17β-Estradiol corrects hemostasis in uremic rats by limiting vascular expression of nitric oxide synthases

MARINA NORIS,1 MARTA TODESCHINI,1 SERGIO ZAPPELLA,1 SAMANTHA BONAZZOLA,1 CARLA ZOJA,1 DANIELA CORNA,1 FLAVIO GASPARI,1 FRANCO MARCHETTI,2 SISTIANA AIELLO,1 AND GIUSEPPE REMUZZI1,2

1Mario Negri Institute for Pharmacological Research and 2Division of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, 24125 Bergamo, Italy

Received 22 June 1999; accepted in final form 24 May 2000

Patients with renal failure have an increased tendency to bleed (33), usually manifested by prolonged bleeding time, a global measure of platelet function and primary hemostasis that often correlates with clinical bleeding (57). Prolonged bleeding time and even clinical bleeding can be corrected by conjugated estrogens which, when given in adequate doses, (24, 35, 56, 61) have a lasting effect on primary hemostasis to the extent that they are widely used in clinical practice to protect uremic patients from the risk of bleeding after surgery (53). Despite extensive research, however (24, 37, 61, 62, 67), the mechanism(s) by which they normalize primary hemostasis in uremia remain elusive.

On the basis of recent evidence in rats made uremic by extensive surgical ablation of renal mass, we suggested that prolonged bleeding time in uremia might be the consequence of excessive formation of nitric oxide (NO), an L-arginine derivative implicated in vasodilatation and immune response, which also inhibits platelet function (19, 38, 42). Plasma concentrations of the stable NO metabolites, nitrites and nitrates (NO2−/NO3−), were higher than normal in uremic rats with prolonged bleeding time (2). In addition, N-monomethyl-L-arginine, a competitive inhibitor of NO synthesis, normalized the prolonged bleeding time of uremic rats and increased ex vivo platelet adhesion (52). This effect was completely reversed by giving the animals the NO precursor L-arginine (52). In the same model we showed that the shortening effect of either conjugated estrogen mixture or its active component, 17β-estradiol (62), on bleeding time was also abolished by L-arginine (64), which can be taken as evidence that conjugated estrogens normalize uremic bleeding by interfering with the pathway of NO synthesis.

The reaction by which L-arginine is converted to L-citrulline and NO is catalyzed by a family of NO synthase (NOS) enzymes which exist in at least three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (63). We used two histochemical approaches, NADPH-diaphorase, that locates NOS catalytic activity, determined by NADPH-diaphorase staining in the thoracic aorta. Endothelial NOS (eNOS) and inducible NOS (iNOS) immunoperoxidase staining in the endothelium of uremic aortas of untreated rats was significantly more intense than in control rats, while in uremic rats receiving 17β-estradiol staining was comparable to controls. Thus 17β-estradiol corrected the prolonged bleeding time of uremic rats and fully normalized the formation of NO by reducing the expression of eNOS and iNOS in vascular endothelium. These results provide a possible biochemical explanation of the well-known effect of estrogens on primary hemostasis in uremia, in experimental animals and humans. Chronic renal failure; conjugated estrogens; bleeding time; endothelium.
thelia, which generate higher than normal amounts of NOS isoenzyme proteins in vessels.

The present experiments were designed to explore whether the fact that conjugated estrogens normalize bleeding time and clinical bleeding and limit the excessive formation of NO in uremia was linked to the process leading to NOS isoenzyme formation from the corresponding genes.

**METHODS**

**Chemicals.** Sodium nitrate, sodium nitrite, sulphamic acid, n-(1-naphthyl)-ethylendiamine dihydrochloride, sodium borate, 17β-estradiol, N-nitro-L-arginine, β-NADPH, nitroblue tetrazolium, Triton X-100, diphenyleneiodonium (DPI), cadmium powder (100 mesh), and all other chemicals were from Sigma (St. Louis, MO). Phosphoric acid, ZnSO₄ × 7H₂O, and diamino benzidine tablets were from Merck (Darmstadt, Germany). [3H]-L-arginine (1.6 TBq/mmol) was purchased from New England Nuclear (Boston, MA). A low-nitrate diet was prepared from commercial standard diet (Rieper, Bolzano, Italy) by extracting the nitrate in water. Briefly, the pulverized diet was resuspended in distilled water at 37°C and whipped, then filtered and dried up. Because water-soluble vitamins and minerals were also lost, the low-nitrate diet was supplemented with minerals, vitamins, choline, DL-methionine, and L-arginine, in amounts recommended by the American Institute of Nutrition.

**Experimental design.** Male Sprague-Dawley rats (Charles River Italy, Calco, Italy), 275–300 g, (n = 29) were made uremic by surgical removal of the right kidney and ligation of two or three branches of the left renal artery according to Olson et al. (47). Seventeen rats were sham operated with manipulation of pedicles and served as controls (CTR). Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U., Suppl. 40, 1992, Circolare No. 8, G.U., 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 1987; Guide for the Care and Use of Laboratory Animals. Washington, DC: National Research Council, 1996).

Animals with renal mass reduction (RMR) and controls were studied three mo after the surgical procedure. Renal function, measured as serum creatinine, urinary protein excretion, bleeding time, and systolic blood pressure were assayed in all RMR and CTR rats.

To obtain an indirect in vivo indicator of systemic NO synthesis, NO₂⁻/NO₃⁻ levels were measured in plasma obtained by centrifugation of heparinized blood from the tail vein from RMR (n = 12) and CTR rats (n = 6). To minimize dietary NO₃ intake rats were fed a low-nitrate diet (<8 nmol/g) and given distilled water to drink, starting three days before the study. In animals fed this diet, plasma levels of NO₂⁻/NO₃ were accurately reflect the endogenous production of NO (14, 17). Plasma levels of the aminoacid L-arginine were also measured.

To investigate whether the shortening effect of conjugated estrogens on bleeding time in RMR rats was related to any effect on vascular NO synthesis, six RMR rats on the low-nitrate diet were intravenously injected with 0.6 mg/kg of 17β-estradiol (Sigma, St. Louis, MO), the major active component of conjugated estrogen mixture (62), and six with vehicle (1 ml of 2% benzyl alcohol solution) (62). Bleeding time, plasma, and urinary NO₂⁻/NO₃⁻ levels were measured immediately before the drug and 24 h later, i.e., at the time of maximum shortening of bleeding time (62). At the same time plasma levels of L-arginine were measured to assess whether 17β-estradiol affected circulating levels of the NO precursor in uremic rats.

To investigate ex vivo vascular NO production five RMR and five CTR rats were killed and the thoracic aortas removed. Aortas were cleaned from fat tissue and rings (~10 mg each) were incubated in duplicate for 24 h in a water bath at 37°C under slow shaking, after addition of 0.5 ml plasma from the same animals and 0.5 μCi [3H]-L-arginine. Vascular NO synthesis was evaluated by measuring the conversion of [3H]-L-arginine to [3H]-citrulline and the data were corrected for mg tissue in each sample. To evaluate the effect of conjugated estrogens on vascular NO synthesis ex vivo, additional rings from the same RMR rats were incubated as above in the presence of 17β-estradiol (100 nM). Aliquots (0.5 ml) of plasma from each animal containing [3H]-L-arginine were also incubated for 24 h and used as blanks.

To see whether conjugated estrogens affected vascular NOS activity in vivo and the expression of iNOS and eNOS, two additional groups of RMR rats were treated with 17β-estradiol (n = 6) or vehicle (n = 6) and killed 24 h later. An additional group of six CTR rats was also studied. The catalytic activity of NOS in the thoracic aorta was assessed by isoenzyme-independent enzymatic oxidation of nitroblue tetrazolium in the presence of NADPH (NADPH-diaphorase). Expression of eNOS and iNOS was evaluated on the same tissues by immunoperoxidase with specific antibodies. In selected RMR (n = 6, three treated with vehicle and three with 17β-estradiol) and CTR (n = 3), animals expression of iNOS and eNOS in thoracic aorta was confirmed by Western Blot analysis.

The NO₂⁻/NO₃⁻ ratio (nmol/g) and given distilled water to drink, starting three days before the study were assayed in all RMR and CTR rats.

To obtain an indirect in vivo indicator of systemic NO synthesis, NO₂⁻/NO₃⁻ levels were measured in plasma obtained by centrifugation of heparinized blood from the tail vein from RMR (n = 12) and CTR rats (n = 6). To minimize dietary NO₃ intake rats were fed a low-nitrate diet (<8 nmol/g) and given distilled water to drink, starting three days before the study. In animals fed this diet, plasma levels of NO₂⁻/NO₃ were accurately reflect the endogenous production of NO (14, 17). Plasma levels of the aminoacid L-arginine were also measured.

To investigate whether the shortening effect of conjugated estrogens on bleeding time in RMR rats was related to any effect on vascular NO synthesis, six RMR rats on the low-nitrate diet were intravenously injected with 0.6 mg/kg of 17β-estradiol (Sigma, St. Louis, MO), the major active component of conjugated estrogen mixture (62), and six with vehicle (1 ml of 2% benzyl alcohol solution) (62). Bleeding time, plasma, and urinary NO₂⁻/NO₃⁻ levels were measured immediately before the drug and 24 h later, i.e., at the time of maximum shortening of bleeding time (62). At the same time plasma levels of L-arginine were measured to assess whether 17β-estradiol affected circulating levels of the NO precursor in uremic rats.

To evaluate ex vivo vascular NO production five RMR and five CTR rats were killed and the thoracic aortas removed. Aortas were cleaned from fat tissue and rings (~10 mg each) were incubated in duplicate for 24 h in a water bath at 37°C under slow shaking, after addition of 0.5 ml plasma from the same animals and 0.5 μCi [3H]-L-arginine. Vascular NO synthesis was evaluated by measuring the conversion of [3H]-L-arginine to [3H]-citrulline and the data were corrected for mg tissue in each sample. To evaluate the effect of conjugated estrogens on vascular NO synthesis ex vivo, additional rings from the same RMR rats were incubated as above in the presence of 17β-estradiol (100 nM). Aliquots (0.5 ml) of plasma from each animal containing [3H]-L-arginine were also incubated for 24 h and used as blanks.

To see whether conjugated estrogens affected vascular NOS activity in vivo and the expression of iNOS and eNOS, two additional groups of RMR rats were treated with 17β-estradiol (n = 6) or vehicle (n = 6) and killed 24 h later. An additional group of six CTR rats was also studied. The catalytic activity of NOS in the thoracic aorta was assessed by isoenzyme-independent enzymatic oxidation of nitroblue tetrazolium in the presence of NADPH (NADPH-diaphorase). Expression of eNOS and iNOS was evaluated on the same tissues by immunoperoxidase with specific antibodies. In selected RMR (n = 6, three treated with vehicle and three with 17β-estradiol) and CTR (n = 3), animals expression of iNOS and eNOS in thoracic aorta was confirmed by Western Blot analysis.
an excitation wavelength of 338 nm and an emission wavelength of 425 nm. Samples were chromatographed on a C18 reverse-phase column (Chromsep 5 µm, 200 mm x 3 mm ID, Chrompack) by using a linear gradient from 100% solvent A (0.025 M KH₂PO₄, at pH 7.2/methanol: 75/25 plus 0.8 ml/l tetrahydrofuran) to 25% solvent B (methanol) in 10 min at a flow rate of 0.5 ml/min.

**Determination of [³H]-citrulline formation from [³H]-L-arginine.** Incubations were stopped by adding one volume of ice-cold 15% trichloroacetic acid (TCA). TCA-treated samples were centrifuged at 10,000 g to precipitate proteins. The supernatant was extracted with ether, vacuum lyophilized and resuspended in 2 ml HEPES, pH 5.5, and purified on 2 ml wet bed volumes of Dowex AG 50 WX-8 (100–200 mesh, Li⁺ form), as described (2). [³H]-L-citrulline was quantitated by liquid scintillation counting in the 4-ml column effluent and identified as described (2).

**Tissue preparation for histochemistry.** The thoracic aorta was surgically dissected and fixed by immersion in 4% p-formaldehyde in PBS overnight at 4°C. A portion of each sample was processed for conventional paraffin inclusion, and the remainder was treated with 10% sucrose in PBS for cryoprotection, then frozen in liquid nitrogen.

**NADPH-diaphorase.** Frozen sections 3 µm thick were cut on a cryostat (HM500-O, Microm, Zeiss Oberkochen, Germany). Sections were air-dried, then washed in PBS 0.05 M pH 7.4 for 5 min at room temperature and permeabilized by immersion in 0.3% Triton X-100/PBS 0.01 M, pH 7.2, at 4°C for 30 min (2). For the NADPH-diaphorase reaction slides were incubated with 1 mM β-NADPH/0.2 mM nitroblue tetrazolium/100 mM Tris-Cl buffer, pH 8.0, containing 0.2% Triton X-100 for 1 h at 37°C. The reaction was stopped by rinsing sections in PBS 0.05 M, pH 7.4.

In all experiments the reproducibility of the reaction was followed on a control tissue section. Negative controls were run without NADPH or in the presence of the NO synthesis inhibitor DPI (41); reactivity was totally NADPH dependent and was abolished by DPI. Slides were observed on a DM/2B microscope (Leitz, Leica, Milan, Italy) by a pathologist blind to the nature of the experiment.

**Immunoperoxidase.** Rabbit polyclonal antibody directed against mouse macrophage iNOS (Transduction Laboratories, Exeter, UK) and mouse monoclonal antibody against human ecNOS (Transduction Laboratories) were used. Both antibodies recognize rat NOS antigens ([58, 59] and Transduction Laboratories catalogue). The specificity of anti-ecNOS and anti-iNOS antibodies was first verified by immunofluorescence. Human microvascular endothelial cells [SV-40 transfected immortalized endothelial cell line (HMEC) (1)] which constitutively express ecNOS but not iNOS, were grown on glass coverslips, fixed in 2% paraformaldehyde and stained with anti-ecNOS or anti-iNOS antibodies at different dilutions (anti-ecNOS, 1:150 and 1:300; anti-iNOS, 1:25 and 1:100) followed by secondary antibody (FITC-conjugated goat anti-mouse IgG 1:50, Jackson ImmunoResearch Laboratories, West Grove, PA, for ecNOS; and indocarboxyanine (Cy₅) conjugated goat anti-rabbit IgG 1:100, Jackson ImmunoResearch Laboratories, for iNOS). Fixed cells were permeabilized for 4 min with Triton X-100 0.1% before anti-iNOS staining. As positive controls, rat peritoneal macrophages were incubated for 18 h at 37°C with lipopolysaccharide (LPS) (20 µg/ml) to upregulate iNOS) in culture dishes containing glass coverslips, then fixed and stained as described above. The slides were observed with an Olympus BH-2 epifluorescence microscope. Anti-ecNOS antibody stained HMEC at the optimal dilution of 1:150, while no signal was found on peritoneal macrophages. At 1:25 dilution anti-iNOS brightly stained rat peritoneal macrophages, but also HMEC, indicating a cross reaction with ecNOS. At 1:100 dilution the antibody still brightly stained macrophages without cross-reacting with ecNOS, as indicated by a very faint staining on HMEC.

Enzyme specificity of anti-iNOS and anti-ecNOS antibodies was confirmed by Western blot analysis of HMEC and rat macrophage extracts, following the manufacturer's instructions. On the basis of these results, in the immunoperoxidase studies described below anti-ecNOS and anti-iNOS antibodies were used at dilutions of 1:150 and 1:100, respectively.

Three-µm paraffin sections from aortic tissue were processed for light microscopy immunohistochemistry by using an avidin-biotin horseradish peroxidase complex technique [avidin-biotin-complex (ABC) method, ABC-Elite, Vector Laboratories, Burlingame, CA] (2). Briefly, the sections were dewaxed, rehydrated, and incubated for 1 h with 0.3% H₂O₂ in methanol to quench endogenous peroxidase. Tissue was permeabilized in 0.1% Triton X-100 in PBS 0.01 M, pH 7.2, for 30 min and aspecificities were blocked by 30 min incubation with nonimmune sera (goat serum for anti-iNOS, horse serum for anti-ecNOS). All the above steps were carried out at room temperature. Slides were then incubated overnight at 4°C in a moist chamber with the primary antibody (anti-iNOS 1:100, anti-ecNOS 1:150) in PBS/1% BSA (Miles, Bayer, Milan, Italy), followed by the secondary antibody (biotinylated goat anti-rabbit IgG, or biotinylated horse anti-mouse IgG). ABC solution, and developed with diaminobenzidine as described. The sections were counterstained with Harris hematoxylin (Biooptica, Milan, Italy).

Negative controls were obtained by omitting the primary antibody on a second section present on all the slides. The slides were observed under the light microscope by a pathologist blind to the nature of the experiment.

**Analysis of NADPH-diaphorase and immunohistochemical data.** Multiple sections from each animal for two separate NADPH-diaphorase or immunoperoxidase reactions were examined by one investigator who was blinded to the identity of sample. Each section was scored for intensity of immunostaining and NADPH-diaphorase staining (absent, faint, moderate, intense, very intense: 0 through 4). These values were then compared with those in appropriate, concurrently run negative controls. At least 8–10 fields per section were examined. The modal value for each section was determined and the mean for each group calculated, as previously described (45).

**Western blotting.** Western blots were performed as previously described (40). After pulverization, frozen arteries were resuspended in 0.5 ml lysis buffer (50 mM β-glycerophosphate, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 0.5% NP-40, 1 mM DTT, 1 mM pefabloc, 20 µM pepstatin, 20 µM leupeptin, 1,000 U/ml apronitin) and sonicated. The whole lysate was stored at ~70°C. Protein concentration was determined by using the Bradford method (Bio-Rad). The proteins (20 µg for each lane) were separated on denaturating sodium dodecyl sulfate 7.5% polyacrylamide gel by electrophoresis and then blotted on nitrocellulose membrane by wet electroblotting for 90 min. Blots were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline (TBS) at pH 7.5 (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20) and then incubated for 2.5 h with anti-iNOS (1:1,000) or anti-ecNOS (1:250) followed by the secondary antibody (biotinylated goat anti-rabbit IgG, or biotinylated horse anti-mouse IgG). ABC solution, and finally developed with diaminobenzidine (Vector Laboratories).
Urinary protein excretion. Proteinuria was determined in 24-h urines by the modified Coomassie blue G-dye binding assay, with BSA as standard (2).

Serum creatinine. Serum was obtained by leaving 1 ml portions of native blood, collected from the tail vein of anesthetized animals, at 37°C for 30 min. Creatinine was measured by the alkaline picrate method (2).

Bleeding time. Bleeding time was determined as previously described (52). Briefly, nonanesthetized rats were placed in a plastic cylinder with several openings, from one of which the tail emerged. Bleeding time was measured by using a standardized Simplate II device (General Diagnostic, Milan, Italy) and expressed in s from the moment the tail was incised until bleeding stopped completely (no rebleeding within 30 s).

Statistical analysis. Results are expressed as means ± SE. Bleeding times, NO$_2$/NO$_3$ plasma concentrations and urinary excretion, plasma l-arginine, and the production of [H]-l-citrulline in aortic tissue were analyzed by the Mann-Whitney U-test, Wilcoxon’s rank sum test, or Kruskal-Wallis test, as appropriate. Data on NADPH-diaphorase staining and ecNOS and iNOS staining were analyzed by the Kruskal-Wallis test, as appropriate. Data on NADPH-diaphorase staining was found on endothelial cells with few traces on the vessel wall both in CTR and RMR rats.

Table 1. Laboratory findings in RMR and CTR rats

<table>
<thead>
<tr>
<th></th>
<th>RMR (n = 29)</th>
<th>CTR (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary Protein, mg/24 h</td>
<td>304.6 ± 38.9*</td>
<td>257.7 ± 1.7</td>
</tr>
<tr>
<td>Serum Creatinine, mg/dl</td>
<td>1.72 ± 0.19a</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>173.4 ± 4.3a</td>
<td>114.2 ± 1.4</td>
</tr>
<tr>
<td>Bleeding Time, s</td>
<td>280 ± 12a</td>
<td>93 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE. RMR, renal mass reduction; CTR, control; SBP, systolic blood pressure. *P < 0.01 vs. CTR rats; Mann-Whitney U-test.
17β-estradiol or vehicle-treated RMR rats, by using immunoperoxidase with specific antibodies. Moderate to intense immunostaining for ecNOS and iNOS (semiquantitative scores ranged from 1 to 3 for both), was evident on the endothelium of thoracic aortas from vehicle-treated RMR rats (Fig. 5, B and E, Table 2), and vascular smooth muscle cells showed weak, focal staining. Expression of iNOS was very faint or absent in the thoracic endothelium of CTR rats (Fig. 5 D).

Statistical analysis indicated that ecNOS and iNOS staining in the endothelium of uremic aortas was significantly more intense than in CTR rats (P < 0.05 for both ecNOS and iNOS, Fig. 5, A, B, D, E, Table 2), consistent with the diaphorase results. In RMR rats receiving 17β-estradiol ecNOS and iNOS endothelial staining was normal (P < 0.01 vs. RMR vehicle, Fig. 5, C and F, Table 2). iNOS staining in vascular smooth muscle cells did not differ in 17β-estradiol-treated and untreated uremic rats.

When the primary antibodies were omitted no staining was observed in adjacent sections in all experiments (Fig. 5 G and H).

Western blot analysis with anti-iNOS antibody of aorta homogenates from RMR rats treated with vehicle showed an immunoreactive band at 130 kDa (Fig. 6), by contrast no signal was found in samples from RMR rats treated with 17β-estradiol and from CTR rats (Fig. 6), which is consistent with immunoperoxidase data. With anti-ecNOS antibody a faint immunoreactive band was evident at 140 kDa in aorta homogenates from RMR rats treated with vehicle and from CTR rats, by contrast no band was found in samples from 17β-estradiol-treated animals (not shown).

**DISCUSSION**

In this study uremic rats with prolonged bleeding time had higher plasma concentrations of the NO metabolites (NO₂/NO₃) than CTR rats. These findings are consistent with evidence obtained in experimental animals (2, 52) and in humans (46), that bleeding abnormalities in uremia were associated with excessive systemic NO synthesis.

We also showed that 17β-estradiol significantly shortened the bleeding time of uremic rats without significant changes in renal function or proteinuria. In the same animals 17β-estradiol almost completely normalized plasma NO₂/NO₃. These results indicate that estrogens normalize primary hemostasis in uremia (24, 35, 56, 61) by correcting the abnormalities in NO synthesis, in line with previous reports that the NO precursor L-arginine eliminated the effect of 17β-estradiol by shortening bleeding time in uremic rats (66).
Effects of 17β-estradiol on primary hemostasis and NO activity in vessels. We found that 17β-estradiol related to an inhibitory effect on endothelial NOS activity in cells and tissues irrespective of the enzyme with NADPH-diaphorase, which detects catalytic NOS. Here we used a histochemical approach, (6, 21, 27) NOS activity and NO release in vascular late (5, 10, 21), and very high doses of estrogens inhibit synthesis. Relevant to this possibility are in vitro data that physiological concentrations of estrogens stimulate (5, 30, 48) which by converting ornithine to putrescine, activates the enzyme arginase, which degrades L-arginine in the urea cycle (25). To test whether estrogens' effects on the NO pathway in uremia were actually related to lowering L-arginine, we measured L-arginine in RMR rats before and after injection of 17β-estradiol. 17β-estradiol did not cause significant reduction in plasma L-arginine so it is extremely unlikely that the effects of 17β-estradiol on primary hemostasis and NO synthesis in RMR rats were dependent on L-arginine.

Alternatively, estrogens may act on vascular NO synthesis. Relevant to this possibility are in vitro data that physiological concentrations of estrogens stimulate (5, 10, 21), and very high doses of estrogens inhibit (6, 21, 27) NOS activity and NO release in vascular endothelium. Here we used a histochemical approach, with NADPH-diaphorase, which detects catalytic NOS activity in cells and tissues irrespective of the enzyme isoform (4, 41, 59), to check in vivo whether the fact that estrogens limited NO formation in uremia was related to an inhibitory effect on endothelial NO activity in vessels. We found that 17β-estradiol dramatically reduced endothelial vascular NO activity, and indeed endothelial diaphorase staining was significantly weaker in uremic rats given 17β-estradiol than in vehicle-treated RMR rats and even than in control rats.

To relate the changes in NOS catalytic activity to differences in NOS isoenzyme expression, we evaluated ecNOS and iNOS protein expression in the thoracic aorta of 17β-estradiol or vehicle-treated RMR rats, by immunohistochemical analysis. ecNOS and iNOS staining in the endothelium of uremic aortas was significantly stronger than in control rats. By contrast, in RMR rats given 17β-estradiol ecNOS and iNOS endothelial staining was normal.

These data demonstrate that conjugated estrogens, at the doses needed to correct primary hemostasis in experimental uremia, markedly reduce vascular endothelial expression of ecNOS and iNOS protein, which may explain the fact that these compounds limit excessive NO synthesis in the systemic circulation. Although the effect of 17β-estradiol on ecNOS and iNOS expression in microvessels was not evaluated in this present study, we observed that ecNOS and iNOS expression was lower in vasa vasorum of aortas from rats receiving the 17β-estradiol compared with untreated uremic rats (not shown), indicating that the drug reduced endothelial NOS isoenzymes expression also in small vessels.

Surprisingly, 17β-estradiol did not modify systolic blood pressure in uremic rats, despite the impressive reduction of vascular NOS isoenzymes. It is well known, however, that estrogens are potent inhibitors of several vasoconstrictor systems, and they inhibit the release of endothelin by endothelial cells (3), reduce plasma angiotensin converting enzyme activity (8), and blunt the vascular pressor response to phenylephrine (23). It is tempting to speculate that in RMR the inhibitory effect of 17β-estradiol on vasoconstrictors might have counterbalanced the increase in vascular tone due to reduction of vascular NO.

Endothelial cells of bovine (21) and rabbit (12) origin appear to have estrogen receptors. In human endothelial cells estrogen receptors were found in cultures from umbilical vein (21) and in aorta (9). Although the ecNOS promoter does not contain a canonical estrogen cis-regulatory element, the left palindromic sites of an estrogen responsive element (GGTCA) and the right half sites (TGACC) were identified in bovine (60) and human (39) ecNOS genes which might be one mechanism by which estrogens regulate ecNOS gene expression.

Table 2 shows the intensity of NADPH-diaphorase staining in RMR rats.

![Graph showing plasma L-arginine concentration](http://ajprenal.physiology.org/)

**Fig. 4. Effect of 17β-estradiol (n = 6) on plasma concentration of L-arginine in RMR rats studied three mo after surgery.** L-arginine was measured before (pre) and 24 h after 17β-estradiol (0.6 mg/kg intravenously) (post). L-arginine values in all RMR rats (n = 12). Horizontal dark-grey bar, range of values for L-arginine plasma concentrations in CTR.

Table 2. Intensity of NADPH-diaphorase staining and ecNOS and iNOS immunostaining in aorta endothelium

<table>
<thead>
<tr>
<th></th>
<th>NADPH-d</th>
<th>ecNOS</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>RMR + vehicle</td>
<td>1.8 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>RMR + 17β-estradiol</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for each group. NADPH-d, NADPH-diaphorase; ecNOS, endothelial NOS; iNOS, inducible NOS. Semiquantitative score: 0: absent, 1: faint, 2: moderate, 3: intense, 4: very intense. *P < 0.05 vs. CTR; †P < 0.01 vs. RMR + vehicle; Kruskall-Wallis test.
pressed by 17β-estradiol at concentrations higher than 1 nM. Thus the 5' half partial sequences may have functional roles as estrogen responsive elements in ecNOS.

Previous findings on how estrogens affect ecNOS are controversial (21, 27) and recent observations showed increased expression and/or activity of ecNOS in 17β-estradiol-treated cultured endothelial cells (11, 21, 31, 32, 36). Similarly, elevated plasma levels of NO₂/NO₃ were found in postmenopausal women given 17β-estradiol as hormone replacement therapy (5, 55). It was suggested that physiological amounts of estrogens stimulate NO production by vascular endothelium.

Fig. 5. Immunoperoxidase staining of ecNOS (A, B, and C) and iNOS (D, E, and F) in thoracic aortas from CTR rats (A and D) and RMR rats 24 h after iv, 17β-estradiol (C and F), or vehicle (B and E). RMR and CTR rats were studied three mo after surgery. Note localization of specific staining for ecNOS and iNOS on arterial endothelium. Moderate to strong reactivity is evident on aortas of RMR rats treated with vehicle (B and E). Signals are weaker on the aortas from RMR rats treated with 17β-estradiol (C and F) and from CTR rats (A and D). No staining is seen with omission of the primary antibodies (G for ecNOS, H for iNOS). Magnification 535x.

Fig. 6. Western blot analysis of iNOS in thoracic aortas from CTR and uremic (RMR) rats treated with vehicle (v) or 17β-estradiol (17β). Aliquots of tissue homogenate lysates (20 μg protein for each lane) were subjected to SDS-PAGE and immunoblotted with anti-iNOS antibody (1:1,000). Extracts from thoracic aortas of RMR rats treated with vehicle show a specific immunoreactive band at 130 kDa, by contrast no band is evident in lanes loaded with samples from CTR rats and from RMR rats treated with 17β-estradiol.
which may contribute to their beneficial cardiovascular actions (13, 34, 64), although alternative, not NO-dependent mechanisms have also been proposed (16, 64).

One possible explanation for the apparent inconsistency between the above studies and our present data is based on two observations: 1) the dose of conjugated estrogens necessary to improve primary hemostasis and reduce clinical bleeding in uremia in experimental animals (62, 67) and humans (35, 56, 61) is approximately fifty times the dose conventionally prescribed for contraception (44), postmenopausal replacement (15), or prevention of osteoporosis (44); 2) as shown by Hall et al. (20), transcriptional regulation of ecNOS by 17β-estradiol has a biphasic response: limited amounts of 17β-estradiol enhanced, while increasing amounts of ligand inhibited ecNOS transcription in endothelial cells. One can therefore reasonably assume that the effect of estrogens on vascular ecNOS expression are complex and depend very much on the concentration of the hormone: physiologic amounts enhance vascular endothelial ecNOS expression whereas pharmacologic doses, as high as the ones needed to improve primary hemostasis and correct bleeding time, inhibit the expression of vascular ecNOS. This possibility is consistent with in vivo findings in rats (6) that low-dose estrogens increased vascular NO release, while high doses significantly reduced basal release of NO and acetylcholine-induced relaxation.

One can speculate on the possible mechanism of the effect of 17β-estradiol on iNOS taking into account the fact that estrogens share with glucocorticoids a steroidal tridimensional structure, a receptor-mediated mechanism of action and certain biological activities (30, 44, 48) including the capacity to reduce uremic bleeding (66). Recent studies in macrophages (43) and endothelial cells in vitro (50), and in vivo in endotoxin-treated rats (51), have shown that dexamethasone potently inhibits iNOS expression by blocking the transcription of the enzyme. Therefore 17β-estradiol may control iNOS content in uremic vessels by a similar mechanism. This is consistent with recent findings that 17β-estradiol in vitro inhibited LPS-stimulated iNOS expression and NO production in a murine macrophage cell line (22) and in rat alveolar macrophages (54). An estrogen receptor antagonist blocked the effect of 17β-estradiol on iNOS (22), indicating that 17β-estradiol inhibited the induction of iNOS by a classic receptor-mediated mechanism. In another study estradiol reduced iNOS expression and NO production in rat aortic endothelial cells stimulated with interleukin-1β (65). The effect was evident at doses as low as 0.1 nM, indicating that, at variance with ecNOS, estrogens inhibit iNOS expression also at physiological concentrations. Consistently, in isolated rat aorta rings, 17β-estradiol caused concentration-dependent inhibition of interleukin-1β-induced iNOS expression and NO production and restored vasoconstriction responsiveness (28). In addition, Kauser et al. showed that administration of 17β-estradiol in vivo to ovariectomized rats attenuated the endotoxin-induced elevation of plasma nitrite levels (29), demonstrating that 17β-estradiol can inhibit excessive NO production by iNOS in vivo.

Like estrogens, the hemostatic effect of dexamethasone in uremic rats was eliminated by L-arginine (66) which further suggests that the two compounds have similar mechanisms of action. Because of a Kᵅ of around 30 μM, the iNOS isoenzyme is totally dependent on the availability of extracellular L-arginine (38, 42). Presumably the addition of the NO precursor in uremic rats overcomes the action of 17β-estradiol and dexamethasone of normalizing iNOS expression in vascular endothelium, by maximizing the catalytic activity of residual iNOS molecules either in the endothelium or in smooth muscle cells.

In conclusion, in uremic rats 17β-estradiol, at a dose that normalizes the prolonged bleeding time, fully corrects the excessive formation of NO by markedly reducing the expression of both ecNOS and iNOS in vascular endothelium. These results provide a possible biochemical explanation for the well-known effect of estrogens on primary hemostasis in uremics. Further studies are needed to provide definitive prove of the cause-and effect relationship between the activity of estrogens on NO biology and uremic bleeding and to sort out the relative importance of ecNOS vs. iNOS and to sort out the relative contribution of iNOS and ecNOS on uremic bleeding.

The authors thank Mrs. Judy Baggott for editing the manuscript. Part of this paper was presented as an abstract at the 31st Annual Meeting of the American Society of Nephrology (October 25–27, 1998, Philadelphia, PA). Sergio Zappella is a recipient of a fellowship from “Fondazione A., a. Valenti.” Samantha Bonazzola is a fellow of the Rotary Club Bergamo Nord.

REFERENCES


