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Estradiol augments while progesterone inhibits arginine transport in human endothelial cells through modulation of cationic amino acid transporter-1

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Bentur OS, Schwartz D, Chernichovski T, Ingbir M, Weinstein T, Chernin G, Schwartz IF. Estradiol augments while progesterone inhibits arginine transport in human endothelial cells through modulation of cationic amino acid transporter-1. *Am J Physiol Regul Integr Comp Physiol* 309: R421–R427, 2015. First published June 10, 2015; doi:10.1152/ajpregu.00532.2014.—Decreased generation of nitric oxide (NO) by endothelial NO synthase (eNOS) characterizes endothelial dysfunction (ECD). Delivery of arginine to eNOS by cationic amino acid transporter-1 (CAT-1) was shown to modulate eNOS activity. We found in female rats, but not in males, that CAT-1 activity is preserved with age and in chronic renal failure, two experimental models of ECD. In contrast, during pregnancy CAT-1 is inhibited. We hypothesize that female sex hormones regulate arginine transport. Arginine uptake in human umbilical vein endothelial cells (HUVEC) was determined following incubation with either 17β-estradiol (E_2) or progesterone. Exposure to E_2 (50 and 100 nM) for 30 min resulted in a significant increase in arginine transport and reduction in phosphorylated CAT-1 (the inactive form) protein content. This was coupled with a decrease in phosphorylated MAPK/extracellular signal-regulated kinase (ERK) 1/2. Progesterone (1 and 100 pM for 30 min) attenuated arginine uptake and increased phosphorylated CAT-1, phosphorylated protein kinase Cα (PKCα), and phosphorylated ERK1/2 protein content. GO-6976 (PKCα inhibitor) prevented the progesterone-induced decrease in arginine transport. Coincubation with both progesterone and estrogen for 30 min resulted in attenuated arginine transport. While estradiol increases arginine transport and CAT-1 activity through modulation of constitutive signaling transduction pathways involving ERK, progesterone inhibits arginine transport and CAT-1 via both PKCα and ERK1/2 phosphorylation, an effect that predominates over estradiol.

estrogen; progesterone; endothelial function

DIMINISHED CAPACITY OF THE endothelium to generate nitric oxide (NO) has emerged as a primary factor provoking endothelial dysfunction (ECD) (7). Abnormal function of the endothelial nitric oxide synthase (eNOS) system that results in ECD includes: decreased eNOS expression, altered NO signaling, destruction of NO by reactive oxygen species, impaired availability of cofactors, and elevated endogenous NOS inhibitors such as asymmetrical dimethylarginine and advanced glycation end products (23). In addition, the delivery of transported arginine to membrane-bound eNOS, selectively by the cationic amino acid transporter-1 (CAT-1), rather than intra- or extracellular arginine concentration, has been suggested to govern eNOS activity. Indeed, we have previously shown in several different animal models, characterized by endothelial dysfunction and decreased eNOS activity, that CAT-1 activity is diminished due to increase in its phosphorylated (inactive) form (10, 12, 24, 27, 31). Interestingly, in two well-established experimental models of ECD (renal failure and aging), arginine transport by CAT-1 decreased only in males while females were protected (22, 28). In contrast, during pregnancy, females lose the ability to maintain adequate endothelial arginine transport velocities by CAT-1, a loss that is associated with increased protein nitration possibly due to posttranslational regulation of CAT-1 by protein kinase Cα (PKCα) (24, 30). Our current experiments, which utilized human umbilical vein endothelial cells (HUVEC), were designed to test the hypothesis that female sex hormones modulate CAT-1 activity, thus playing a role in both the gender- and pregnancy-related effects on the L-arginine-eNOS system.

MATERIALS AND METHODS

Cell culture and chemicals. Fresh unpassaged HUVEC (female) were obtained from Promo Cell (Heidelberg, Germany). Upon arrival, the cells were cultured according to the manufacturer's instructions in endothelial cell M2 growth medium containing 2% fetal calf serum, 100 μg/ml penicillin-streptomycin, and 0.05 μg/ml amphotericin B, at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed three times weekly. Cells were used for experiments when they were 70–80% confluent, between passages 3 and 6. Twenty four hours before the experiments, the incubation medium was changed to 5% bovine serum albumin. 17β-Estradiol (E_2) and progesterone were dissolved in ethanol (final concentration $<10^{-5}$%). MAPK inhibitors UA-126 [extracellular signal-regulated kinase (ERK) inhibitor] and SP-600125 [c-Jun NH_2-terminal kinase (JNK) inhibitor], and GO-6976 (PKCα inhibitor) (3), were all dissolved in dimethyl sulfoxide (final concentration $<10^{-3}$%). All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless indicated otherwise.

L-Arginine uptake in HUVEC. Uptake of radiolabeled L-arginine in HUVEC was measured according to previously described methods (26). Cells were seeded onto six-well plates (Corning) at a density of 10^6 cells/well. When confluent, cells were washed with 1 ml HEPES buffer, pH 7.4 at 37°C. L-[H^3]arginine and L-arginine, in a final concentration of 100 μM, were added to a total volume of 1 ml for 1 min. The duration of 1 min was chosen since it was within the linear portion of the uptake curve (data not shown). Transport was terminated by rapidly washing the cells with ice-cold PBS buffer (4 times, 1 ml/well). The cells were then dried and solubilized in 1 ml of 0.5% SDS in 0.5 N NaOH. Lysate (700 μl) was used to monitor radioactivity by liquid scintillation spectrometry (Betamatic; Kontron). The remaining 300 μl were used for protein content determination by the Lowry method (Lowry Assay Kit; Sigma). To correct for nonspecific uptake or cell membrane binding, additional studies were performed in which HUVEC were incubated with 10 mM unlabeled arginine in HEPES buffer, and the associated radioactivity was subtracted from

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each data point. Results are expressed as means ± SE of at least five different experiments.

Nitrite/nitrate determination. The level of nitrite (NO_2)/nitrate (NO_3) concentrations in the conditioned medium was measured using a nitric oxide detection kit (ENZO Life Sciences) according to the manufacturer's instructions. In brief, following filtration in a 10-kDa spin column (Biovision) 50 μl of the culture medium were diluted with 50 μl of reaction buffer containing 50 μM L-N^6-(1-iminoethyl)lysine hydrochloride [L-NIL, a selective inducible NOS (iNOS) inhibitor] and mixed with 25 μl NADH and 25 μl nitrate reductase. After 30 min incubation during which NO_3 is reduced to NO_2, Griess reagents [sulfanileamide in hydrochloric acid and *N*-(1-naphthyl)ethylenediamine in hydrochloric acid] were added to react with NO_2 to yield a diazochromophore. Total NO_2 was measured spectrophotometerically at 540 nm. Each sample was analyzed in triplicate, and the concentration of NO_2 was calculated by using a calibration curve. Each measurement was corrected for protein content (using the Lowry assay). Results of at least six experiments were used for statistical significance.

Protein quantification by Western blotting. Endothelial CAT-1, PKCα, phosphorylated PKCα (p-PKC-α), MAPK ERK, phosphorylated ERK (p-ERK), MAPK JNK, and phosphorylated JNK (p-JNK) protein expression were determined by immunoblotting. Cells were separately placed in ice-cold PBS lysis buffer (pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4.5 μM leupeptin, and 5 μM aprotinin; ICN Biomedicals), 0.01% Triton X-100, and 0.1% SDS and then mechanically homogenized and left on ice for 45 min. Homogenates were subsequently centrifuged [13,000 revolutions/min (rpm), 10 min, at 4°C], and cell lysates were stored in aliquots at −80°C. A membrane fraction was obtained by adding to the pellet an equal volume of lysis buffer supplemented by Tween 20 (0.25%) to solubilize. The protein content of each sample was determined by the method of Lowry. Equal amounts of protein (30 μg) were prepared in a sample buffer (2% SDS, 0.01% bromophenol blue, 25% glycerol, 0.0625 M Tris·HCl, pH 6.8, and 5% mercaptoethanol) and analyzed on a 7.5% SDS-PAGE. The gel was transferred onto Hybond ECL nitrocellulose membranes (Amersham) and blocked in PBS-Tween 20 (PBS-T) containing 5% nonfat dried milk, at room temperature. Membranes were then incubated with rabbit anti-human CAT-1, ERK, p-ERK, p-PKCα, and mouse monoclonal antihuman PKC-α, JNK, and p-JNK antibodies (all from Santa Cruz Biotechnology) for 1 h at room temperature, washed, and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies in PBS-T for 1 h. Membranes were subsequently washed three times, 5 min each, in PBS-T. Membranes were then stripped and reprobed with monoclonal anti-β-actin or glyceraldehyde-3-phosphate dehydrogenase antibodies as an internal control. The reactive bands corresponding to CAT-1, ERK, p-ERK, p-PKCα, PKCα, JNK, and p-JNK were detected by enhanced chemiluminescence (Kodak X-Omat AR film) and quantified by densitometry.

Immunoprecipitation studies. Aliquots of endothelial cell lysate (1 ml) from the different experimental groups were used for immunoprecipitation. Each cell lysate sample was incubated with 20 μl of anti-CAT-1 antibodies for 2 h at 4°C. Optimal antibody concentration was determined by titration (data not shown). This was followed by addition of 20 μl of protein A/G agarose and incubation overnight at 4°C on a rotating device (Santa Cruz Biotechnology). Pellets were collected by centrifugation at 3,000 rpm for 30 s, 4°C. The supernatants were discarded, and each pellet was subsequently washed three times with PBS. After the final wash, the pellets were resuspended in 40 μl of 2× electrophoresis sample buffer, boiled for 3 min, and subjected to immunoblotting, as described above, with antibodies against CAT-1 or the phosphorylated tyrosine residue of CAT-1 (Santa Cruz Biotechnology). To estimate the phosphorylation of CAT-1 in the different groups, the density of bands for CAT-1 and its phosphorylated form was analyzed as above. Results are adjusted for CAT-1 levels and expressed in arbitrary units.

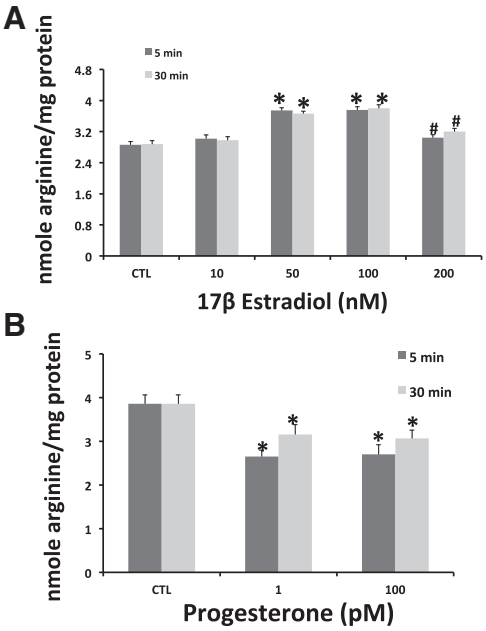

Fig. 1. Effect of 17β-estradiol and progesterone on the uptake of radiolabeled arginine (L-[^{3}H]arginine) by human umbilical vein endothelial cells (HUVEC). *A*: cells exposed to 17β-estradiol (10–200 nM) for 5 and 30 min. *B*: cells exposed to progesterone (1,100 pM) for 5 and 30 min. Data are presented as means ± SE of 8 different experiments. *$P < 0.05$ vs. control (*) and 100 nM 17β-estradiol (#). CTL, controls.

Statistical analysis. Values shown in Figs. 1–7 are means ± SE from data obtained from 4 to 10 experiments. ANOVA test was applied for comparisons of means, and then Bonferroni's test was performed. *P* values <0.05 were considered to be statistically significant.

RESULTS

Based on our previous reports, the kinetic properties of the arginine transport system in endothelial cells resemble those of CAT-1 (10).

The first set of experiments was designed to explore the effect of estradiol and progesterone on CAT-1-related arginine transport. Incubating cells with E_2 (50–100 nM) for 5 or 30 min significantly augmented arginine transport while lower (10 nM) or higher (200 nM) concentrations had no effect (Fig. 1*A*). Arginine uptake was unchanged during longer incubation periods (1, 6, and 24 h) (data not shown). Exposing the cells to progesterone at concentrations of 1 and 100 pM for 5 and 30 min significantly attenuated arginine transport (Fig. 1*B*). To explore whether the hormonal effect on arginine transport is mediated through CAT-1, CAT-1 protein levels were studied. We have found that CAT-1 abundance did not alter by exposure to either E_2 (50 nM) or progesterone (1 pM) for 30 min. To unveil a posttranslational effect on CAT-1 activity, hence on arginine transport velocities, we performed immunoprecipitation studies for phosphorylated CAT-1. E_2 induced a profound decrease in p-CAT1 after 30 min while treatment with progesterone for 5 or 30 min resulted in a significantly augmented p-CAT1 protein content (Fig. 2, *A* and *D*).

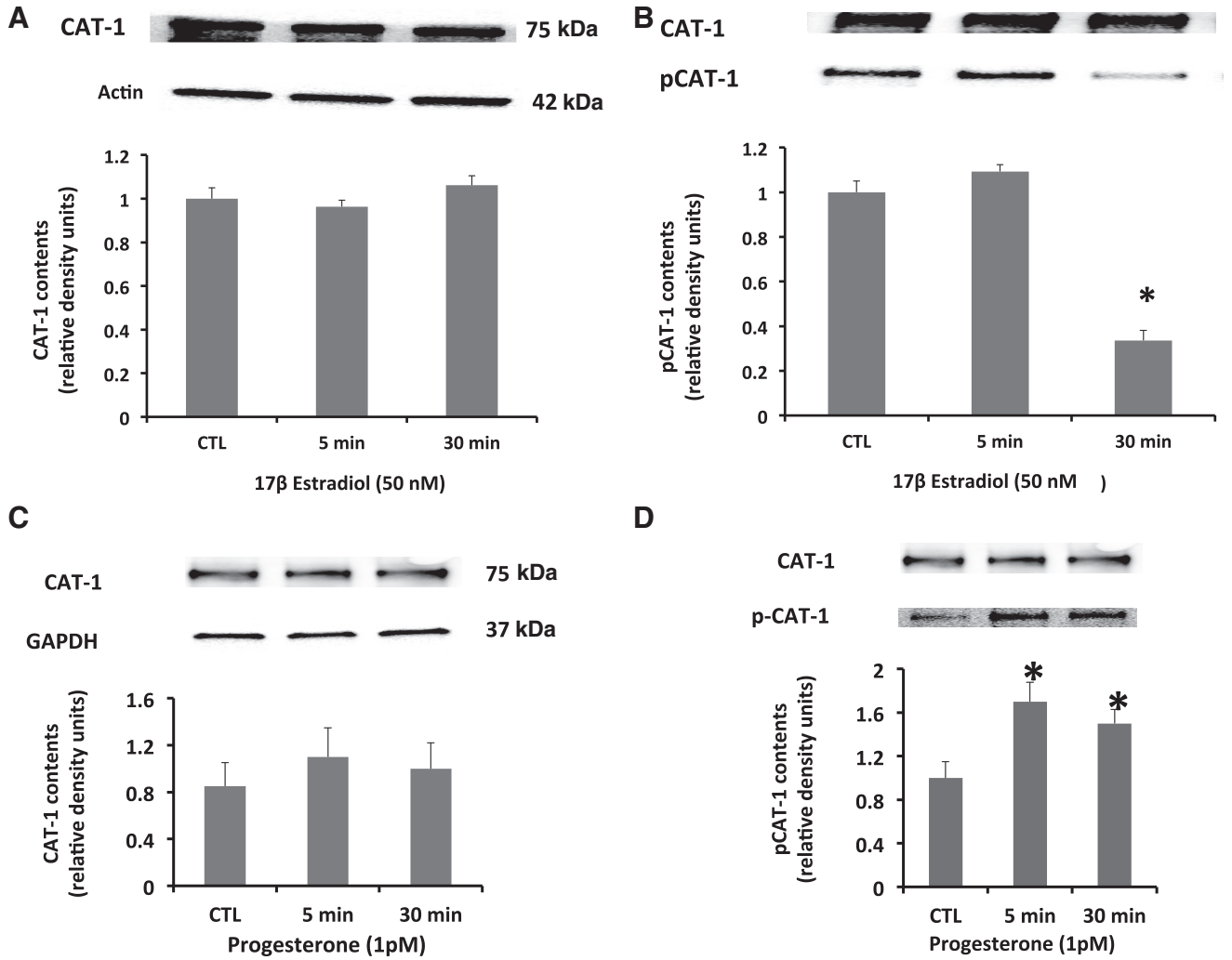

Fig. 2. Representative Western blot analysis and densitometric analysis showing regulation of cationic amino acid transporter-1 (CAT-1) and phosphorylated CAT-1 protein level in HUVEC treated with 50 nM 17β-estradiol (*A* and *B*) or 1 pM progesterone (*C* and *D*) for 5 and 30 min. Data are presented as means ± SE of 3 different experiments *$P < 0.05$ vs. control.

NO_2/NO_3 generation by cells exposed to either E_2 or progesterone for 30 min in the presence of L-NIL (a selective iNOS inhibitor), with or without L-NAME (a nonselective NOS inhibitor), was used to evaluate NO production by eNOS. The concentration of NO_2/NO_3 was significantly higher following E_2 treatment than in control, while progesterone substantially decreased NO_2/NO_3 generation (Fig. 3).

To identify a specific signal transduction pathway by which E_2 and progesterone affect arginine uptake, we chose to examine PKC-α, JNK, and ERK1/2, all of which have been previously shown to modulate eNOS or CAT-1 signaling pathways (5, 6, 8, 15–19) and are regulated by these hormones (5, 13, 14, 25). Initially, arginine uptake was measured following incubation of HUVEC with inhibitors of these signal transduction pathways. UO-126 (ERK1/2 inhibitor) and SP-600125 (JNK inhibitor) significantly augmented arginine uptake while GO-6976 (PKCα inhibitor) had no effect (Fig. 4*A*). Next, arginine uptake was determined when cells were exposed to the same agents in the presence of either E_2 or progesterone for 30 min. We have found that arginine transport was significantly increased following coincubation of E_2 and SP-600125 (JNK inhibitor), while neither E_2 and GO-6976 nor E_2 and UO-126 (ERK1/2 inhibitor) had any effect compared with E_2 alone (Fig. 4*B*). Cotreatment with progesterone and either GO-

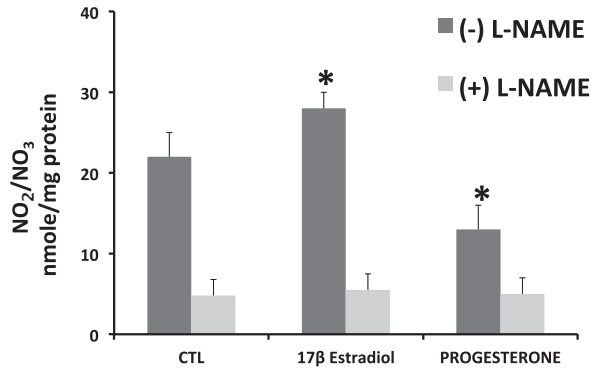

Fig. 3. Nitrite (NO_2)/nitrate (NO_3) generation by cells exposed to progesterone or 17β-estradiol for 30 min in the presence of L-N^6-(1-iminoethyl)lysine hydrochloride (50 μM, a selective iNOS inhibitor), with or without L-NAME. Data are presented as means ± SE of 6 different experiments.

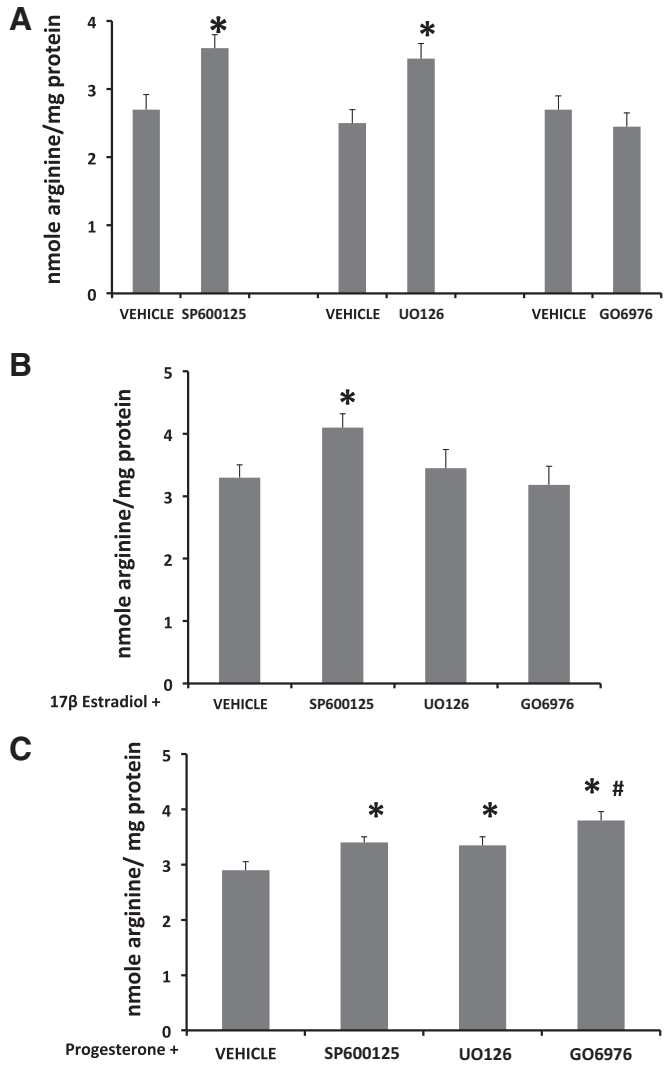

Fig. 4. Effect of 10 μM UO126 [extracellular signal-regulated kinase (ERK) 1/2 inhibitor], 20 μM SP-600125 [c-Jun NH_2-terminal kinase (JNK) inhibitor], and 1 μM GO-6976 [protein kinase Cα (PKC-α) inhibitor] on uptake of radiolabeled arginine (L-[^{3}H]arginine) by HUVEC with or without coexposure to 50 nM 17β-estradiol or 1 pM progesterone for 30 min. *A*: cells exposed solely to the inhibitors. *B*: concurrent exposure to inhibitor and 17β-estradiol. *C*: concurrent exposure to inhibitor and progesterone. Data are presented as means ± SE of 5 different experiments. $P < 0.05$ vs. control (*) and UO-126 and SP-600125 (#).

6976, UO-126, or SP-600125 attenuated the decrease in arginine transport compared with progesterone only. The effect of GO-6976 was more pronounced than the other two (Fig. 4*C*). Subsequently, Western blotting was performed for PKCα, JNK, and ERK1/2 and their phosphorylated forms, following incubation of HUVEC with either E_2 or progesterone for 30 min. Cell exposure to E_2 resulted in a significant decrease in ERK phosphrolyation (Fig. 5) while no changes were seen in PKCα or JNK (data not shown). Progesterone significantly augmented phosphorylated ERK and phosphorylated PKCα protein levels while JNK remained unchanged (Fig. 6, *A–D*).

Finally, coincubating HUVEC simultaneously with both E_2 and progesterone for 30 min resulted in a significantly diminished arginine transport (Fig. 7).

DISCUSSION

The present study demonstrates, for the first time to our knowledge, that the two main female sex hormones, estradiol and progesterone, exert an opposite effect on NO generation in HUVEC through modulation of arginine transport. E_2 was found to augment, whereas progesterone attenuates, arginine transport velocities and NO synthesis. We have also found that these effects are mediated through posttranslational modulation of CAT-1. An exception is the early (5 min) effect of E_2 by which the mechanism remains elusive. Large epidemiological studies show that premenopausal women have less cardiovascular disease and lower cardiovascular morbidity and mortality than men of the same age and that these cardioprotective benefits disappear after menopause (15). It is widely thought that estrogen exerts protective effects on the cardiovascular system, at least in part, through augmenting NO generation by eNOS (33). We have previously shown in two experimental models of ECD, namely, aging and renal failure, that in the female gender, in contrast to males, arginine transport is not downregulated (22, 27–30). The current experiments, explor-

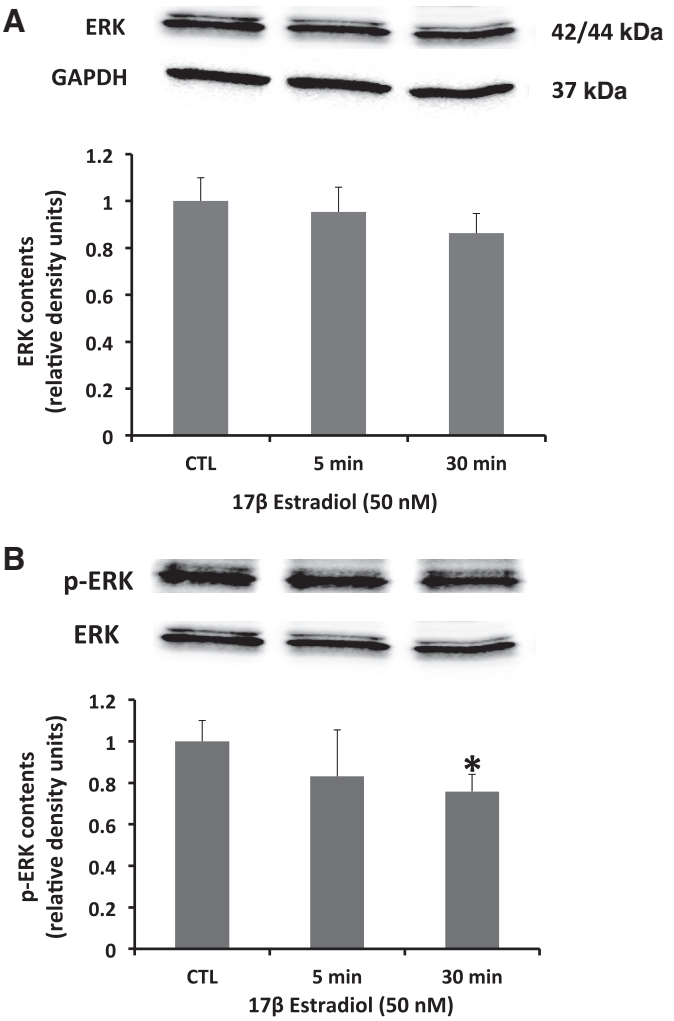

Fig. 5. Representative Western blot analysis and densitometric analysis showing protein content and regulation of ERK (*A*) and phosphorylated (p)-ERK (*B*) in HUVEC treated with 50 nM 17β-estradiol for 5 and 30 min. Results are means ± SE of 4 different experiments. *$P < 0.05$ vs. control.

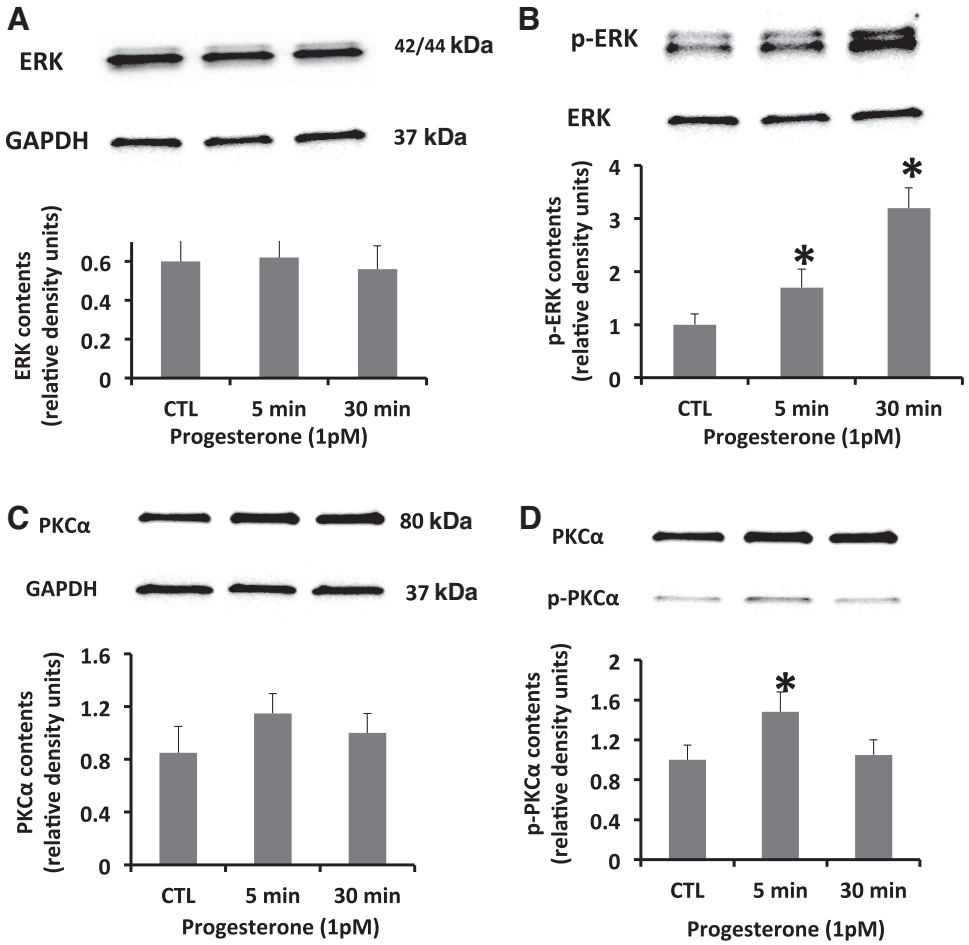

Fig. 6. Representative Western blot analysis and densitometric analysis showing protein content and regulation of ERK (*A*), p-ERK (*B*), PKCα (*C*), and p-PKCα (*D*) in HUVEC treated with 1 pM progesterone for 5 and 30 min. Results are means ± SE of 4 different experiments. *$P < 0.05$ vs. control.

ing a positive effect of E_2 on arginine transport and the NOS system, can therefore provide a mechanism to explain the aforementioned phenomenon. In contrast, during pregnancy, arginine transport is significantly inhibited in spite of elevated serum estrogen concentrations (24, 30). This discrepancy can be explained by two observations made in our current studies. First, E_2 failed to augment arginine transport when concentrations were increased, implying that there is a well-defined E_2 concentration range in which arginine transport is augmented. Increasing the concentration beyond that level results in loss of this effect. This finding is in agreement with previous studies that have demonstrated that estrogens (mainly E_2) exhibit a biphasic effect on various biological systems (1, 11, 34). Second, progesterone provokes an opposite effect on this system. Moreover, when cells were incubated with both hormones, arginine transport was downregulated, suggesting that progesterone dominates over E_2. Several studies have demonstrated that progesterone antagonizes the effects of E_2 on eNOS, yet this issue remains controversial (2, 4, 8, 16, 19, 20, 35). In this regard, our findings provide a novel mechanism to explain the opposing effects of these two hormones on endothelial function. Accordingly, E_2 augments arginine transport, and this may provide an explanation of the endothelial resilience in females compared with males. During pregnancy, however, the negative effects of progesterone overcome those of estrogens, resulting in decreased endothelial arginine transport.

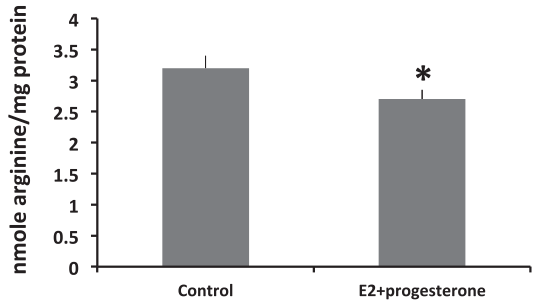

Fig. 7. Effect of concurrent exposure to 50 nM 17β-estradiol and 1 pM progesterone for 30 min on radiolabeled arginine (L-[^{3}H]arginine) uptake by HUVEC. Data are presented as means ± SE of 8 different experiments. *$P < 0.05$ vs. control.

We have tried to elucidate a molecular mechanism to explain our findings. Changes in arginine uptake due to both E_2 and progesterone were associated with directional changes in the relative amount of phosphorylated CAT-1 protein. The enhanced arginine transport induced by E_2 was associated with a decrease in phosphorylated CAT-1 while the attenuated arginine transport by progesterone was associated with increased CAT-1 phosphorylation. An exception was the effect of E_2 after 5 min, which did not appear to relate to changes in CAT-1 phosphorylation. One can argue that there are several pathways by which E_2 affects arginine metabolism, and these were not revealed by the current studies. We have previously reported,

in four different experimental models characterized by diminished arginine transport, namely hypercholesterolemia, chronic renal failure, pregnancy, and aging in the male rat, a posttranslational modulation of CAT-1 that was associated with upregulation of PKCα. Namely, PKCα increased the fraction of phosphorylated CAT-1, which is the inactive form (12, 24, 28, 29, 31). Therefore, we chose to look at changes in PKCα phosphorylation following incubation with either E_2 or progesterone. Indeed, progesterone produced a significant increase in PKCα phosphorylation that can account for the decrease in CAT-1 activity. However, E_2 had no effect. A thorough review of the literature reveals that both MAPKs JNK and ERK1/2 can be modulated by E_2 (13, 14, 25) and positively affect endothelial function (6, 9, 17, 32), which could indicate a potential CAT-1 involvement. Therefore, we pursued with experiments looking at these proteins as a possible link between E_2, progesterone, and CAT-1. Inhibitors for both ERK1/2 and JNK were found to augment arginine transport, implying that these enzymes exhibit a constitutive negative effect on CAT-1 activity. However, only ERK1/2 was influenced by both E_2 and progesterone. E_2 decreased ERK1/2 phosphorylation, whereas progesterone did the opposite. The enhanced arginine transport velocities induced by E_2 were not affected by incubation with an ERK1/2 antagonist, which further supports the notion that E_2 effect on CAT-1 is mediated by ERK inhibition.

In conclusion, while estradiol augments arginine transport through modulation of CAT-1 protein via ERK1/2 downregulation, progesterone diminishes CAT-1 activity through activation of both ERK1/2 and PKCα. These findings may shed light on sexual dimorphism and on the behavior of this system during pregnancy.

Perspectives and Significance

CAT-1, the selective arginine supplier of eNOS, is an important regulator of NO generation by endothelial cells. In the current studies we have shown that estradiol increases, whereas progesterone decreases, NO generation through modulation of CAT-1 activity. These data provide a novel mechanism to explain the effects of female sex hormones on the endothelium and support the therapeutic use of arginine under certain conditions.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: O.S.B., T.C., M.I., and I.F.S. performed experiments; O.S.B., D.S., T.C., and I.F.S. analyzed data; O.S.B. and D.S. prepared figures; O.S.B., D.S., and I.F.S. drafted manuscript; O.S.B., D.S., T.C., M.I., T.W., G.C., and I.F.S. approved final version of manuscript; D.S. and I.F.S. conception and design of research; D.S., T.C., and I.F.S. interpreted results of experiments; D.S., T.W., G.C., and I.F.S. edited and revised manuscript.

REFERENCES

1. **Banerjee SN, Sengupla K, Banerjee S, Saxena NK, Banerjee S.** 2-Methoxyestradiol exhibits a biphasic effect on VEGF-A in tumor cells and upregulation is mediated through ER-α: a possible signaling pathway associated with the impact of 2-ME_2 on proliferative cells. *Neoplasia* 5: 417–426, 2003.
2. **Chataigneau T, Zerr M, Chataigneau M, Hudlett F, Hirn C, Pernot F, Schini-Kerth VB.** Chronic treatment with progesterone but not medroxyprogesterone acetate restores the endothelial control of vascular tone in the mesenteric artery of ovariectomized rats. *Menopause* 11: 225–263, 2004.
3. **Chen F, Yo Y, Haigh J, Jhonson S, Lucas R, Stepp D, Futton DJR.** Regulation of NADPH oxidase 5 by protein kinase C isoforms. *Plos One* 9: e88405, 2014.
4. **Cox MW, Weiping F, Chai H, Paladugu R, Lin PH, Lumsden AB, Yao Q, Chen C.** Effects of progesterone and estrogen on endothelial dysfunction in porcine coronary arteries. *J Surg Res* 124: 104–111, 2005.
5. **Duckles SP, Miller VM.** Hormonal modulation of endothelial NO production. *Pflugers Arch* 459: 841–851, 2010.
6. **Flores C, Rojas S, Aguayo C, Parodi J, Mann G, Pearson JD, Casanello P, Sobrevia L.** Rapid stimulation of L-arginine transport by D-glucose involves p42/44(mapk) and nitric oxide in human umbilical vein endothelium. *Circ Res* 92: 64–72, 2003.
7. **Furchgott RF, Zawadzki AJV.** The obligatory role of endothelial cells in the relaxation of arterial smooth muscles by acetylcholine. *Nature* 288: 373–376, 1980.
8. **Gerhard M, Walsh BW, Tawakol A, Haley EA, Creager SJ, Seely EW, Ganz P, Creager MA.** Estradiol therapy combined with progesterone and endothelium-dependent vasodilation in postmenopausal women. *Circulation* 98: 1158–1163, 1998.
9. **González M, Flores C, Pearson JD, Casanello P, Sobrevia L.** Cell signalling-mediating insulin increase of mRNA expression for cationic amino acid transporters-1 and -2 and membrane hyperpolarization in human umbilical vein endothelial cells. *Pflugers Arch* 448: 383–394, 2004.
10. **Grupper A, Shashar M, Bahry D, Pri-Paz Y, Bentur O, Levi S, Chernichovski T, Chernin G, Schwartz IF.** Cyclosporine attenuates arginine transport, in human endothelial cells, through modulation of cationic amino acid transporter-1. *Am J Nephrol* 37: 613–619, 2013.
11. **Hayashi T, Ishikawa T, Yamada K, Kuzuya M, Naito M, Hidaka H, Iguchi A.** Biphasic effect of estrogen on neuronal constitutive nitric oxide synthase via Ca(2+)- calmodulin dependent mechanism. *Biochem Biophys Res Commun* 203: 1013–1019, 1994.
12. **Ingbir M, Schwartz IF, Reshef R, Shtabsky A, Chernichovski T, Isserlin BA, Chernin G, Levo Y, Schwartz IF.** Rosiglitazone attenuates aortic arginine transport, through inhibition of PKCα, in uremic rats. *Am J Physiol Renal Physiol* 295: F471–F477, 2008.
13. **Kim-Schulze S, Lowe WL Jr, Schnaper HW.** Estrogen stimulates delayed mitogen-activated protein kinase activity in human endothelial cells via an autocrine loop that involves basic fibroblast growth factor. *Circulation* 98: 413–421, 1998.
14. **Klinge CM, Blankenship KA, Risinger KE, Bhatnagar S, Noisin EL, Sumanasekera WK, Zhao L, Brey DM, Keynton RS.** Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells. *J Biol Chem* 280: 7460–7468, 2005.
15. **Lerner DJ, Kannel WB.** Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. *Am Heart J* 111: 383–930, 1986.
16. **Levine RL, Chen SJ, Durand J, Chen YF, Oparil S.** Medroxyprogesterone attenuates estrogen-mediated inhibition of neointima formation after balloon injury of the rat carotid artery. *Circulation* 94: 2221–2227, 1994.
17. **Lovren F, Pan Y, Shukla PC, Quan A, Teoh H, Szmitko PE, Peterson MD, Gupta M, Al-Omran M, Verma S.** Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis in vitro and in vivo: translational implications for atherosclerosis. *Am J Physiol Endocrinol Metab* 296: E1440–E1449, 2009.
18. **McDonald KK, Zharikov S, Block ER, Kilberg MS.** A caveolar complex between the cationic amino acid transporter 1 and endothelial nitricoxide synthase may explain the "arginine paradox." *J Biol Chem* 272: 31213–31216, 1997.
19. **McNeill AM, Zhang C, Stanczyk FZ, Duckles SP, Krause DN.** Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels, effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone. *Stroke* 33: 1685–1691, 2002.
20. **Miller VM, Vanhoutte PM.** Progesterone and modulation of endothelium-dependent responses in canine coronary arteries. *Am J Physiol Regul Integr Comp Physiol* 261: R1022–R1027, 1991.
21. **Mineo C, Ying YS, Chapline C, Jaken S, Anderson RG.** Targeting of protein kinase C alpha to caveola. *J Biol Chem* 141: 601–610, 1998.
22. **Nesher N, Frolkis I, Schwartz D, Chernichovski T, Levi S, Pri-Paz Y, Chernin G, Shtabsky A, Ben-Gal Y, Paz Y, Schwartz IF.** L-Arginine improves endothelial function, independently of arginine uptake, in aortas

from chronic renal failure female rats. *Am J Physiol Renal Physiol* 306: F449–F456, 2014.
23. **O'Riordan E, Chen J, Brodsky SV, Smirnova I, Li H, Goligorsky MS.** Endothelial cell dysfunction: the syndrome in making. *Kidney Int* 67: 1654–1658, 2005.
24. **Reshef R, Schwartz D, Ingbir M, Shtabsky A, Chernichovski T, Isserlin BA, Chernin G, Levo Y, Schwartz IF.** A profound decrease in maternal arginine uptake provokes endothelial nitration in the pregnant rat. *Am J Physiol Heart Circ Physiol* 294: H1156–H1163, 2008.
25. **Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR.** Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci USA* 97: 5930–5935, 2000.
26. **Schwartz D, Schwartz IF, Gnessin E, Wollman Y, Chernichovsky T, Blum M, Iaina A.** Differential regulation of the glomerular arginine transporters (CAT-1 and CAT-2) in lipopolysaccharide treated rats. *Am J Physiol Renal Physiol* 284: F788–F795, 2003.
27. **Schwartz IF, Ayalon R, Chernichovski T, Reshef R, Chernin G, Weinstein T, Litvak A, Levo Y, Schwartz D.** Arginine uptake is attenuated through modulation of cationic amino-acid transporter-1, in uremic rats. *Kidney Int* 69: 298–303, 2006.
28. **Schwartz IF, Chernichovski T, Krishtol N, Grupper A, Laron I, Schwartz D.** Sexual dimorphism in glomerular arginine transport affects nitric oxide generation in old male rats. *Am J Physiol Renal Physiol* 297: F80–F84, 2009.
29. **Schwartz IF, Chernichovski T, Schwartz D.** Aortic arginine transport is attenuated, through post-translational modulation of CAT-1 by PKCalpha, in old male rats. *Vasc Med* 15: 55–59, 2010.
30. **Schwartz IF, Grupper A, Soetendorp H, Hillel O, Laron I, Chernichovski T, Ingbir M, Shtabski A, Weinstein T, Chernin G, Shashar M, Hershkoviz R, Schwartz D.** Attenuated glomerular arginine transport prevents hyperfiltration and induces HIF-1α in the pregnant uremic rat. *Am J Physiol Renal Physiol* 303: F396–F404, 2012.
31. **Schwartz IF, Ingbir M, Chernichovski T, Reshef R, Chernin G, Litvak A, Weinstein T, Levo Y, Schwartz D.** Arginine uptake is attenuated through post translational regulation of cationic amino-acid transporter-1, in hyperlipidemic rats. *Atherosclerosis* 194: 357–363, 2007.
32. **Vásquez R, Farías M, Vega JL, Martin RS, Vecchiola A, Casanello P, Sobrevia L.** D-Glucose stimulation of L-arginine transport and nitric oxide synthesis results from activation of mitogen-activated protein kinases p42/44 and Smad2 requiring functional type II TGF-beta receptors in human umbilical vein endothelium. *J Cell Physiol* 212: 626–632, 2007.
33. **Virdis A, Taddei S.** Endothelial aging and gender. *Maturitas* 71: 326–330, 2012.
34. **Voss AK, Fortune JE.** Estradiol-17β has a biphasic effect on oxytocin secretion by bovine granulose cells. *Biol Reprod* 48: 1404–1409, 1993.
35. **Wakatsuki A, Okatani Y, Ikenoue N, Fukaya T.** Effect of medroxyprogesterone acetate on endothelium-dependent vasodilation in postmenopausal women receiving estrogen. *Circulation* 104: 1773–1778, 2001.
36. **Wyatt AW, Steinert JR, Wheeler-Jones CP, Morgan AJ, Sugden D, Pearson JD, Sobrevia L, Mann GE.** Early activation of the p42/p44MAPK pathway mediates adenosine-induced nitric oxide production in human endothelial cells: a novel calcium-insensitive mechanism. *FASEB J* 16: 1584–1594, 2002.