Estrogen modulates paracellular permeability of human endothelial cells by eNOS- and iNOS-related mechanisms

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Cho, Michael M., Nicholas P. Ziats, Dipika Pal, Wulf H. Utian, and George I. Gorodeski. Estrogen modulates paracellular permeability of human endothelial cells by eNOS- and iNOS-related mechanisms. Am. J. Physiol. 276 (Cell Physiol. 45): C337–C349, 1999.—Estradiol had a biphasic effect on permeability across cultures of human umbilical vein endothelial cells (HUVEC): at nanomolar concentrations it decreased the HUVEC culture permeability, but at micromolar concentrations it increased the permeability. The objective of the present study was to test the hypothesis that the changes in permeability were mediated by nitric oxide (NO)-related mechanisms. The results revealed dual modulation of endothelial paracellular permeability by estrogen. 1) An endothelial NO synthase (eNOS)-, NO-, and cGMP-related, Ca2+-dependent decrease in permeability was activated by nanomolar concentrations of estradiol, resulting in enhanced Cl− influx, increased cell size, and increases in the resistance of the lateral intercellular space (RiLIS) and in the resistance of the tight junctions (RTJ); these effects appeared to be limited by the ability of cells to generate cGMP in response to NO. 2) An inducible NO synthase (iNOS)- and NO-related, Ca2+-independent increase in permeability was activated by micromolar concentrations of estradiol, resulting in enhanced Cl− efflux, decreased cell size, and decreased RiLIS and RTJ. We conclude that the net effect on transendothelial permeability across HUVEC depends on the relative contributions of each of these two systems to the total paracellular resistance.

Endothelial cells form a barrier between the blood and tissue parenchyma and control the movement of blood cells, plasma fluid, and solutes between the vascular compartment and the extracellular space. The main route of transport across endothelia is via the intercellular (paracellular) space, and the driving forces are transendothelial hydrostatic and hypertonic gradients (41).

Endothelial cells are targets for the actions of the female hormone estrogen. Studies in vivo suggest that estrogens may regulate the permeability of endothelia in different vascular beds (44), but data are lacking about the effects of estrogen on endothelial paracellular permeability. We (15) and others (39) have recently developed experimental conditions to study transport phenomena across low-resistance epithelia in vitro (e.g., cultured endothelial cells), and we used cultured human umbilical vein endothelial cells (HUVEC) to study the effects of estrogen on paracellular permeability and the mechanisms involved.

In endothelia, some of the effects of estrogen are mediated by the nitric oxide (NO) system. NO can modulate the permeability of epithelial and endothelial tissues, but there is considerable controversy concerning the role of NO as a mediator of vascular permeability (e.g., Refs. 3, 24, 25). Some studies suggested the involvement of downstream messengers from NO such as cGMP (23). Estrogens can upregulate NO activity (e.g., Refs. 46, 49) in endothelial cells (20) and in women (37), but there are no definitive conclusions with regard to the mechanisms involved (2, 47) and whether NO increases (42) or decreases (48) the permeability. In preliminary experiments with HUVEC, we found that Nω-nitro-L-arginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, modulates the paracellular permeability, suggesting that NO regulates the paracellular permeability across HUVEC. In those studies, the magnitude of the L-NAME effect was dependent on previous treatment of the cells with estrogen, suggesting that estrogen controls endothelial paracellular permeability by modulating the activity of NO. One of our objectives was to test the hypothesis that the effects of estrogen on HUVEC permeability are mediated by NO-related mechanisms.

METHODS

Isolation of HUVEC. The experiments were conducted on passage 3 HUVEC, which retain morphological and physiological characteristics of the native umbilical vein endothelium (51). We used cells obtained from females, to avoid gender-related differences in the response to estrogen. HUVEC were isolated from fresh human umbilical veins of newborn female using previously described methods (51). Briefly, segments of umbilical cords were washed with saline, and the distal end was clamped. An umbilical vein was filled with 20–30 ml of solution, composed of 1 mg/ml collagenase (type CLS, Worthington Biochemical, Freehold, NJ) in Hanks’ balanced salt solution (HBSS) containing Ca2+ and Mg2+. The proximal portion of the vessel was ligated, and the vessel was incubated for 10–20 min at room temperature. The solution contained within the vessel, including cells detached from the umbilical vein, was collected and rinsed in culture medium composed of medium 199 (M.A. Bioproducts, Walkersville, MD) containing 20% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 5 µg/ml insulin, 5 ng/ml transferrin, 5 µg/ml selenium, 75 µg/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 100 ng/ml heparin, and penicillin-streptomycin-fungizone solution. The mixture containing cells was centrifuged at 800 g for 5–10 min at room temperature. The cells in the pellet (mostly HUVEC) were resuspended in culture medium and plated...
onto human fibronectin (5 µg/cm²; Chemicon International, Temecula, CA)-coated T-75 tissue culture flasks (Corning, Corning, NY). Cells were maintained at 37°C in 91% O₂-9% CO₂ in a humidified incubator and refed fresh culture medium every 3 days. Cells were grown to confluence (~2 wk; typical confluent density of 2–3 × 10⁶ cells/ flask). Identification of endothelial cells was confirmed by immunohistochimical staining for factor VIII antigen (factor VIII/von Willebrand factor) (51).

For some experiments with estrogens, cells were shifted to a steroid-free medium. This medium was composed of phenol-red-deficient medium 199 (M. A. Bioproducts) containing 20% heat-inactivated FBS that was previously treated with charcoal to remove steroids, and supplemented with all the other reagents as described above for the standard culture medium. Preparation of charcoal-treated serum was described (7). Briefly, dextran-coated charcoal was dissolved at 8% in 0.15 M NaCl, autoclaved, mixed by stirring, and spun, and the pellet was resuspended as 1 g/L.5 ml in H₂O. FBS (HyClone) was mixed with the activated charcoal-dextran at 20:1 (vol/ vol) and incubated for 45 min at 55°C. At the completion of incubation, the mixture was spun twice at 800 g for 20 min and the supernatant (serum) was decanted and collected.

Preparation of cultures on filters. Anocell filters (ceramic base; Anocell-10, Anotec, Oxon, UK; surface area of 0.6 cm², pore size of 0.02 µm, and width of 50 µm) were coated on their upper (luminal) surface with 5 µg/cm² collagen type I and incubated at 37°C overnight. The excess collagen solution was aspirated, the filter was dried at 37°C, and both sides of the filter were rinsed three times with HBSS. 3T3 murine fibroblasts were irradiated as described (16) and were used as feeders to improve plating efficiency of HUVEC on filters. 3T3 fibroblasts were plated on the luminal surface of the filter at a high density (3 × 10⁶ cells/cm²) and preincubated for 15 min at 37°C in a modified Ringer buffer composed of (in mM) 120 NaCl, 5 KCl, 10 NaHCO₃ (before saturation with 95% O₂-5% CO₂), 1.2 CaCl₂, 1 MgSO₄, 5 glucose, and 10 HEPES (pH 7.4), with 0.1% BSA in volumes of 4.7–5.2 ml in both the luminal and subluminal compartments.

Determination of Ppyr. The Ppyr was determined from unidirectional (luminal to subluminal) fluxes across filters mounted vertically in the modified Ussing-diffusion chamber as described (16), to prevent hydrostatic gradients. Pyranine is a trisulfonic acid with a molecular mass of 0.51 kDa; it was chosen as a probe to assess paracellular permeability because it traverses epithelia via the paracellular pathway, and its concentration can be measured down to nanomolar levels by fluorescence techniques (16). Cytolysis of HUVEC that were previously incubated with 0.1 mM pyranine did not increase pyranine fluorescence significantly above background (not shown). Ppyr was determined from unidirectional (luminal to subluminal) fluxes: pyranine was added to the luminal compartment, and the amount of pyranine in the subluminal compartment was measured after 10 min. The transendothelial permeability coefficient (Ppyr) was calculated as described (16).

Determination of GTE. GTE was chosen as an end point to assess paracellular permeability because this method is sensitive to small changes in transendothelial permeability, and it allows for real-time measurements of changes in GTE. The permeability of the paracellular pathway is 10⁴ to 10⁶ times higher than that of the plasma membranes (34), and the former determines the overall conductive properties of the cultured endothelium. Changes in GTE therefore reflect changes in paracellular permeability. The electrophysiological methods, including appropriate measures to prevent artifacts, were previously described by us (15) and by others (34). Changes in GTE were determined continuously across filters mounted vertically in a modified Ussing chamber as described (15). This chamber, which also served as a diffusion chamber, was originally designed by Dr. Grass (Precision Instrument Design), was obtained through Costar (no. 3430, Cambridge, MA), and was custom modified by Analytical Bioinstrumentation (Case Western Reserve University School of Medicine, Cleveland, OH). The potential electrodes consist of Ag-AgCl₂ in 2 M KCl, are enclosed in Teflon tubing ending with a porcelain porous plug, and are situated at close proximity (~1 mm) to the center of the luminal and subluminal surfaces of the filter. This enabled us to measure precisely the transendothelial potential difference (PD). The current electrodes were made of Pt-Pt black and installed via existing inlets. Electrical measurements were made with a conventional four-electrode voltage clamp (DVC 1000, World Precision Instruments, Sarasota, FL), with a fluid resistance compensation range of 0–1,000 Ω, which was determined each time with a blank filter. GTE was determined continuously from successive measurements of the transendothelial electrical current (I); obtained by measuring the current necessary to clamp the offset potential to zero and normalized to the 0.6-cm² surface area of the filter) and the transendothelial PD (lumen negative), switching between 1 (pulses of 200–1,400 μA·cm⁻²) and PD at a rate of 20 Hz: GTE = ΔI/ΔPD. The volumes and content of the buffer solutions used in the Ussing chamber experiments were kept constant. The temperature in the chamber was maintained at 37°C, and the medium in each compartment was continuously bubbled with an air lift of 95% O₂-5% CO₂, which flowed parallel to the surface of the culture. The output of the voltage clamp was recorded in parallel on a strip-chart recorder (Linseis L6514, Cleveland, OH) and on an IBM PC 286-30 equipped with a DI-220 analog-to-digital converter board, a 1995 version of AT/MA CODAS hardware and software (DATA Q, Akron, OH), and a Bernoulli 90-megabyte hard disk. The CODAS
board allows acquisition of up to 50,000 samples/s with gap-free display. AT-playback provides for data analysis.

The experimental design of the electrophysiological measurements, including calibrations and controls, the significance of the voltage and I, and the conditions for optimal determinations of $G_{\text{TE}}$ across low-resistance epithelia (e.g., HUVEC) were described and discussed (15). In HUVEC cultures, the electrical resistance of the filter itself as measured in the Ussing chamber is $\approx 10 \, \Omega \cdot \text{cm}^2$, which is 1.5- to 2-fold higher than the resistance generated by the cells. To determine the endothelium-generated resistance, the following steps were taken. First, in every experiment, we included additional controls, such as blank filters without cells and filters containing cells grown in regular medium with known $G_{\text{TE}}$ on the basis of previous studies (15). Second, each experiment began by adjustment of the PD across a blank filter without cells to zero, to allow for subtraction of the filter-generated resistance. Third, at the conclusion of some experiments, Ca$^{2+}$ in the medium was lowered to zero by adding 2 mM EGTA to disrupt intracellular connections, including tight junctions (13, 17, 21), and to abolish the paracellular resistance. Under these conditions, the $G_{\text{TE}}$ is similar to the conductance of the filter itself.

Determinations of dilution potential. Determinations of the transepithelial dilution potential ($V_{\text{dil}}$) were performed in the Ussing chamber as described (15). $V_{\text{dil}}$ were determined by measuring the effect of lowering NaCl in the luminal solution on changes in voltage generated across the endothelial culture. This was done by replacing the Ringer buffer in the luminal compartment (130 mM NaCl) with low-NaCl (10 mM) solution. The latter buffer was similar to the Ringer solution except that it lacked the 120 mM NaCl and was supplemented with 240 mM sucrose to compensate for osmolality. The methods of electrophysiological data evaluation were previously described and discussed (15). $V_{\text{dil}}$ was the measured PD (voltage in subluminal solution – voltage in luminal solution) after lowering of NaCl in the luminal solution, corrected for the asymmetry of the potential electrodes. The Henderson diffusion equation (15) was used to interpret the $V_{\text{dil}}$ in terms of ionemic permeabilities. With the assumption that Na$^+$ and Cl$^-$ are the major permeant ions and all other component currents can be neglected, the $V_{\text{dil}}$ can be expressed in terms of the ratio of the mobilities of the monomers as $u_C/\Delta u_{Na}$, where $u_C$ and $\Delta u_{Na}$ are the mobilities of Na$^+$ and Cl$^-$ in the intercellular space.

For experiments using cells on filters, all reagents were added from concentrated stocks ($>1000$ of either 1% ethanol or saline to both the luminal and subluminal solutions.

Assay of NO. Release of NO was determined as the accumulation of nitrite ($\text{NO}_2^-$) and nitrate ($\text{NO}_3^-$) in the extracellular medium by a modified Greiss method (30, 10). Before experiments, cells were washed three times with PBS (37°C) and incubated in Ringer solution for 2 h at 37°C. At the end of incubation, the buffer was collected and centrifuged to remove dead cells. Aliquots (20 µl) were mixed 1:1 vol/vol with 2× Greiss reagent (composed of 0.5% sulfanilamide and 0.05% naphthylelamine dihydrochloride in 2.5% H$_3$PO$_4$) and incubated for 10 min at room temperature. The fluorescence was read at 365-nm excitation and 450-nm emission against a NaNO$_2$ standard curve, using the buffer as blank fluorescence. The detection limit of the assay was 2 µM. Results were expressed as picomoles per minute per milligram protein. Total cellular protein was determined as described (16).

Determinations of cGMP. Cells were washed with ice-cold PBS, the buffer was aspirated, and 1.0 ml of 6% TCA was added and vigorously pipetted. The cell homogenate was collected in a microcentrifuge tube; this step was repeated, and the two homogenates were combined. The tube was vortexed for 1 min and then centrifuged at 9,000 g for 15 min at room temperature, and the aqueous phase was stored at $-20\degree$C until assayed. The cGMP content within the aqueous phase was assayed using a commercially available RIA kit (Amersham, Arlington Heights, IL), after addition of a 25:1 ratio of acetylation reagent (1 vol of acetic anhydride with 2 vol of triethylamine) to each 500-µl sample. Results were expressed as picomoles per minute per milligram protein.

Fluorescence of attached cells. The fluorescence experiments were conducted in a newly designed fluorescence chamber (18). In this apparatus, a filter with cells was placed in an enclosed dark chamber maintained at a fixed temperature and under conditions that permit selective perfusion of the luminal and subluminal compartments. The cells were illuminated over the apical surface, and the intensity of the emitted light from the apical surface was measured as described (18). Cells on filters were incubated in culture medium with 7 µM fura 2-AM plus 0.25% Pluronic F-12 for 45 min at 37°C. After the incubation, cells were washed twice and reincubated with fresh culture medium for 10 min at 37°C to permit hydrolysis of the esters and to retain the polar molecules intracellularly. Measurements of fura 2 fluorescence were made at the isosbestic wavelengths (360-nm excitation and 510-nm emission; $F_{360}/F_{510}$) (40). Under these conditions, the leakage, photobleaching, and metabolism of fura 2 are minimal (18).

The theoretical background for the changes in $F_{360}/F_{510}$ was recently discussed (18). Similar to the principle of $F_{360}/F_{510}$ fura 2 microfluorescence imaging of attached cells (7), changes in $F_{360}/F_{510}$ are not influenced by cytosolic Ca$^{2+}$ but rather reflect changes in the intracellular concentration of the fura 2 and subsequently reflect changes in cell volume. The explanation is that, in attached and confluent cells, changes in volume are the result of changes both in the cross-sectional plane (the x-y plane, parallel to basal lamina) and in height (the z-axis, from basal lamina to the apical surface). It was suggested that in the new fluorescence chamber the emitted light stems from a single (or from a few) section(s) at the x-y plane. Changes in cell volume, and in particular in cell height, will draw more fura 2 molecules into, or away from, the monitored x-y plane(s), depending on whether the cell volume decreases or increases, respectively. Therefore, if the cell volume decreases, the signal will be stronger; if the cell volume increases, the signal will be weaker.

Viability of cells attached on filters. Viability of cells attached on filters was determined by incubation of cells with 10 µg/ml propidium iodide. Positive staining with propidium iodide indicates late apoptosis or necrosis. A stock solution of propidium iodide (100 µg/ml) was prepared in PBS and kept in the dark at room temperature for experiments, HUVEC on filters were washed with Ringer buffer and reincubated for 3 min at room temperature with propidium iodide; cultures were washed with PBS, and the fluorescence (540-nm excitation, 625-nm emission) was determined immediately in the fluorescence chamber at room temperature. Propidium iodide fluorescence was normalized to maximal fluorescence obtained in permeabilized cells that were preincubated with 5 µg/ml digitonin.

Preparation of total RNA. Total RNA from HUVEC on filters was isolated in situ on the filter with the Qiagen kit (Qiagen, Chatsworth, CA), using lysis buffer plus β-mercaptoethanol at 350 µl/10$^7$ cells. The final total RNA pellets were resuspended in 50 µl of diethyl pyrocarbonate water and quantitated by measuring optical density at 260 nm (OD$_{260}$).
ESTROGEN EFFECTS ON ENDOTHELIAL PERMEABILITY

The purity of the RNA sample was judged by determination of OD_{260}/OD_{280}.

RT-PCR. A Perkin-Elmer DNA thermal cycler (Cetus, Norwalk, CT) was utilized for the assays using a RT-PCR kit (Boehringer Mannheim, Indianapolis, IN). Total RNA (1.5 µg) denatured at 65°C for 5 min was reverse transcribed in a final volume of 20 µl of reaction mixture containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 10 mM MgCl_2, 1 mM dNTPs, 5 µM oligo(dT)_{15} (Promega, Madison, WI), 40 units RNase inhibitor, and 25 units avian myeloblastosis virus (AMV) RT (Boehringer Mannheim, Mannheim, Germany). For mock reaction, a similar tube was made up without the oligo(dT) and without the AMV RT. The reaction was allowed to proceed at 42°C for 60 min and was terminated by heating to 99°C for 2 min. The sample was diluted 1:5 with deionized distilled water. PCR was then performed in a 50-µl volume using 5 µl of the diluted sample, 5× PCR buffer (Amersham), 1 µM each primer, 0.01 mM dNTPs, 5 units Taq polymerase (Amersham), and 1.4 µM Taq antibody (Clontech) in a Perkin-Elmer 460 DNA thermal cycler (Cetus). Samples were heated for 5 min at 94°C, and then the following conditions were applied: for endothelial NOS (eNOS), 35 cycles of 1 min of denaturation step at 94°C, 1 min of annealing step at 62°C, and 2 min of extension step at 72°C; for inducible NOS (iNOS), 35 cycles of 1 min at 94°C, 2 min at 56°C, and 2 min at 72°C; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. Samples were then kept 7 min at 72°C, cooled at 4°C (soak file), and frozen at −80°C to facilitate removal of the mineral oil. The following oligonucleotide primers were used: for eNOS (26), forward (sense) 5′-CAG TGT CCA ACA TGC TGC TGG AAA TTG-3′ and reverse (antisense) 5′-TAA AGG TCT TCT TCT TGT GAG CTC C-3′; for iNOS (11), forward (sense) 5′-GCC TCG CTC TGG AAA GA-3′ and reverse (antisense) 5′-TCC ATG CAG ACA ACC TT-3′; and for GAPDH, forward (sense) 5′-TGA GGG TCG GAC TCA ACC GAT TTG GT-3′ and reverse (antisense) 5′-GTG GTG GTC CTC ATG GCC CAC ATG-3′. The sequences used to amplify the primers were synthesized by the Molecular Biology Core Laboratory (Case Western Reserve University School of Medicine) and were prepared as 10 µM stocks. Amplified samples (20 µl) were analyzed on 1.5% agarose gel, stained with ethidium bromide, and photographed. Parallel experiments were routinely done using DNase-I before RT, to exclude amplification of genomic cDNA contaminants. The DNA molecular mass markers were from HinII digest of OX174 DNA (United States Biochemical, Cleveland, OH) or 1-kb ladder (GIBCO, Grand Island, NY).

Densitometry. The X-ray films were analyzed with a laser densitometer (SdsCan 5000; United States Biochemical) and normalized relative to GAPDH RNA.

Statistical analysis of data. Data are presented as means ± SD. Significance of differences among means within groups was estimated by ANOVA. Significance of differences among means of the ratio u_0/u_4 was estimated by a randomization test for two independent nonparametric samples (36). Changes in trends were calculated using GB-STAT version 5.3 (1995, Dynamic Microsystems, Silver Spring, MD) and analyzed with ANOVA. Best fit of regression equations (least squares criterion) was achieved with SlideWrite Plus 1995 (Advanced Graphics Software, Carlsbad, CA), which uses the Levenberg-Marquardt algorithm, and analyzed using ANOVA. Variance among experiments (interassay variability) ranged 15%, but variance within experiments (intra-assay variability) ranged <5%, and all trends were consistent among experiments.

Chemicals and supplies. Anocell-10 filters were obtained from Anotec (Oxon, UK). All chemicals were obtained from Sigma Chemical (St. Louis, MO), unless stated otherwise.

RESULTS

Estradiol effects on G_{TE} and on P_{pyr}. HUVEC grown on filters formed epithelia that significantly decreased G_{TE} and P_{pyr} compared with levels across blank filters without cells (Table 1). Preplating of 3T3 fibroblasts had no significant effect on permeability (Table 1). In EGTA-treated cells, the levels of G_{TE} and of P_{pyr} were similar to those across blank filters (Table 1), indicating that lowering extracellular Ca^{2+} to <0.1 mM abolished the paracellular resistance, probably by disrupting the tight junctions (13, 17, 21). The levels of G_{TE} and P_{pyr} that were conferred by HUVEC were ~140 mS·cm^{-2} and 11 cm·s^{-1}·10^{-6} (for fluxes of 0.51 kDa), respectively (Table 1, Fig. 1). These values compare well with previously published results in endothelial cells (3, 5, 27, 29, 33, 41) and confirm that HUVEC form confluent endothelia on filters and restrict the free movement of solutes through the intercellular space.

Estradiol had no effect on G_{TE} and P_{pyr} levels across filters seeded with only 3T3 fibroblasts (not shown), and these levels were not different from those across blank filters (Table 1). Treatment with 17β-estradiol had a biphasic effect on G_{TE} across filters with HUVEC: at low concentrations (~1 nM), G_{TE} decreased from 129 ± 8 to 107 ± 5 mS·cm^{-2} (Fig. 1A; P < 0.02); at concentrations of >10 nM, the conductance increased, and at 1 µM G_{TE} was 175 ± 19 mS·cm^{-2} (Fig. 1B; P < 0.01). Estradiol had a similar biphasic effect on P_{pyr} treatment with 1 nM estradiol decreased the P_{pyr} from 16.0 ± 1.5 cm·s^{-1}·10^{-6} and treatment with 1 µM estradiol increased P_{pyr} significantly to 21.0 ± 1.5 cm·s^{-1}·10^{-6} (Fig. 1B; P < 0.01). These results indicate that low concentrations of estradiol decrease the paracellular permeability across HUVEC cultures, whereas high concentrations of estradiol increase it.

Effects of NOS inhibitors on G_{TE}. In HUVEC grown in steroid-free medium, L-NAME increased G_{TE} acutely by 31 mS·cm^{-2} (Fig. 2); preincubation of cells with L-Arg blocked the L-NAME effect (Treatment 2). In cells treated with 1 nM estradiol L-NAME increased G_{TE} by only 8 mS·cm^{-2}, and in cells treated with 1 µM estradiol...
L-NAME decreased G_{TE} by 8 mS⋅cm⁻² (Fig. 2, Table 2). L-NAME is a relatively nonspecific NOS inhibitor (10); to better understand the effect of NOS inhibition on G_{TE}, cells were treated also with N⁵-nitro-L-arginine (L-NNA), a more selective NOS inhibitor for the Ca²⁺-dependent eNOS (9), or with N⁶-monomethyl-L-arginine (L-NMMA), which inhibits mainly the Ca²⁺-independent iNOS (28).

L-NNA had an effect on G_{TE} similar to that of L-NAME in control cells, but in cells treated with 1 nM estradiol L-NNA increased G_{TE} to a greater degree than L-NAME. L-NNA failed to modulate L-NAME across cells treated with 1 µM estradiol (Fig. 2, Table 2).

L-NMMA also modulated G_{TE}; in control cells L-NMMA increased G_{TE} to a lesser degree than L-NAME (Fig. 2, Table 2). L-NMMA had no effect on G_{TE} in cells treated with 1 nM estradiol, and in cells treated with 1 µM estradiol L-NMMA decreased G_{TE} significantly more than L-NAME (Fig. 2, Table 2). Collectively, these results indicate that NOS inhibitors modulate acutely the permeability across HUVEC: the effects depend on the type of NOS inhibitor used, on prior treatment L-Arg Hb 1 nM 1 µM

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Values are means ± SD of changes in G_{TE} (mS⋅cm⁻²); n = 3–4 filters. ND, not determined. Passage 3 HUVEC were plated on filters and after 24 h were switched to steroid-free medium for additional 48 h in absence (control) or presence of 17β-estradiol at indicated concentrations. Changes in G_{TE} in response to N⁵-nitro-L-arginine (L-NAME), N⁶-nitro-L-arginine (L-NNA), N⁶-monomethyl-L-arginine (L-NMMA), sodium nitroprusside (SNP) (all at 1 mM), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; 10 µM), and SIN-1 (0.1 mM) were determined as in Figs. 2 and 4, after levels of G_{TE} had stabilized, by subtracting baseline G_{TE} from conductance levels after addition of agonist. Some filters with cells were pre-treated with 10 µM KT-5823 for 15 min before experiments as in Fig. 4, with 1 mM L-Arg 30 min before experiment, or with 50 µM hemoglobin (Hb) added with SNP. All agonist-induced changes were significant (P < 0.01) compared with baseline levels of G_{TE}, except those marked no significant change (NSC). *Differences within agonist categories (P < 0.03–0.01). Pretreatment with L-Arg or Hb blocked responses to L-NAME and to SNP, respectively (P < 0.04 in both cases).

Fig. 1. Means ± SD of transendothelial electrical conductance (G_{TE}; A) and of endothelial permeability to pyranine (P_{pyr}; B) across human umbilical vein endothelial cells (HUVEC) on filters. Passage 3 HUVEC were plated on filters and after 24 h were switched to steroid-free medium for additional 48 h in absence [control (C)] or presence of 17β-estradiol (E₂) at indicated concentrations. There were 3 filters for each time point. *P < 0.01 compared with control.
treatment of cells with estrogen, and on the dose of estrogen.

L-NAME, L-NNA, and L-NMMA had no effect on GTe and Poyr levels across filters seeded with 3T3 fibroblasts only (not shown).

Estrogen effects on NO release and on cellular levels of cGMP. Levels of NO in the extracellular fluid bathing filters seeded with 3T3 fibroblasts were undetectable by our method, regardless of treatment with estradiol (not shown). In contrast, HUVEC plated on filters released measurable amounts of NO into the extracellular fluid (2.5 ± 0.2 pmol·min⁻¹·mg protein⁻¹ in control cells; Fig. 3A). At concentrations of <1 nM, estradiol had no significant effect on NO release into the extracellular fluid by HUVEC; however, at estradiol concentrations of more than 10 nM, there was a dose-dependent increase in NO release (Fig. 3A). Lowering extracellular Ca²⁺ from 1.2 to <0.1 mM by adding aliquots of 0.3 M EGTA increased GTe across HUVEC cultures but had no effect on cell viability for up to 3 h after addition of the Ca²⁺ chelator; furthermore, replenishment of extracellular Ca²⁺ to 1.2 mM reversed the increase in permeability (not shown). Lowering extracellular Ca²⁺ tended to increase NO release, but it did not influence the estrogen effect. Incubation of cells with 1 mM L-NMMA inhibited the estradiol-induced increase in NO release; L-NNA had no significant effect (Fig. 3A).

The dose-response effect of estradiol on the increase in permeability correlated with the effects of estradiol on the increase in NO (Figs. 1A and 3A). This correlation suggested that NO mediates the increase in permeability. In contrast, the dose-response effect of estradiol on the decrease in permeability did not correlate with the effect of estrogen on NO (Figs. 1A and 3A). Lack of such correlation suggested that another factor, in addition to NO, mediates the decrease in permeability. To determine whether the estrogen-induced decrease in permeability is mediated by cGMP, we measured the accumulation of cGMP in HUVEC and the effect of treatment with estradiol.

Levels of cGMP in the extracellular fluid bathing filters seeded with 3T3 fibroblasts only were undetectable by our method, regardless of treatment with estradiol (not shown). In contrast, levels of cGMP in filters seeded with HUVEC were measurable and amounted to 52 ± 5 pmol·min⁻¹·mg protein⁻¹ in control cells (Fig. 3B). In HUVEC cultures, estradiol increased cGMP in a dose-related manner: effects began at 0.1 nM and reached saturation at 10 nM. The dose-response curve was sigmoidal, and the calculated EC₅₀ of estradiol was ~1 nM. Pretreatment with sodium nitroprusside (SNP) shifted the curve to the left (Fig. 3B). Lowering extracellular Ca²⁺ from 1.2 to <0.1 mM increased the estradiol EC₅₀ to ~10 nM and decreased the maximal increase in cGMP at saturating concentrations of estradiol (100 nM) from 150 to 100 pmol·min⁻¹·mg protein⁻¹ (Fig. 3B). These results indicate that lowering extracellular Ca²⁺ decreased the potency and efficacy of the effects of estradiol on cGMP.

Incubation of cells with 1 mM L-NNA decreased the maximal increase in cGMP in response to 105 pmol·min⁻¹·mg protein⁻¹ without affecting the EC₅₀ of estradiol; L-NMMA tended to decrease cGMP at estradiol concentrations of >10⁻¹⁰ M, but the response did not reach statistical significance (Fig. 3B). In cells pretreated with SNP, levels of cGMP were high, and estradiol had only small additional effect (Fig. 3B). These results indicate that the estrogen-induced decrease in permeability that occurs with low concentrations of estradiol correlates with an increase in cellular cGMP.

Collectively, the results shown in Figs. 1–3 suggest that the decrease in permeability is stimulated by nanomolar concentrations of estradiol and is mediated by a Ca²⁺-dependent, NO- and cGMP-related mechanism, and that the increase in permeability is stimulated by micromolar concentrations of estradiol and is mediated by a Ca²⁺-independent NO mechanism.

Effects of 8-bromoguanosine 3′,5′-cyclic monophosphate and of SNP on GTe. To clarify the roles of cGMP and of NO on GTe across HUVEC, two additional
experiments were conducted. First we determined the effects of 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) and of SNP on permeability. 8-Br-cGMP is a stable, cell-permeant analog of cGMP, and it can mimic cellular effects of cGMP; SNP is a NO donor, and it can mimic cellular effects of NO (30, 31).

8-Br-cGMP decreased \( G_{TE} \) acutely across cells grown in steroid-free medium, as well as across cells treated with low (1 nM) or with high (1 µM) concentrations of estradiol. In all cases, \( G_{TE} \) decreased by \(-29 \text{ mS} \cdot \text{cm}^{-2} \) (Fig. 4A, Table 2). SNP increased \( G_{TE} \) acutely across cells grown in steroid-free medium by \(-32 \text{ mS} \cdot \text{cm}^{-2} \) (Fig. 4A, Table 2). The effect of SNP was blocked by preincubation of cells with hemoglobin (Table 2); furthermore, the NO donor molsidomine, also called N-[ethoxycarbonyl]-3-[4-morpholinyl]sydnone imine (SIN-1), also increased \( G_{TE} \) (Table 2). These results indicate that the effect of SNP was mediated by released NO. SNP also increased \( G_{TE} \) across cells treated with 1 nM or with 1 µM estradiol, and the effect was similar to that across cells grown in steroid-free medium (Fig. 4A, Table 2). These results indicate that the effects of 8-Br-cGMP and of SNP on \( G_{TE} \) do not depend significantly on previous treatment with estradiol.

In the second experiment, cells were pretreated for 15 min with 10 µM KT-5823, which blocks the cGMP-dependent protein kinase (22), and then treated with 8-Br-cGMP or with SNP. Pretreatment with KT-5823 had no significant effect on \( G_{TE} \) or on the responses to SNP. However, it abolished the decreases in permeability induced by 8-Br-cGMP (Fig. 4B, Table 2). Pretreatment of cells for 15 min with 10 µM staurosporine, an inhibitor of protein kinase C, had no significant effects on \( G_{TE} \) or on the responses to 8-Br-cGMP or SNP (not shown). These results suggest that the effect of cGMP on permeability in HUVEC involves activation of a cGMP-dependent protein kinase.

Neither 8-Br-cGMP nor SNP had any effect on \( G_{TE} \) across filters seeded only with 3T3 fibroblasts (not shown).

**Estrogen effects on eNOS and iNOS RNA.** Likely sources for the NO involved in the estrogen effects shown in Figs. 2 and 3 are eNOS and iNOS (30, 31). To test this hypothesis, we determined the effects of estrogen treatment on the expression of eNOS and iNOS RNA. Total RNA was isolated from cells treated with 1 nM or 1 µM estradiol and analyzed by RT-PCR, using specific primers for the respective NOS isoforms. RT-PCR analysis of RNA from the murine 3T3 fibroblasts using the primers of human eNOS and iNOS did not reveal expression of NOS. In contrast, HUVEC expressed RNA of both eNOS and iNOS isoforms (Fig. 5). Treatment with estradiol had no significant effect on the expression of RNA for the ubiquitous enzyme GAPDH, but it modulated the expression of RNA of eNOS and iNOS relative to GAPDH (Fig. 5). Analysis of the results using densitometry revealed that treatment with 1 nM estradiol increased the expression of eNOS/GAPDH RNA by 6-fold (\( P < 0.05 \)), but treatment with 1 µM estradiol increased it only 1.5-fold (\( P > 0.1 \)). These results indicate that estradiol had a biphasic effect on the expression of eNOS RNA; at nanomolar concentrations the hormone increased eNOS RNA, but at micromolar concentrations estradiol had no significant effect on eNOS RNA. Treatment with 1 nM estradiol increased the expression of iNOS/GAPDH RNA by 1.5-fold, and treatment with 1 µM estradiol increased it by 5-fold, significantly more than the effect in cells treated with 1 nM estradiol (\( P < 0.05 \), Fig. 5). Thus, in contrast to eNOS, the estrogen-induced increase in iNOS RNA occurred mainly at micromolar concentrations of the hormone.

Effects on \( V_{all} \) and on \( u_C/u_Na \). The results shown in Fig. 1 indicate that estradiol modulates the paracellular permeability across HUVEC. The paracellular permeability is usually determined by two main mechanisms: the tight junctional resistance (\( R_{TJ} \)) and resistance of the lateral intercellular space (\( R_{LIS} \)) (34). To determine whether estrogen modulates the \( R_{TJ} \), we studied the effect of treatment with estrogen on \( u_C/u_Na \) in the paracellular space (34). Treatment with estradiol had a biphasic effect on \( u_C/u_Na \); levels of \( u_C/u_Na \) were lower in cells treated with 1 nM estradiol (1.28 ± 0.01)
Table 3; levels of cells (Fig. 6) thus resembled the lack of effect of estrogen treatment on the responses to 8-BrcGMP and to SNP (Table 3); the results were grown in a 100-cm² culture dish and were shifted to steroid-free medium for 48 h in absence or presence of indicated concentrations of 17β-estradiol. RT-PCR assays revealed expression of single 486-, 500-, and 932-bp cDNA bands of eNOS, iNOS, and GAPDH, respectively. In mock reactions lacking oligo(dT) and avian myeloblastosis virus (see METHODS), no detectable bands were found (not shown). Experiment was repeated twice.

Fig. 5. Effects of estradiol on expression of endothelial NOS (eNOS), inducible NOS (iNOS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA in HUVEC. Preconfluent passage 3 HUVEC were grown in a 100-cm² culture dish and were shifted to steroid-free medium for 48 h in absence or presence of indicated concentrations of 17β-estradiol. RT-PCR assays revealed expression of single 486-, 500-, and 932-bp cDNA bands of eNOS, iNOS, and GAPDH, respectively. In mock reactions lacking oligo(dT) and avian myeloblastosis virus (see METHODS), no detectable bands were found (not shown). Experiment was repeated twice.

than in untreated cells (1.35 ± 0.01; Fig. 6A, inset; Table 3; P < 0.05). In cells treated with 1 µM estradiol, levels of Uc/UNa were similar to those across untreated cells (Fig. 6A, inset). These results indicate a biphasic effect on Uc/UNa, similar to the biphasic effects of estradiol on GT and on PDE (Fig. 1).

To clarify this result, we determined the effects of L-NAME, 8-BrcGMP, and SNP on Uc/UNa. All three agents acutely modulated the ratio of Uc/UNa: L-NAME and SNP increased the ratio, whereas 8-BrcGMP decreased the ratio (Fig. 6A). These results indicate that L-NAME and SNP decrease the cation selectivity and that 8-BrcGMP increases it. The effects of estradiol on changes in Uc/UNa in response to L-NAME, L-NMMA, 8-BrcGMP, and SNP also were determined. L-NMMA increased the ratio of Uc/UNa in untreated cells and in cells treated with 1 nM estradiol but not in cells treated with 1 µM estradiol. L-NMMA decreased the ratio of Uc/UNa in cells treated with 1 µM estradiol but not in cells treated with 10 nM estradiol (Table 3). These results resemble the effects of estradiol on changes in GT induced by L-NMMA and by L-NMMA (Fig. 2). Treatment with estradiol had no effect on the Uc/UNa responses to 8-BrcGMP and to SNP (Table 3); the results thus resembled the lack of effect of estrogen treatment on changes in GT in response to these agents (see Fig. 4A, Table 2).

Collectively, the results shown in Figs. 4 and 6 and Tables 2 and 3 indicate that the effects of estradiol on the changes in GT in response to L-NNA, L-NMMA, 8-BrcGMP, and SNP correlate with the changes induced by these agents on cation selectivity.

Modulation of cell size. To determine whether estrogen also modulates the Rlis, we determined the effects of 8-BrcGMP and of SNP on cell size. The rationale was as follows. 1) 8-BrcGMP and SNP appear to mediate, respectively, the estrogen-induced decrease and increase in permeability (Figs. 1 and 4A). 2) In an intact epithelium, such as HUVEC, the Rlis usually depends on the geometry of the intercellular space and in turn on the volume of the surrounding cells that form this space (34). HUVEC on filters were loaded with the fluorescent dye fura 2, and changes in cell size were determined by measuring changes in fluorescence in the Ca²⁺-insensitive wavelengths (F 360/510). To quantify the changes in F 360/510, measurements of fluorescence were expressed relative to the fluorescence obtained when cells were perfused with Ringer buffer.

Experiments done on filters seeded only with 3T3 fibroblasts revealed that the cells were able to load fura 2, but the fluorescence signal was ~100-fold weaker than that obtained from filters plated with HUVEC on top of 3T3 fibroblasts (not shown), indicating that the changes in fluorescence are contributed mainly by the HUVEC.

We have previously shown that changes in F 360/510 induced by changing the buffer osmolarity in attached human cervical cells were linear in the range from 0.5 to 1.5 F 360/510 (14). A similar relationship was also found in HUVEC, using the same method (not shown).

8-BrcGMP decreased F 360/510 across HUVEC in a time-related manner [Fig. 6B; half time (t½) of ~1.5 min]. It was previously shown by us (18) and by others (7) that a decrease in F 360/510 correlates with an increase in cell size, and the response to 8-BrcGMP shown in Fig. 6B is interpreted as an increase in HUVEC cell size. SNP had an opposite effect, and it increased F 360/510, i.e., it decreased cell size (Fig. 6B; t½ of ~1 min); in those experiments, the background autofluorescence of SNP was minimal and did not affect the interpretation of the results (not shown). The fluorescence method was not sensitive enough to determine actual changes in cell size. Collectively, the results shown in Fig. 6B indicate that 8-BrcGMP induces an acute increase in HUVEC size, whereas SNP induces an acute decrease in cell size, and they therefore suggest that 8-BrcGMP increases Rlis and SNP decreases Rlis.

Role of extracellular Cl⁻. One of the mechanisms by which cells can change their size acutely is by modulating transcellular osmotic gradients secondary to changes in transcellular ion transport. To determine whether the effects of 8-BrcGMP and of SNP involve acute changes in ion transport, we determined the effects of lowering luminal NaCl on the GT responses to 8-BrcGMP or to SNP. Changes in GT under conditions of asymmetrical NaCl concentrations were determined by...
correcting the measured levels of $G_{TE}$ for fluid conductivity (15). Lowering luminal NaCl attenuated the 8-BrcGMP-induced decrease in $G_{TE}$ and augmented the SNP-induced increase in conductance (Fig. 7, Table 4). Lowering NaCl in the luminal solution of filters seeded only with 3T3 fibroblasts had no effect on $G_{TE}$ (not shown). These results suggest that Na$^+$ and/or Cl$^-$ may be involved in the changes in cell size induced by 8-BrcGMP or by SNP.

To clarify these findings, two additional experiments were done. First, we studied the effects of Cl$^-$ channel blockers on changes in $G_{TE}$. In filters seeded only with 3T3 fibroblasts, diphenylamine-2-carboxylate (DPC; 0.5 mM) had no effect on $G_{TE}$. In filters with HUVEC, DPC also had no significant effect on baseline $G_{TE}$, but it attenuated the 8-BrcGMP-induced decrease in $G_{TE}$, and it augmented the SNP-induced increase in conductance (Fig. 7, Table 4). Similar results were obtained with 250 µM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; not shown). Furthermore, when blockers

Table 3. Modulation of $u_{Cl}/u_{Na}$ across HUVEC cultures on filters

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>L-NNA</th>
<th>L-NMMA</th>
<th>8-BrcGMP</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.35</td>
<td>1.41*</td>
<td>1.37</td>
<td>1.30*</td>
<td>1.42*</td>
</tr>
<tr>
<td>Estradiol (1 nM)</td>
<td>1.28</td>
<td>1.35*</td>
<td>1.28</td>
<td>1.24*</td>
<td>1.35*</td>
</tr>
<tr>
<td>Estradiol (1 µM)</td>
<td>1.34</td>
<td>1.34</td>
<td>1.28*</td>
<td>1.28*</td>
<td>1.41*</td>
</tr>
</tbody>
</table>

Values are mean baseline monion mobilities ($u_{Cl}/u_{Na}$) levels and changes in $u_{Cl}/u_{Na}$ in response to L-NNA (1 nM), L-NMMA (1 mM), 8-BrcGMP (10 µM), and SNP (1 mM); n = 3–4 filters for each value, and SD ranged from 0.01 to 0.02. Directional potentials was established by lowering luminal NaCl, and changes in $u_{Cl}/u_{Na}$ were determined as described in Methods. All reagents were added to both luminal and subluminal solutions. *P < 0.01 compared with baseline.

Fig. 6. A: effects of estradiol (inset), SNP (1 mM), L-NAME (1 mM), and 8-BrcGMP (10 µM) on ratio of monion mobilities ($u_{Cl}/u_{Na}$) across HUVEC cultures. Cells for experiment in inset were prepared as in Fig. 2. Passage 3 HUVEC on filters were grown in regular medium for 3 days. B: effects of 8-BrcGMP (10 µM) and SNP (1 mM) on fura-2 fluorescence in isosbestic wavelengths for Ca$^{2+}$ (360-nm excitation and 510-nm emission; $F_{360/510}$) in HUVEC attached on filters. Cells were plated on filters and grown in regular medium for 3 days. Cells were loaded with fura-2, and measurements of fluorescence were made in a fluorescence chamber designed to house filter insert. When indicated, cells were perfused in both luminal and subluminal compartments with a solution containing an agonist. Measurements of fluorescence were expressed as arbitrary units (AU) relative to fluorescence obtained when cells were perfused with 290 mosmol/l buffer. The half time of changes in $F_{360/510}$ was determined by extrapolation. Experiments were repeated 3 times.

Fig. 7. Low luminal (L) NaCl and diphenylamine-2-carboxylate (DPC) modulate changes in $G_{TE}$ induced by 8-BrcGMP and by SNP. Passage 3 HUVEC were plated on filters and grown in regular medium for 3 days. Control experiments were done in regular Ringer buffer. For low luminal NaCl, luminal solution was replaced with a low-NaCl buffer (see Methods). For DPC treatment, cells were pretreated for 10 min with DPC. 8-BrcGMP (10 µM), SNP (1 mM), and DPC (0.5 mM) were added to both luminal and subluminal solutions from 1,000 stocks. Results are summarized in Table 2.
were administered to the luminal solution, the effects of DPC (Table 4) and of NPPB (not shown) were greater than when the agents were administered to the subluminal solution.

Second, we studied the effects of Na\(^+\) transport blockers on G\(_{TE}\). Neither 10 µM amiloride or 100 µM benzamil (Na\(^+\) channel blockers at these concentrations) nor 1 mM amiloride (Na\(^+\)/H\(^+\), and possibly Na\(^+\)/Ca\(^{2+}\) exchanger blocker at this concentration) or 100 µM furosemide (inhibitor of Na\(^+\)-Cl\(^-\) and Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporters) (12) changed baseline G\(_{TE}\) or the responses to 8-BrcGMP or SNP (not shown).

Collectively, these results suggest that transcellular transport of Cl\(^-\) via the apical membrane is necessary for the full magnitude of changes in G\(_{TE}\) in response to 8-BrcGMP and to SNP.

**DISCUSSION**

Estradiol had a biphasic effect on paracellular permeability across HUVEC cultures: at nanomolar concentrations it decreased the permeability, whereas at micromolar concentrations it increased the permeability. Our results suggest that the decrease in permeability was mediated by a Ca\(^{2+}\)-dependent eNOS-NO-cGMP mechanism for five reasons. 1) 8-BrcGMP decreased the permeability, whereas KT-5823, a cGMP-dependent protein kinase inhibitor, blocked the effect. 2) The effect of estradiol on cGMP correlated with the decrease in permeability. 3) Incubation of cells in low extracellular Ca\(^{2+}\) decreased the efficacy and potency of the estrogen effect on cellular cGMP. 4) L-NMMA, a Ca\(^{2+}\)-dependent NOS inhibitor, but not L-NMMA, increased the permeability and attenuated the estrogen-induced increase in cGMP. L-NMMA also increased acutely the permeability, and treatment with 1 nM estradiol augmented the response to L-NMMA. A possible explanation is that estrogen upregulated and L-NMMA inhibited a NO-related mechanism proximal to cGMP. 5) Micromolar but not micromolar concentrations of estradiol increased the expression of eNOS RNA, which is a Ca\(^{2+}\)-dependent NOS (30, 31).

Our results also suggest that the estrogen-dependent increase in permeability was mediated by a Ca\(^{2+}\)-independent iNOS-NO mechanism for the following reasons. 1) SNP and SIN-1, NO donors, produced an acute increase in permeability that was blocked by hemoglobin but not by KT-5823. 2) The effect of estradiol on NO release correlated with the increase in permeability. 3) The increase in NO release was not affected by lowering extracellular Ca\(^{2+}\). 4) L-NMMA (which inhibits mainly the Ca\(^{2+}\)-independent NOS), but not L-NNA, blocked the NO release that was induced with high concentrations of estradiol. L-NMMA also decreased the permeability in cells treated with 1 µM estradiol, but not in cells grown in steroid-free media or in cells treated with nanomolar estradiol. 5) Estradiol increased iNOS RNA, but, in contrast to the biphasic effect on eNOS RNA, micromolar concentrations of estradiol produced a significant increase in iNOS RNA.

The results of the experiments using L-NNA and L-NMMA should be interpreted with caution, because these agents may have effects other than those previously suggested (9, 10, 28) or have nonspecific effects. In cells grown in steroid-free medium, L-NNA did increase G\(_{TE}\) but had no significant effect on cGMP. This may suggest that L-NNA acts on permeability via an additional pathway unrelated to cGMP. On the other hand, the data in other experiments support our hypothesis, and our results agree with previous publications in the field (9, 10, 28).

Our results showed that the eNOS-NO-cGMP and iNOS-NO systems differed in their sensitivity to estradiol with regard to the expression of eNOS and iNOS RNA, NO activity, and the cellular accumulation of cGMP. The expression of eNOS RNA and the accumulation of cGMP were maximal at nanomolar estradiol concentrations; the latter conclusion is supported by the finding that, in cells pretreated with SNP at a high concentration (1 mM), already 0.1 nM estradiol had no additional effect on cGMP. In contrast, iNOS RNA expression and NO release did not reach saturation even at micromolar estradiol. SNP increased cGMP significantly in cells grown in steroid-free medium, but 0.1 nM estradiol did increase cGMP further. Although the additional effect of estradiol was mild compared with that of SNP and with that of 10 nM estradiol, the results overall suggest that SNP per se does not increase cGMP maximally. Regardless, however, our data showed that levels of cGMP in cells treated with ≥10 nM estradiol were similar to those in cells treated with SNP plus 0.1 nM estradiol, suggesting that saturation of the soluble guanylate cyclase had occurred.

Although changes in the activities of either cGMP or NO can explain the biphasic effect of estrogen on permeability, the limiting factor for the estrogen-dependent decrease in permeability appears to be cGMP: 8-BrcGMP decreased the permeability even in cells treated with 1 µM estradiol; in these cells, levels of cGMP were presumably maximal, because (as stated above) cGMP accumulation was saturated already at 10 nM estradiol. Our results therefore suggest that the limiting factor for the estrogen-dependent decrease in...
permeability is the NO-induced turnover of guanylate cyclase.

One of our objectives was to determine the mechanism by which estrogen modulates the paracellular resistance across HUVEC. The results suggest that estrogen and cGMP (the presumed mediator of the estrogen-induced decrease in permeability) and NO (the presumed mediator of the estrogen-dependent increase in permeability) modulate both $R_{TJ}$ and $R_{LIS}$. A possible explanation, based on the Ussing-Zerahn model of transepithelial transport (43), is that the changes in cell size (i.e., in $R_{LIS}$) lead to changes in $R_{TJ}$. According to the Ussing-Zerahn model, movement of molecules in the intercellular space (paracellular pathway) is restricted by the $R_{TJ}$ and $R_{LIS}$. The regions of the tight junction are considered high-resistance elements, due to the occlusion of the intercellular space by the tight junctional complexes. In contrast, $R_{LIS}$ is considered a low-resistance element, and it is determined by the proximity of the plasma membranes of neighboring cells and by the length of the intercellular space from the tight junctions to the basal lamina (34). The intercellular space, both within the regions of the tight junctions and outside these regions, can be modeled as a series of narrow tubes, whose diameters depend on the proximity of plasma membranes of neighboring cells. Because the resistance to flow in a tube depends on the fourth power of the radius of the tube, even minor changes in the cross-sectional area of the intercellular space can greatly affect the resistance. In attached cells, changes in the geometry of the intercellular space are usually secondary to changes in cell size in the opposite direction. The present results showed that both 8-Br-cGMP and SNP induced acute changes in the size of HUVEC, and it is therefore suggested that the changes in cell size affect both the $R_{LIS}$, i.e., the resistance of the intercellular space outside the regions of the tight junctions, and the $R_{TJ}$, i.e., the resistance of the intercellular space within the tight junctions.

Acute changes in cell size can be the result of two main mechanisms: rearrangement of cytoskeletal proteins (35) and acute changes in osmotic gradients across the plasma membrane that shift water out of or into the cell (8). The changes in $G_{TE}$ induced by 8-Br-cGMP and by SNP were influenced by the concentrations of extracellular Na$^+$ and Cl$^-$. Na$^+$ transport blockers, including furosemide (an inhibitor of the Na$^+$-Cl$^-$ and Na$^+$-K$^+$-Cl$^-$ cotransporters), had no effect on the responses, but Cl$^-$ channel blockers modulated the changes in conductance: DPC and NPPB, as well as low luminal NaCl, blocked the decrease in $G_{TE}$ in response to 8-Br-cGMP, but they augmented the increase in conductance in response to SNP. Furthermore, most of the effects of DPC and NPPB were observed when the agents were administered in the luminal solution. These results suggest that Cl$^-$ transport via the apical plasma membrane determines the full magnitude of changes in $G_{TE}$ in response to 8-Br-cGMP and to SNP. Consequently, the effect of 8-Br-cGMP can be the result of augmented Cl$^-$ influx via apically located Cl$^-$ channels, followed by water influx to compensate for hyperosmolality. The end result would be an increase in cell size. The finding that low luminal NaCl attenuated the response to 8-Br-cGMP can be explained by a decrease in the driving force for Cl$^-$ influx (lower extracellular-intracellular Cl$^-$ gradient). The effect of SNP can be explained by a similar mechanism, but in the opposite direction: SNP may have induced Cl$^-$ secretion via apically located Cl$^-$ channels, with subsequent loss of cellular water and a decrease in cell size. Low luminal NaCl may have augmented the response by increasing the driving force for Cl$^-$ efflux.

On the basis of these results, we suggest that estradiol activates two NO-related mechanisms in HUVEC. At nanomolar concentrations, estradiol upregulates mainly the eNOS. The generated NO activates guanylate cyclase and produces cGMP. cGMP stimulates enhanced Cl$^-$ and water influx, an increase in cell size, an increase in $R_{LIS}$ and in $R_{TJ}$, and subsequently a decrease in permeability. At nanomolar concentrations, estrogen also upregulates NO production by inducing iNOS; however, the total amount of NO produced at these low concentrations of estradiol is low and could not be detected by the Greiss method. At micromolar concentrations, estradiol stimulates a substantial increase in NO from iNOS: one of the targets of NO, the guanylate cyclase, becomes saturated already at nanomolar estradiol concentrations, and this allows NO to act on other cellular targets, including Cl$^-$ channels. Our results suggest that, in contrast to cGMP, which activates Cl$^-$ influx, NO itself or acting via another mediator stimulates Cl$^-$ efflux. This leads to water efflux, to decreases in cell size, in $R_{LIS}$, and in $R_{TJ}$, and subsequently to an increase in permeability. The mechanisms by which cGMP and NO activate influx and efflux of Cl$^-$, respectively, in HUVEC are unknown.

The relevance of the present results to understanding how estrogens modulate transendothelial transport in vivo should be discussed with regard to the experimental model (cultured endothelial cells) and with regard to the concentrations of estradiol that were used in the present study. The measured permeability of cultured HUVEC was similar to that of other cultured endothelia but was higher than that determined for endothelia in vivo. This topic has been recently discussed (3, 5, 27, 29, 33, 41), and one explanation for the higher permeability in vitro is the lack of matrix and stromal cells that are present in vivo and can contribute to the paracellular resistance. Despite these considerations, experiments with cultured HUVEC can yield important mechanistic information, as in the present study.

The estrogen-induced decrease in permeability occurred at physiological (nanomolar) concentrations of estradiol in women. Because estrogen response elements were previously described in the eNOS gene (4), the modulation of the eNOS can be explained by the classical nuclear estrogen receptor mechanism (1). In contrast, the increase in permeability required micromolar concentrations of estradiol, which are supraphysiological for women. Free estrogens usually do not reach
micromolar levels in the plasma; however, in certain conditions tissues may be exposed to high levels of estrogens. For instance, women treated with the birth control pill consume high doses of synthetic estrogens. It has been shown that one of the effects of the pill is tissue edema, as a result of increased transudation of fluid from the plasma through the capillary endothelium into the extracellular space (38). Based on the present study, it is possible that this effect is the result of high-dose, estrogen-induced increase in endothelial permeability. Another example is pregnancy, which is also a condition of high-estrogen milieu. During pregnancy, the permeability of the utero-placental tissues is high; a possible explanation is that the high levels of estrogens increase the permeability of both fetal (e.g., HUVEC) and maternal endothelial cells.

An argument raised by a number of previous studies was that micromolar estrogen concentrations can modulate cell function via nongenomic mechanisms; examples are changes in intracellular pH and in cytosolic late cell function via nongenomic mechanisms; ex- in cytosolic late cell function via nongenomic mechanisms; ex- in cytosolic late cell function via nongenomic mechanisms; ex- in cytosolic late cell function via nongenomic mechanisms; ex-

To summarize, the present results in HUVEC revealed dual modulation of endothelial paracellular permeability by estrogen. 1) An eNOS-, NO-, and cGMP-dependent decrease in permeability is activated by nanomolar concentrations of estradiol, resulting in increased Cl− influx, increased cell size, and increased RLT and RTJ; these effects appear to be limited by the ability of cells to generate cGMP in response to NO. 2) An iNOS- and NO-dependent increase in permeability was activated by micromolar concentrations of estradiol, resulting in enhanced Cl− efflux, decreased cell size, and decreased RLT and RTJ. The net effect on transendothelial permeability will depend on the relative contributions of each of these two systems. Understanding the mechanisms by which estrogens modulate endothelial permeability may be important for development of drugs that can target the specific mechanisms involved in the actions of estrogen and may provide the pharmacological means to selectively regulate the permeability.

This study was supported in part by grants from Bristol Myers Squibb (US Pharmaceuticals) to G. I. Gorodeski and by National Heart, Lung, and Blood Institute Grant HL-48771 to N. P. Ziats. Address for reprint requests: G. I. Gorodeski, University Macdonald Hospital, Cleveland, Ohio 44106.

Received 28 May 1998; accepted in final form 20 October 1998.

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