaction, neutrophils were centrifuged and lysed with cold ethanol and the supernatant was evaporated to dryness under N₂.

As previously described^{18,27}, the extracts were resuspended in 20 mM Hepes/KOH pH 5.5 and
applied to columns of Dowex AG50WX8 (Na+ form), which were subsequently eluted with water (L-citrulline fraction) and 0.5 M NaOH (L-arginine fraction). The [3H]-L-citrulline fraction was quantified by liquid-scintillation counting. The percentage of separation between L-arginine and L-citrulline was 92±3%.

CD18 antigen expression and adhesion assays

To further address the physiological implication of the NO produced by 17β-estradiol-incubated neutrophils from men, two different approaches were followed. First, we determined the presence of the CD18 antigen in the surface of neutrophils by flow cytometry. For immunofluorescence flow cytometry assays, neutrophils from men were incubated with 10^8 mol/L 17β-estradiol or the solvent ethanol (final concentration <0.01%) for 6 hours at 37°C. Then, neutrophils were incubated with a monoclonal antibody against CD18 antigen (0.003 μg/μL) (Dako, Glostrup, Denmark) for 30 min followed, after extensive washing, by a further incubation with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (The Binding Site, Birmingham, England). As previously described 30, the intensity of fluorescence was measured by using an EPICS flow cytometer sorter (coulter) at 480 nm excitation and 520 nm emission. A minimum of 6000 cells were analyzed for each sample. The data were displayed as one-parameter histograms plotting the logarithm of the green fluorescence vs cell number.

Secondly, the ability of neutrophils to adhere to plastic surface (tissue culture dish 35x10 mm, Costar, Cambridge, Mass) was also tested. As above mentioned, neutrophils were incubated with 10^8 mol/L 17β-estradiol or the solvent (ethanol, <0.01%) for 6 hours at 37°C. In the last 45 min, 2 μCi/mL 51 Cr (Amersham Life Science, Ltd) was added to the incubation medium to label the neutrophils. To remove the unbound 51Cr, the neutrophil suspension was centrifuged and resuspended in RPMI-1640 medium containing 0.25% bovine serum albumin (3x10^6 cell/0.5 mL). Adhesion experiments were then done by adding 0.5 mL 51 Cr-neutrophils on a plastic surface for 30 min at 37°C under static conditions. Unattached neutrophils were washed twice with 0.5 mL RPMI-1640 medium and the adherent neutrophils were harvested by using a scraper and lysed in 0.5 mL of 5 mol/L NaOH as described 30. The radioactivity was quantified in a gamma counter measuring the 51Cr levels. The number of neutrophils adhered in each experiment was estimated from the total radioactivity of the samples containing 3x10^6 cells/0.5 mL which were not added to the plastic surface. All experiments were performed in triplicate.
Statistical Methods

Results are expressed as means ± S.E.M. Unless otherwise stated, each value corresponds to a minimum of 6 different experiments performed. To determine the statistical significance we have performed ANOVA with Bonferroni's correction for multiple comparisons or a Student's t test, paired or unpaired. A P value <0.05 was considered statistically significant.