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 Renal Int 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 estrogen 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 (E2) or progesterone. Exposure to E2 (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 Ca (PKCa), and phosphorylated ERK1/2 protein content. GO-6976 (PKCa 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 PKCa and ERK1/2 phosphorylation, an effect that predominates over estradiol.

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with NO2 to yield a diazochromophore. Total NO2 was measured as nitrate (NO3) 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 1-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 NO3 is reduced to NO2, Griess reagents [sulfanilamide in hydrochloric acid and N-(1-naphthyl)ethylenediamine in hydrochloric acid] were added to react with NO2 to yield a diazochromophore. Total NO2 was measured spectrophotometrically at 540 nm. Each sample was analyzed in triplicate, and the concentration of NO2 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, PKCo, phosphorylated PKCo (p-PKCo-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-PKCo, and mouse monoclonal anti-human PKCo-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-PKCo, PKCo, JNK, and p-JNK were detected by enhanced chemiluminescence (Kodak X-Omat AR film) and quantified by densitometry.

**Immuno precipitation studies.** Aliquots of endothelial cell lysate (1 ml) from 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.

**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 E2 (50–100 nM) for 5 or 30 min significantly attenuated arginine transport while lower (10 nM) or higher (200 nM) concentrations had no effect (Fig. 1A). 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. 1B). 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 E2 (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. E2 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).
NO$_2$/NO$_3$ generation by cells exposed to either E2 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 E2 treatment than in control, while progesterone substantially decreased NO$_2$/NO$_3$ generation (Fig. 3).

To identify a specific signal transduction pathway by which E2 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 (PKC-α inhibitor) had no effect (Fig. 4A). Next, arginine uptake was determined when cells were exposed to the same agents in the presence of either E2 or progesterone for 30 min. We have found that arginine transport was significantly increased following coincubation of E2 and SP-600125 (JNK inhibitor), while neither E2 and GO-6976 nor E2 and UO-126 (ERK1/2 inhibitor) had any effect compared with E2 alone (Fig. 4B). Cotreatment with progesterone and either GO-

![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.](image)

![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-N6-(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.](image)
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. 4C). Subsequently, Western blotting was performed for PKCα, JNK, and ERK1/2 and their phosphorylated forms, following incubation of HUVEC with either E2 or 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 (#).

Finally, coincubating HUVEC simultaneously with both E2 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. E2 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 E2 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-
ing a positive effect of E2 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, E2 failed to augment arginine transport when concentrations were increased, implying that there is a well-defined E2 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 E2) 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 E2. Several studies have demonstrated that progesterone antagonizes the effects of E2 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, E2 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.

We have tried to elucidate a molecular mechanism to explain our findings. Changes in arginine uptake due to both E2 and progesterone were associated with directional changes in the relative amount of phosphorylated CAT-1 protein. The enhanced arginine transport induced by E2 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 E2 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 E2 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 E2 or progesterone. Indeed, progesterone produced a significant increase in PKCα phosphorylation that can account for the decrease in CAT-1 activity. However, E2 had no effect. A thorough review of the literature reveals that both MAPKs JNK and ERK1/2 can be 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 E2 and progesterone. E2 decreased ERK1/2 phosphorylation, whereas progesterone did the opposite. The enhanced arginine transport velocities induced by E2 were not affected by incubation with an ERK1/2 antagonist, which further supports the notion that E2 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


REFERENCES


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We found three primary results:

1. A profound decrease in maternal arginine uptake provokes endothelial nitration in the pregnant rat.
2. Arginine uptake is attenuated through modulation of cationic amino-acid transporter-1, in uremic rats.
3. Arginine uptake is attenuated, through post-translational modulation of CAT-1 by PKCalpha, in old male rats.

These findings highlight the importance of arginine transport in various physiological and pathological conditions, including pregnancy and uremia.