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Estrogen Increases Endothelial Carbon Monoxide, Heme Oxygenase 2, and Carbon Monoxide-Derived cGMP by a Receptor-Mediated System

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Carbon monoxide, a gaseous activator of soluble guanylyl cyclase formed by a subtype of the enzyme heme oxygenase designated heme oxygenase-2 in vascular endothelium, has been found to dilate blood vessels independently from nitric oxide. Because of the parallels between nitric oxide and carbon monoxide, we speculated that estrogen might affect carbon monoxide production in vascular endothelium. Endothelial cells of human origin (umbilical vein and uterine artery) were incubated for 4 or 24 h with 10^{-12}–10^{-6} M 17β-estradiol. 17β-Estradiol, at a concentration such as that attained during the ovulatory phase of the menstrual cycle (10^{-10} M), administrated for 4 h led to a 2-fold increase in intracellular carbon monoxide production and heme oxygenase-2 protein levels ($P < 0.05$). A reporter assay, measuring the formation of cGMP as the direct product of carbon monoxide-induced activation of soluble guanylyl cyclase in endothelial cells, also revealed a 56% increase in cellular cGMP after treatment with 10^{-10} M E_2 17β-estradiol ($P < 0.05$). By contrast, higher 17β-estradiol concentrations had no significant respective effects due to nitric oxide synthase inhibition of carbon monoxide release. This 17β-estradiol effect appeared to be ER dependent, as preincubation with tamoxifen (10^{-6} M) blocked the stimulatory effect of 17β-estradiol in each instance. Our preliminary data indicate a potential role for carbon monoxide as a biological messenger molecule in estrogen-mediated regulation of vascular tone. (*J Clin Endocrinol Metab* 86: 3833–3839, 2001)

THE INCIDENCE OF cardiovascular disease, the leading cause of mortality in western societies, is higher in men than in premenopausal women, but increases in postmenopausal women (1). An abundance of epidemiological data supports a role for estrogens in this atheroprotective effect, prompting recommendations for their widespread use in postmenopausal replacement therapy (2).

However, the mechanism by which estrogen mediates vasoprotection remains to be fully clarified. It is traditionally thought to be due to potentially favorable changes in blood lipids and lipoproteins (3), but a number of human (4, 5) as well as animal studies strongly suggest a direct effect on the vascular system (6, 7) acting via vascular ER (4, 5).

17β-Estradiol (E_2) accounts for the endothelial expression of rate-limiting enzymes in the biosynthesis of the two important vasodilators, prostacyclin and nitric oxide (NO). E_2 potentiates the effect of endothelin-1 on prostacyclin production in human umbilical vein endothelial cells (HUVECs) (8) and causes increased transcription of the endothelial nitric oxide synthase (eNOS) gene (9) as well as activation of eNOS (10) and NO release (11) in endothelial cells via nongenomic mechanisms.

Abbreviations: CO, Carbon monoxide; CrMP, chromium mesoporphyrin; E_2, 17β-estradiol; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO, heme oxygenase; HAUEC, human uterine artery endothelial cells; HUVEC, human umbilical vein endothelial cell; L-NAME, N^G-nitro-L-arginine-methyl-ester; NO, nitric oxide; TAM, tamoxifen.

Heme oxygenase (HO) is the rate-limiting enzyme for heme degradation in mammals (12). It decomposes heme into biliverdin and releases free iron and carbon monoxide (CO). To date, three isoforms of HO have been characterized: HO1, HO2, and HO3 (13, 14). HO1 is widely expressed and is inducible by a host of stimuli that produce oxidative stress (15). On the other hand, HO2 occurs in neuronal populations and vascular endothelial cells (16), and the only known inducer of HO2 is adrenal glucocorticoid (17). HO3 has recently been identified in the rat heart, kidney, brain, and liver (14). Both vascular endothelium and smooth muscle express heme oxygenases (16, 18), and HO-catalyzed formation of CO has been documented in blood vessels (19). Upon endothelial release, CO diffuses to the underlying vascular smooth muscle cells to elicit relaxation by increasing cGMP production independently from NO (18, 20, 21), to cause hyperpolarization by opening potassium channels (22), and to decrease the production of vasoconstrictors such as endothelin (23).

NO is a well established effector molecule of E_2-mediated vasoprotection. Because of the apparent parallels between NO and CO, we sought to clarify preliminarily whether at physiological levels E_2 modulates CO production, HO2 protein and mRNA levels, and levels of cGMP, a direct product of NO- and CO-induced activation of soluble guanylate cyclase in HUVEC and human uterine artery endothelial cells (HAUEC) in both the presence and absence of estrogen and antiestrogen.

Materials and Methods

Endothelial cell culture

HUVEC and HAUEC were isolated from female umbilical veins and hysterectomized uteri, respectively as previously described (24). Cells were passaged on gelatin-coated 25-cm^2 flasks (Costar, Cambridge, MA) in phenol red-free medium 199 (Sigma, St. Louis, MO) containing 20% heat-inactivated FCS (HyClone Laboratories, Inc., Logan, UT) and supplemented with penicillin (1000 IU/ml), streptomycin (1 mg/ml), fungizone (25 μg/ml; penicillin-streptomycin-fungizone solution, JRH Biosciences, Lenexa, KS), endothelial cell (EC) growth supplement (50 μg/ml; Technoclone, Vienna, Austria), and heparin (5 IU/ml; Hoffman-LaRoche Inc., Basel, Switzerland). Cells were used within four passages and were identified as endothelial by their characteristic cobblestone morphology, the presence of factor VIII antigen, and uptake of acetylated low density lipoprotein. Forty-eight hours before the experiments, heparin and endothelial cell growth supplement were removed from the medium. ECs were treated with E_2 (Sigma) over a range of concentrations and time points, as indicated in *Results* and the figure legends. The role of ER in the response to E_2 was determined during incubations performed in the simultaneous presence of 10^{-6} M tamoxifen (TAM; Sigma) added 1 h before E_2. To examine the role of the NOS system on endothelial CO release, different dilutions of the nonspecific NOS inhibitor N^G-nitro-L-arginine-methyl-ester (L-NAME; Sigma) were added to cells 30 min before E_2 treatment.

CO detection system

CO release by vascular ECs was determined by spectrophotometric detection of CO-hemoglobin (25) in cell culture supernatants. In brief, supernatant was collected from confluent cells at the end of the incubation period. The percentage of COHb was determined spectrophotometrically using a U-3000 spectrophotometer (Hitachi, Tokyo, Japan), and the amount of pure CO was calculated and expressed as micrograms per liter total supernatant.

Western blotting

For cytosolic fractions, cells were lysed in Nonidet P-40 lysis buffer and further processed as previously described (26). In brief, the concentration of protein in aliquots of the lysates was measured with a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL). Twenty micrograms of cellular extracts were used for experiments, and 5 μg of a rat spleen or a rat brain microsomal preparation (Chemicon, Temecula, CA) were used as positive controls for the detection of HO1 or HO2 protein, respectively. After quantification, proteins were electrophoresed through the use of standard SDS-PAGE on 8–18% gradient gels and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were blocked in a solution consisting of 5% nonfat dry milk and 5% BSA (Sigma) in 0.1 mol/liter Tris-HCl, 0.15 mmol/liter NaCl, and 0.01% Nonidet P-40, pH 7.5. Immunoreactions were performed with an anti-HO1 or anti-HO2 polyclonal antibody (StressGen, Victoria, Canada; dilution of both antibodies, 1:2,000). An appropriately diluted polyclonal rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) instead of the primary AB was used as a negative control for each AB. This was followed by a horseradish peroxidase-conjugated goat antirabbit IgG (Pierce Chemical Co.; dilution, 1:20,000). Specific reaction products were detected by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech).

Quantitative real-time RT-PCR

Total RNA was extracted from cultured cells as previously described (27). Briefly, total RNA was prepared by ultracentrifugation of a guanidine-isothiocyanate lysate through a cesium trifluoroacetate cushion. Precipitated RNA was removed, diluted with absolute ethanol, pelleted by centrifugation, washed with 70% ethanol, dissolved in ribonuclease-free water, and stored at −80 C. The integrity of RNA was assessed by agarose gel electrophoresis. RT was performed on 500 ng total RNA using 200 U Moloney murine leukemia virus reverse transcriptase (ViennaLab Labordiagnostika GmbH, Vienna, Austria) and a commercially available reagent kit (Random Primed RT-Mix, ViennaLab Labordiagnostika GmbH). Synthesized cDNA was stored in aliquots at −80 C.

The amplification primers and the TaqMan probe for the HO2 real-time PCR (Table 1) resulting in a 71-bp amplicon (position 841–911) (28) were designed with Primer-Express software (PE Biosystems, Foster City, CA). 6-Carboxyfluorescein was used as the reporter dye, and 6-carboxytetramethylrhodamine was used as the quencher dye. Oligonucleotide synthesis and purification were performed by VBC-Genomics Bioscience Research GmbH (Vienna, Austria). The reaction was carried out in a 25-μl total volume containing 2 μl cDNA, 25 pmol of each amplification primer, 5 pmol probe, and 12.5 μl 2 × TaqMan Universal Mix (PE Applied Biosystems). The reaction conditions were 50 C for 2 min, 95 C for 10 min (activation of the AmpliTaq-Gold polymerase), and then 40 cycles of 15 sec at 95 C (denaturation), followed by 60 sec at 60 C (annealing and extension). To correct variations linked to differences in the amount of RNA taken for the reaction or to different levels of inhibition during RT or PCR, we normalized HO2 expression using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a ubiquitously expressed housekeeping gene, as a reference gene. The expression of this housekeeping gene was quantified with the GAPDH Control Reagents Kit from PE Applied Biosystems according to the manufacturer's guidelines. All HO2 and GAPDH experiments were carried out in triplicate, and several negative controls were included. Fluorescence emission was continuously monitored and analyzed by a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems) with GeneAmp 5700 SDS software (version 1.1).

Standard curves

For the generation of HO2 and GAPDH standard curves, we used 2-fold serial dilutions of a HUVEC cDNA sample showing the highest HO2 mRNA expression levels in HO2 real-time RT-PCR as templates. Standard curves were constructed and calculated using GeneAmp 5700 SDS software by plotting the threshold cycle (PCR cycle at which a specific fluorescence becomes detectable) *vs.* the log of the cDNA starting quantity of each dilution step. These standard curves allowed us to interpolate the unknown HO2 and GAPDH mRNA expression levels in each analyzed sample.

cGMP measurement

HAUEC were cultured under standard culture conditions as described above, incubated with 3-isobutyl-1-methylxanthine (Sigma; final concentration, 0.5 mM) to inhibit phosphodiesterase activity and with the nonselective NOS inhibitor L-NAME (final concentration, 0.1 mM) to avoid demonstrating a potential effect of NO on cGMP levels. Both inhibitors were administrated 30 min before incubation with E_2. Cells were solubilized in lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 1% Triton X-100 (Sigma)], and 10 mM EDTA. Cell fragments were collected by centrifugation at 10,000 × *g*. Quantification of intracellular cGMP levels in cell lysates was assessed using cGMP RIA (Amersham Pharmacia Biotech) according to the supplier's instructions, including an acetylation step. The protein content of the lysates was assessed using a Lowry-based protein assay (DC protein assay, Bio-Rad Laboratories, Inc., Richmond, CA). Duplicate measurements were performed on all samples.

Statistical analysis

The values are expressed as the mean ± SD. A preliminary analysis revealed that the datasets conformed to a normal distribution. Comparisons between groups were made using the exact version of the Wilcoxon test in SAS (SAS/STAT, 1989) (29). Adjustment for multiple testing was performed using the Bonferroni-Holm procedure (30). Statistical significance was defined as $P < 0.05$.

TABLE 1. Primer and probe sequences

Name	Sequence
HO2 forward primer	5′-CCTGTACACGATGGGAAAGGA-3′
HO2 reverse primer	5′-TCCAGCCCTTTGTCTTGTTCA-3′
HO2 TaqMan probe	5′-ACATGCGTAAATGCCCTTTCTACGCTG-3′

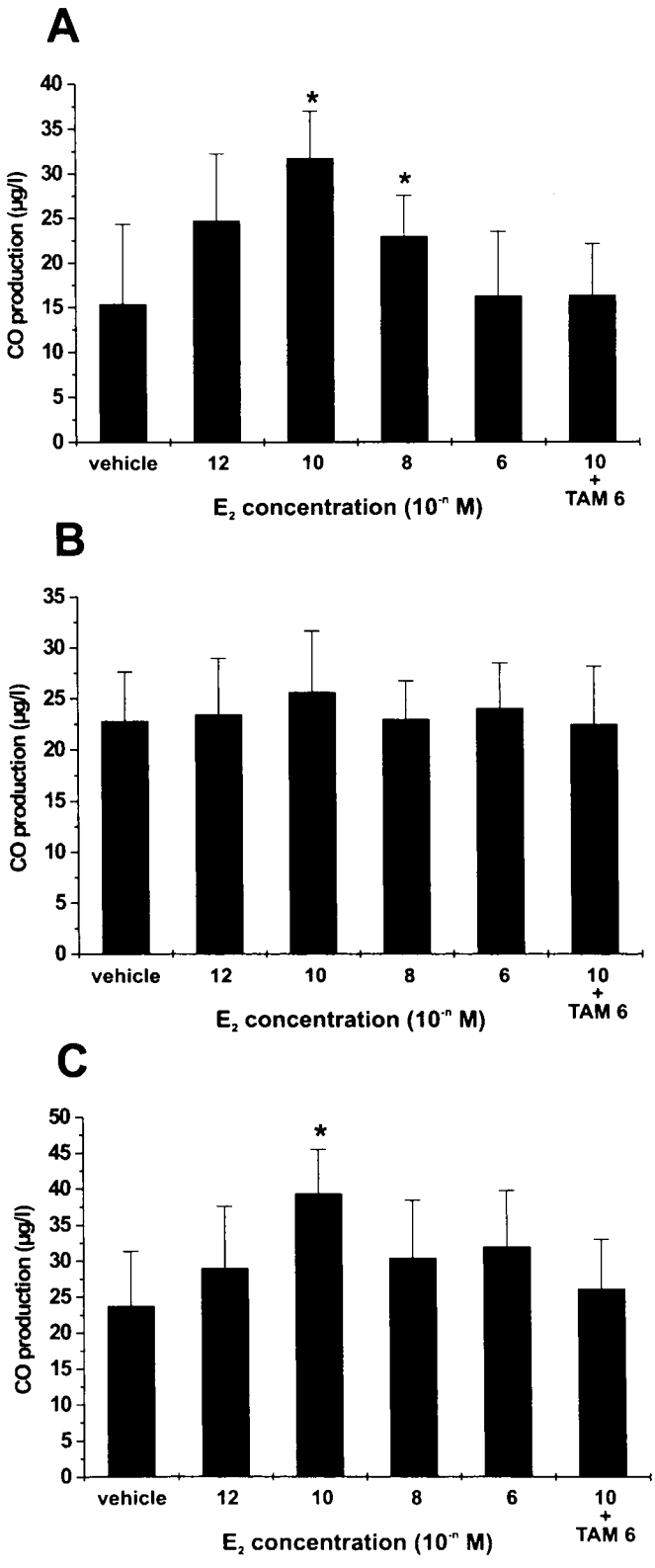

FIG. 1. The effect of E_2 incubation on CO release of HUVEC (A and B) and HAUEC (C). Cells were treated with ethanol (<0.1%; vehicle for E_2, used as a negative control) or E_2 for 4 h (A and C) or 24 h (B), or were pretreated with TAM 1 h before E_2. *Bars* represent the mean ± SD of six (HUVEC) and five (HAUEC) experiments. *, $P < 0.05$ *vs.* control.

Results

Effect of E_2 on CO levels

Four-hour incubation of HUVEC and HAUEC with E_2 increased CO levels in the cell culture supernatant in a biphasic pattern, from 15 and 24 µg/liter at baseline to 32 and 39 µg/liter at 10^{-10} M E_2, respectively ($P < 0.05$), whereas TAM pretreatment fully blocked this effect in both instances (Fig. 1, A and C). Neither 1 h (not shown) nor 24 h (Fig. 1B) of E_2 treatment affected the levels of CO released.

As depicted in Fig. 2, preincubation of HAUEC with a high L-NAME concentration (10 mM) elicited an E_2-dependent increase in CO release at 10^{-10} M E_2, which remained elevated at 10^{-6} M E_2 *vs.* baseline ($P < 0.05$), whereas lower concentrations of L-NAME (0.1 mM) had no significant effect on CO levels.

Effect of E_2 on HO1 and HO2 protein

Using an antibody specific for detection of HO1 protein with an approximate molecular mass of 32 kDa, HUVEC were shown to be negative for HO1 protein (Fig. 3). By contrast, using a specific HO2 antibody that detects a 36-kDa HO2 protein, HUVEC were positive for HO2 protein, even under untreated conditions (Fig. 3). Furthermore, similar to CO levels, 4-h incubation of HUVEC and HAUEC with E_2 induced HO2 protein levels in a biphasic pattern (Fig. 3), reaching significance at 10^{-10} M E_2 ($P < 0.05$), showing 96% and 120% increases compared with incubation of cells with vehicle, respectively (Fig. 4). TAM pretreatment fully prevented the E_2-mediated increase in HO2 protein levels (Figs. 3 and 4), demonstrating a receptor-dependent mechanism of E_2-mediated increase in HO2 protein in those cells. However, at 1 h (not shown) or 24 h (Figs. 3 and 4B) of treatment, E_2 did not change the levels of HO2 protein.

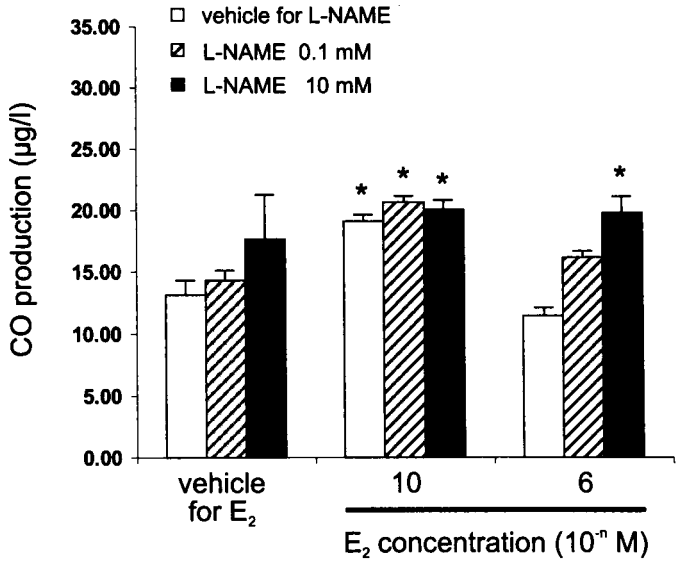

FIG. 2. Effect of L-NAME (0.1 and 10 mM final concentrations) on HAUEC CO release in both the absence and presence of 4-h E_2 treatment of cells. Data are expressed as the mean ± SD of three independent experiments. *, $P < 0.05$ relative to baseline levels before administration of L-NAME or E_2.

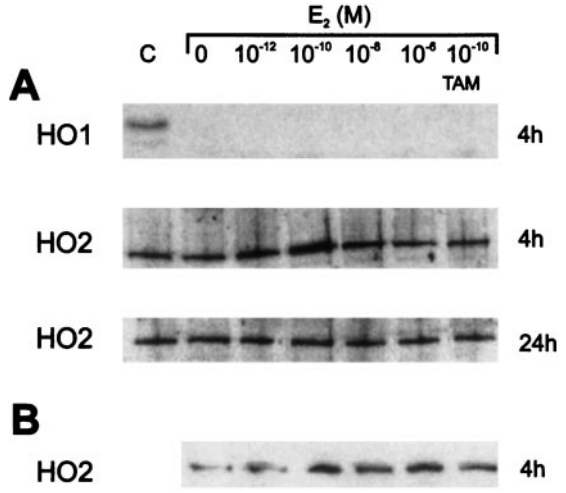

FIG. 3. The effect of E_2 on HO1 and HO2 protein levels in HUVEC (A) and HAUEC (B) shown by Western blot analysis. Positive controls for both HO1 and HO2 antibodies and cells treated with ethanol (negative control, 0) or E_2 for 4 or 24 h or pretreated with TAM 1 h before E_2 were then analyzed for HO1 and HO2 protein, respectively. Similar findings were observed in seven (HUVEC) or five (HAUEC) independent experiments.

E_2 induced HO2 gene expression in HUVECs

After 1 h of treatment, E_2 induced HO2 mRNA expression in HUVECs in a biphasic pattern, reaching significance only at a concentration of 10^{-10} M E_2 ($P < 0.05$), showing a 17% increase compared with the control value (ethanol, <0.1%, used as a vehicle for E_2; Fig. 5). To ascertain ER involvement in the noted E_2 response, HUVECs were incubated with 10^{-6} M of the partial ER antagonist TAM 1 h before E_2 treatment. TAM pretreatment fully prevented E_2-stimulated HO2 gene expression (Fig. 5), whereas TAM alone, used to evaluate a potential agonistic effect of this compound, showed no effect on HUVEC HO2 mRNA expression (not shown). These data clearly demonstrate that E_2 at a concentration such as that attained during the ovulatory phase of the menstrual cycle up-regulates HO2 mRNA levels in HUVEC by a receptor-dependent mechanism. However, at 4 h (Fig. 5) or 24 h (not shown) of treatment, E_2 did not change the levels of HO2 mRNA.

Effect of E_2 on cellular cGMP

In subsequent experiments the functional significance of increased cellular CO production was assessed by measuring the intracellular formation of cGMP, a direct product of NO- and CO-induced activation of guanylate cyclase. Experiments were performed after NOS inhibition to avoid potential interactions between the NO and CO systems (12, 31). Four-hour incubation of HAUEC with 10^{-10} M E_2 increased cGMP levels from 1.8 to 2.8 fmol/1.5×10^3 cells (56% increase from baseline; $P < 0.05$). This effect of E_2 was fully abolished by pretreatment of cells with TAM (Fig. 6). However, after stimulation of cells with higher E_2 concentrations, cGMP levels returned to baseline (Fig. 6).

Discussion

The mechanisms by which E_2 exerts direct protective effects on the vasculature are incompletely understood. In the

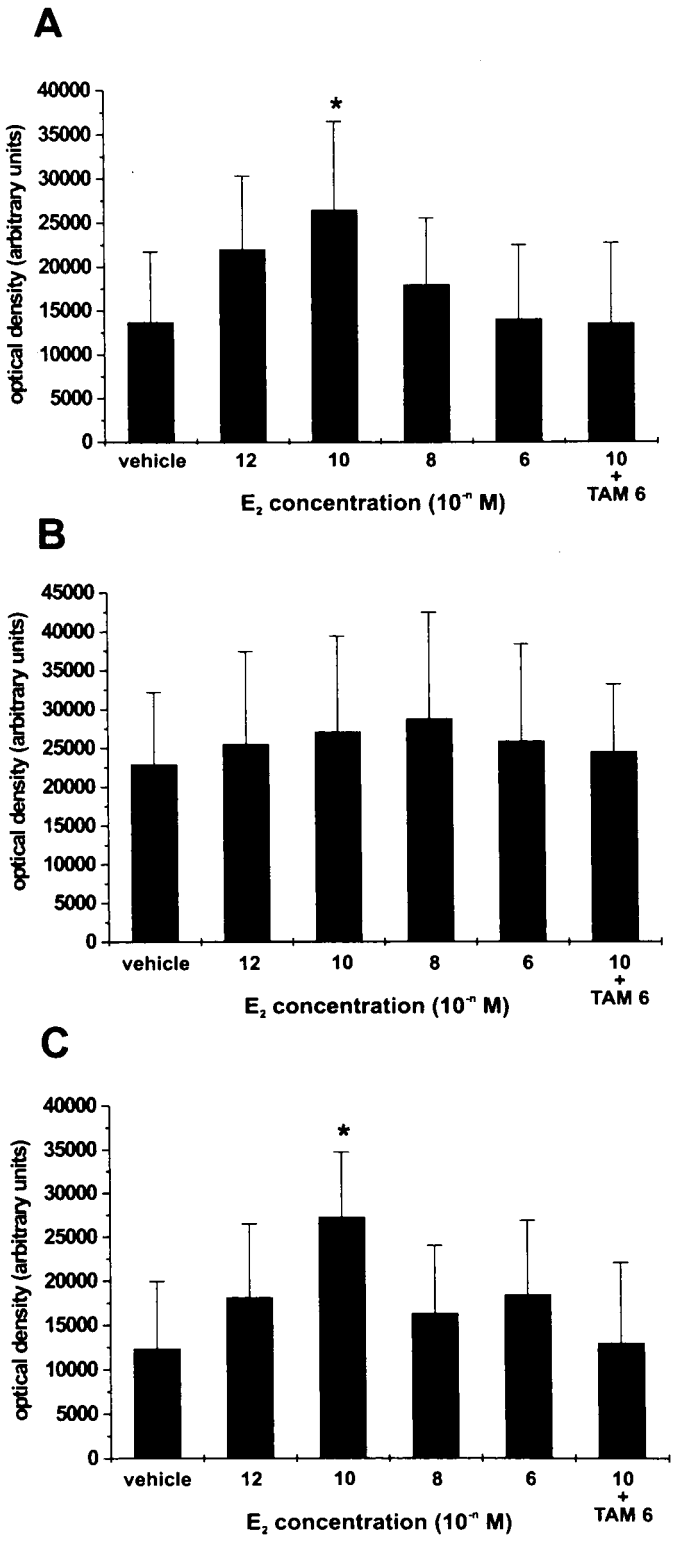

FIG. 4. Densitometric analysis of HO2 protein in HUVEC (A and B) and HAUEC (C). Cells were treated with ethanol (vehicle for E_2), or E_2 for 4 h (A and C) or 24 h (B) or were pretreated with TAM 1 h before E_2 and then analyzed for HO2 protein, respectively. *Bars* represent arbitrary densitometric units and are shown as the mean ± SD of seven (HUVEC) and five (HAUEC) independent experiments. *, $P < 0.05$ *vs.* control.

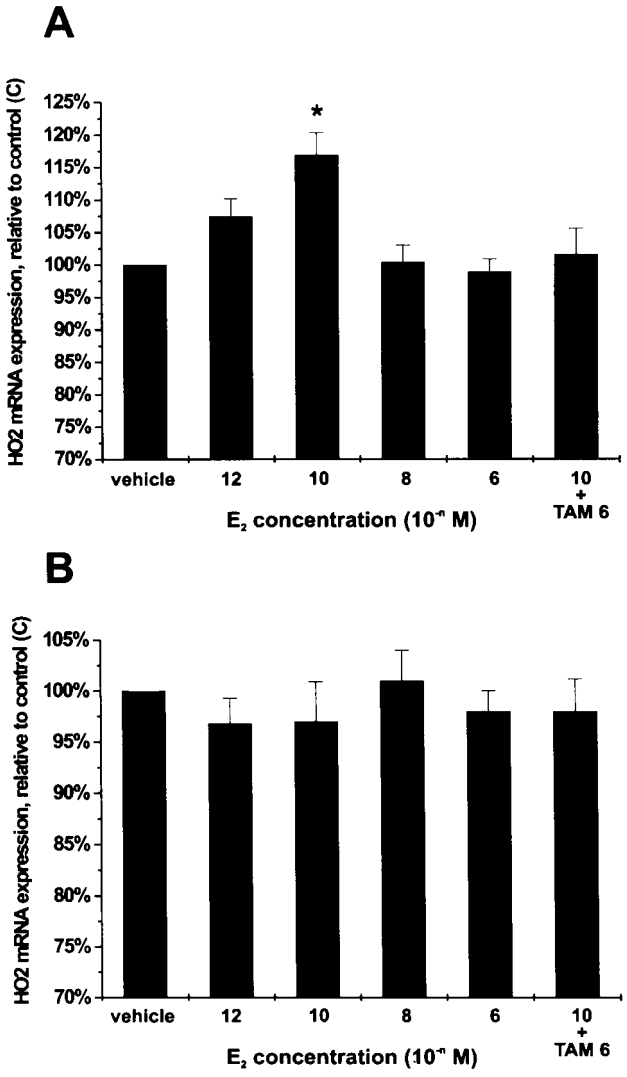

FIG. 5. Quantitative real-time RT-PCR analysis of HO2 mRNA expression in HUVECs is shown. Cells were treated with ethanol (vehicle for E_2) or E_2 for 1 h (A) or 4 h (B) or were pretreated with TAM 1 h before E_2 and then analyzed for HO2 mRNA expression levels, respectively. *Bars* represent HO2 mRNA expression levels relative to controls (C) and are shown as the mean ± SD of four independent experiments. *, $P < 0.05$ *vs.* control.

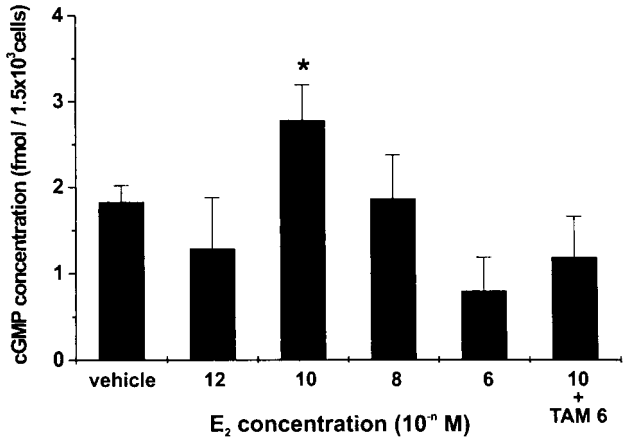

FIG. 6. Intracellular cGMP concentrations were determined in HAUEC after 4-h stimulation with ethanol (vehicle for E_2) or increasing E_2 concentrations or after TAM pretreatment before E_2. The data represent cGMP concentrations (femtomoles per 1.5×10^3 cells; mean ± SD) of at least four independent experiments performed in duplicate. *, $P < 0.05$ *vs.* control.

present preliminary study we focus on a novel pathway introducing CO as a potential new candidate mediating atheroprotective effects of E_2. From the amount of CO detected in the medium, we first found that E_2-treated endothelial cells of different human origins (HUVEC and HAUEC) released more CO in their culture medium than control cells. The required duration of E_2 exposure was 4 h at a concentration such as that attained during the ovulatory phase of the menstrual cycle, and the effect could be inhibited by the E_2 antagonists TAM (shown here) and ICI 182,780 (preliminary observations). These data suggest a traditional mechanism of estrogen action involving the functional ERs that have been characterized in this population of cells (32, 33). However, to our knowledge no estrogen-responsive elements or at least half-palindromic sites thereof have been reported in the promoter region of the HO2 gene, suggesting a regulation of CO release by E_2 at the level of protein synthesis rather than at the gene level. We therefore explored the endothelial HO2 protein and mRNA.

HO2 protein levels in cells followed a similar course by estrogen treatment. These results support an enhancement by E_2 on the protein level of HO2. This would probably occur during protein synthesis or even gene transfer. Quantitative real-time RT-PCR confirmed results at the gene level, except the effect was less pronounced and occurred at 1 h, but not 4 h, of stimulation. From the fact that increased HO2 transcription precedes increased protein levels, it is tempting to speculate that mRNA has been lost in the presence of the more stable protein. The less pronounced effect on the gene (17% increase in HUVEC) compared with the protein level (96% and 120% increase for HUVEC and HAUEC, respectively) further suggests that parts of the HO2 regulation may occur at the protein level independent of gene regulation.

We further observed that E_2 elicits CO release with a biphasic concentration dependence, where CO release peaked (32 and 39 μg/L CO, corresponding to 1.1 and 1.3 μmol/L CO) at ovulatory E_2 levels, but decreased at higher E_2 levels. These effects of E_2 are confirmed at the protein and, even less, at the mRNA level. Pertaining to these data, a recent study documented maximal vasodilation (>20% compared with control) in perfused rat afferent juxtamedullary arterioles at CO levels in the superfusate of 1.0 μmol/liter (34), which exactly corresponded to the increased CO levels we observed in HUVEC and HAUEC after appropriate E_2 stimulation. Despite the fact that HUVEC/HAUEC and perfused rat arterioles are noncomparable systems, it is intriguing to speculate that ovulatory E_2 levels might affect vascular tone by CO concentrations previously shown to maximally reducing vascular tone.

In contrast to causing a peak CO release after 4 h of treatment, a 24-h treatment course with ovulatory E_2 concentrations no longer increased cellular CO release. As the CO detection system involves measurement of COHb, in contrast to our finding, one might expect COHb levels to accumulate in the medium with time after cell stimulation. By

contrast, we could not find such an accumulation of COHb, which is consistent with a previous report demonstrating a 7-fold increase in vascular smooth muscle cell-derived COHb in coculture with HUVEC after 12-h exposure to hypoxia compared with only a 2-fold increase in COHb after 48-h hypoxia (35). Albeit not further discussed in the latter report, we speculate that the lack of COHb accumulation in medium over time observed here and by others (35) most likely reflects dissociation of CO from COHb (36, 37).

We finally were interested in whether CO release could also be attributable to HO1 protein, but, as expected, failed to detect any HO1 protein in HUVECs, which is consistent with recent data obtained from rats showing that HO1 expression was present in the medial, but not endothelial, layer of carotid arteries (38).

There is strong evidence to support the emerging paradigm that CO, like NO, elicits vasodilation (20, 39–41). However, CO was reported to be less than 1/1000th as potent as NO as a relaxant (20), raising the question of whether vascular CO release by E_2 could be of biological significance in terms of effective vasodilation. Recently, chromium mesoporphyrin (CrMP), a selective HO inhibitor, has been shown to increase the myogenic tone of the small muscular branch of rat femoral arteries, but not of large arterial vessels such as the aorta or the femoral artery, in organ bath experiments (21), an effect that could be blunted by coadministration of CO. These researchers further demonstrated that intravascular pressure is required to elicit vasoconstriction by CrMP, that stepwise increases in vascular pressure amplified the constrictor responses of CrMP, and that unpressurized muscle arterioles could not be contracted by CrMP. From these data the researchers suggested that production of CO by vascular HO subserves a vasodilatory mechanism that contributes to the regulation of basal tone in resistance vessels. However, from those data and from the data obtained by Thorup *et al.* (34) demonstrating the most pronounced vasodilation in renal afferent arterioles at 1.0 μM CO, the biological significance of CO in regulating vascular tone under normal circumstances remained unclear. We have demonstrated here that E_2 at normal ovulatory levels can exert endothelial CO release at a concentration comparable to that previously reported to elicit the most pronounced vasodilation.

We further observed high concentrations of E_2 had a lesser or no effect on CO and HO2 levels compared with ovulatory E_2 concentrations. This inhibitory effect on further CO release and HO2 levels by high doses of E_2 might reflect the ability of E_2 to maximally induce vasodilation even at higher concentrations shown to markedly stimulate NO release (42), thereby preventing further endothelial CO production, previously shown to reduce arteriolar diameter (34).

Notably, however, the mechanism of how exposure to high levels of E_2 prevents further CO release remained to be defined. As high levels of E_2 are a potent inducer of endothelial NO release (42), a possible explanation might be the existence of a compensatory interrelationship between the eNOS and HO2 systems in endothelial cells as previously described (43). Using cultured rat endothelial cells, the latter researchers demonstrated that eNOS mRNA was up-regulated in the presence of the heme oxygenase inhibitor zinc protoporphyrin IX, and HO2 mRNA was up-regulated in the presence of a NOS inhibitor. To test whether NOS inhibition in the cells we studied is capable of affecting endothelial CO release, we exposed HAUEC to increasing concentrations of the NOS inhibitor L-NAME in both the absence and presence of E_2. Increasing the amount of L-NAME stimulated both basal and E_2-mediated CO release. This effect was significant at a high E_2 level, but was less pronounced at an ovulatory E_2 concentration, suggesting that marked NO release after high dose E_2 administration (42) serves to limit the availability of CO. Such an NO-dependent decrease in CO levels might be required to prevent the reduction in arteriolar diameter that was previously shown to result from unopposed release of high amounts of CO (34). However, a possible limitation of our finding is that we did not measure cell culture supernatant NOx levels, *e.g.* using the Griess detection method, because the entire supernatant in each instance had to be used for CO detection assay. Due to this limitation, we sought to use L-NAME at a maximal concentration 10 times that previously shown to avoid interactions between the NO and CO systems (44).

Another explanation for how E_2 prevents from further CO release could be that high levels of E_2 inhibit HO2 activity and thereby CO production via a nonreceptor-dependent system, as was suggested for eNOS activity and NOx levels in HUVEC and bovine aortic endothelial cells (4), but no strong evidence for this can be provided here.

Our data showing E_2 to be effective in increasing endothelial CO levels are supported by subsequent experiments, demonstrating an increase in HAUEC cGMP levels after an ovulatory E_2 dose, thus providing evidence for a functional relevant heme oxygenase-CO-cGMP system in human uterine artery endothelium. However, it is not apparent at present whether ovulatory E_2 levels effectively reduce vascular myogenic tone by an endothelial heme oxygenase-CO-cGMP system. Hence, careful and detailed *in vivo* studies using, for example, ovariectomized animals with E_2 replacement should therefore serve to corroborate our data.

In conclusion, our study shows a previously unknown, perhaps physiological function for estrogen in terms of its vasoprotective properties. Our preliminary data indicate that estrogen at a concentration corresponding to the ovulatory phase of the menstrual cycle can serve as a CO-inducing agonist in human endothelial cells. This might be of importance when considering treatment with selective ER modulators such as TAM, which was shown here to blunt the effects of estrogen on endothelial CO release, in the case of underlying vascular disease.

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