Estradiol augments while progesterone inhibits arginine transport in Human Endothelial cells, through modulation of Cationic Amino Acid Transporter-1.

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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 (E2) or progesterone. Exposure to E2 (50 and 100nM) for 30 minutes 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/ERK1/2. Progesterone (1 and 100 pM for 30 minutes) attenuated arginine uptake and increased phosphorylated CAT-1, phosphorylated PKCα and phosphorylated ERK1/2 protein content. GO6976 (PKCα inhibitor) prevented the progesterone induced decrease in arginine transport. Co-incubation with both progesterone and estrogen for 30 minutes 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 phosphrolyation, an effect which predominates over estradiol.
Introduction

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 (ADMA) and advanced glycation end products (AGEs) (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 which 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 GmbH, 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 (FCS), 100 µg/ml penicillin/streptomycin, 0.05 µg/ml Amphotericin B, at 37 °C in a humidified atmosphere containing 5% CO2. The medium was changed 3 times weekly. Cells were used for experiments when they were 70–80% confluent, between passages 3 and 6.

Twenty four hours prior to the experiments, the incubation medium was changed to 5% bovine serum albumin (BSA). 17 β estradiol (E2) and progesterone were dissolved in ethanol (final concentration < 10^{-5}%). MAPK inhibitors UA 126 (ERK inhibitor) and SP 600125 (JNK inhibitor), and GO 6976 (PKCα inhibitor) (3) were all dissolved in dimethyl sulfoxide (DMSO) (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 6-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 100µM, 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. 700 μl of the lysate 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
each data point. Results are expressed as mean ± SE of at least five different experiments.

**Nitrite/nitrate determination**

The level of nitrite/nitrate 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 Kd spin column (Biovision INC. CA,
USA) 50 μl of the culture medium was diluted with 50 μl of reaction buffer containing 50
μM L-N6-(1-iminoethyl) lysine hydrochloride (L-NIL, a selective inducible NOS
inhibitor), and mixed with 25 μl NADH and 25 μl nitrate reductase. Following 30’
incubation during which nitrate is reduced to nitrite, Griess reagents (Sulfanileamide in
hydrochloric acid and N-(1-naphthyl ethylenediamine in hydrochloride acid) were added to
react with nitrite to yield a diazochromophore. Total nitrite was measured
spectrophotometrically at 540 nm. Each sample was analysed in triplicates and the
concentration of nitrite 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, PKC-α, phosphorylated PKC-α (p-PKC-α), MAPK extracellular-signal regulated kinase (ERK), phosphorylated ERK (p-ERK), MAPK Jun N-terminal kinase (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, 5 μM aprotinin) (ICN Biomedicals, Inc.), 0.01% Triton X-100 and 0.1% SDS, then mechanically homogenized and left on ice for 45 min. Homogenates were subsequently centrifuged (13,000 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, 5% mercaptoethanol) and analyzed on a 7.5% SDS-PAGE gel. The gel was transferred onto Hybond ECL nitrocellulose membranes (Amersham Corp.) and blocked in PBS-T containing 5% non-fat dried milk, at room temperature. Membranes were then incubated with rabbit anti-human CAT-1, ERK, p-ERK, p-PKC-α and mouse monoclonal anti human PKC-α, JNK, p-JNK antibodies (all from Santa Cruz Biotechnology, Inc., Calif., USA) for 1 h at room temperature, washed, and incubated with secondary HRP-conjugated goat anti rabbit and goat anti mouse antibodies in PBS-T for 1 hr. Membranes were subsequently washed 3 times, 5 min each, in PBS-T. Membranes were then stripped and reprobed with monoclonal anti-β-actin or
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies as an internal control. The reactive bands corresponding to CAT-1, ERK, p-ERK, p-PKC-α, PKC-α, 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 hr 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, Inc., Calif., USA). Pellets were collected by centrifugation at 3,000 rpm for 30 sec, 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 2x 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 were analyzed as above. Results are adjusted for CAT-1 levels, and expressed in arbitrary units.
Values shown in the figures are mean± SE from data obtained from 4-10 experiments. ANOVA test was applied for comparisons of mean, 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 progestrone on CAT-1-related arginine transport. Incubating cells with 17 β estadiol (E2) (50-100nM) for 5 or 30 min significantly augmented 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 hours) (data not shown).

Exposing the cells to progesterone at concentrations of 1 and 100 pM for 5 and 30 minutes significantly attenuated arginine transport (fig1b). 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 (50nM) or progesterone (1pM) for 30 min. In order to unveil a post-translational 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 minutes, while treatment with progesterone for 5 or 30 minutes resulted in a significantly augmented p-CAT1 protein content (fig 2a,d).

Nitrite/nitrate generation by cells exposed to either E2 or progesterone for 30 minutes in the presence of L-NIL (a selective iNOS inhibitor), with or without L-NAME (a non selective NOS inhibitor) was used to evaluate NO production by eNOS. The concentration of NO2/NO3 was significantly higher following E2 treatment than in control, while progesterone substantially decreased nitrite/nitrate generation (fig 3).
In order 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 SP600125 (JNK inhibitor) significantly augmented arginine uptake while GO 6976 (PKCα 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 minutes. We have found that arginine transport was significantly increased following co-incubation of E2 and SP600125 (JNK inhibitor), while neither E2 and GO 6976 nor E2 and UO 126 (ERK1/2 inhibitor) had any effect when compared to E2 alone (fig 4b). Co-treatment with progesterone and either GO6976, UO 126 or SP600125 attenuated the decrease in arginine transport when compared to progesterone only. The effect of GO 6976 was more pronounced than the other two (fig 4c). Subsequently, Western blotting was performed for PKC-α, JNK, and ERK1/2 and their phosphorylated forms, following incubation of HUVEC with either E2 or progesterone for 30 minutes. Cell exposure to E2 resulted in a significant decrease in ERK phosphorylation (fig 5) while no changes were seen in PKC-α or JNK (data not shown). Progesterone significantly augmented phosphorylated ERK and phosphorylated PKCα protein levels while JNK remained unchanged (fig 6a-d). Finally, co-incubating HUVEC simultaneously with both E2 and progesterone for 30 minutes 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 minutes) 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 cardio-protective benefits disappear after menopause (16). 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 down regulated (22,27,28,29,30). The current experiments, exploring 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 an elevated serum estrogen concentrations (24,30). This discrepancy can be explained by two observations made in our current studies. Firstly, 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 which have demonstrated that estrogens (mainly E2) exhibit a biphasic effect on various biological systems \((1,11,34)\). Secondly, 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 17-β estradiol on eNOS, yet this issue remains controversial \((2,4,8,15,19,20,35)\). 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, when compared to 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 minutes, 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 post-translational modulation of
CAT-1 which was associated with upregulation of PKC-α. Namely, PKC-α 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 PKC-α phosphorylation following incubation with either E₂ or progesterone. Indeed, progesterone produced a significant increase in PKC-α phosphorylation which can account for the decrease in CAT-1 activity. However, E₂ had no effect. A thorough review of the literature reveals that both MAPK's JNK and ERK1/2 can be modulated by E₂ (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 E₂, 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 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 down-regulation, progesterone diminishes CAT-1 activity through activation of both ERK1/2 and PKC-α. 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 provides a novel mechanism to explain the effects of female sex hormones on the endothelium and supports the therapeutic use of arginine under certain conditions.
REFERENCES


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LEGENDS

Figure 1. Effect of 17β estradiol and progesterone on the uptake of radiolabeled arginine ([³H] L-arginine) by HUVEC. a. Cell exposed to 17β estradiol (10-200nM) for 5 and 30 min. b. Cells exposed to progesterone (1,100pM) for 5 and 30 min. Data are presented as the mean ± SE of 8 different experiments. *p<0.05 vs. control. #p<0.05 vs. 17β estradiol 100nM. Abbreviations: CTL: controls.

Figure 2. Representative Western blot analysis and densitometric analysis showing regulation of CAT-1 and phosphorylated CAT-1 protein level in HUVEC treated with 17β estradiol 50nM (a,b) or progesterone 1pM (c,d) for 5 and 30 min. Data are presented as the mean ± SE of 3 different experiments *p<0.05 vs. control. Abbreviations: CTL: controls.

Figure 3. NO₂/NO₃ generation by cells exposed to progesterone or 17 β estradiol for 30 minutes in the presence of L-NIL (50 µM, a selective iNOS inhibitor), with or without L-NAME. Data are presented as the mean ± SEM of six different experiments. Abbreviations: CTL: control.
Figure 4. Effect of UO126 (ERK1/2 inhibitor, 10µM), SP600125 (JNK inhibitor, 20µM), and GO 6976 (PKC-α inhibitor 1µM) on uptake of radiolabeled arginine ([³H] L-arginine) by HUVEC with or without co-exposure to 17β estradiol (50nM) or progesterone (1pM) for 30 minutes. 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 the mean ± SE of 5 different experiments. *p<0.05 vs. control. #p<0.05 vs. UO126 and SP600125. Abbreviations: CTL: controls.

Figure 5. Representative Western blot analysis and densitometric analysis showing protein content and regulation of a. ERK. b. p-ERK in HUVEC treated with 17β estradiol 50nM for 5 and 30 min. Results are mean ± SE of four different experiments. *p<0.05 vs. control. Abbreviations: CTL: controls.

Figure 6. Representative Western blot analysis and densitometric analysis showing protein content and regulation of a. ERK. b. p-ERK c. PKC-α. d. p-PKCα, in HUVEC treated with progesterone 1pM for 5 and 30 min.. Results are mean ± SE of four different experiments. *p<0.05 vs. control. Abbreviations: CTL: controls.

Figure 7. Effect of concurrent exposure to 17β estradiol (50 nM) and progesterone (1pM) for 30 minutes on radiolabeled arginine ([³H] L-arginine) uptake by HUVEC. Data are presented as the mean ± SE of 8 different experiments. *p<0.05 vs. control.
Fig 1a

17β Estradiol (nM)

n mole arginine/mg protein

CTL  10  50  100  200

5 min  30 min

# #
Fig 1b

nmol arginine/mg protein vs Progesterone (pM)

- CTL
- 1
- 100

5 min vs 30 min comparison with statistical significance marked by asterisks (*)
Fig 2a

CAT-1

Actin

17β Estradiol (50 nM)

CTL

5 min

30 min

CAT-1 contents (relative density units)

75 kD

42 kD
Fig 2b

**CAT-1**

**pCAT-1**

![Image of Western blot with density units](image)

![Bar graph showing pCAT-1 contents with relative density units](image)

- **CTL**
- **5 min**
- **30 min**

**17β Estradiol (50 nM)**

* Indicates significant change
CAT-1 contents (relative density units)

Fig 2c

CAT-1 75 kD

GAPDH 37 kD

Progesterone (1pM)
**fig2d**

**CAT-1**

**p-CAT-1**

![Image of Western blots](image)

**Graph**

- **x-axis:** Progesterone (1pM)
- **y-axis:** pCAT-1 contents (relative density units)
- **Bars:**
  - **CTL:** 0.8
  - **5 min:** 1.6
  - **30 min:** 1.2

* denotes statistical significance compared to the control (CTL) group.
Fig 4a

nmole arginine/mg protein

VEHICLE 600125SP  VEHICLE 126UO  VEHICLE 6976GO
ERK content (relative density units) for 17β Estradiol (50 nM) treatment:

- CTL: 1.0
- 5 min: 0.9
- 30 min: 0.8

GAPDH (37 kD) and ERK (42/44 kD) expression levels were monitored through Western blot analysis.
Fig 5b

**p-ERK**

**ERK**

![Bar graph showing p-ERK contents (relative density units) for CTL, 5 min, and 30 min treatments with 17β Estradiol (50 nM). The graph indicates a decrease in p-ERK content from CTL to 30 min with a significant decrease marked by an asterisk.](image-url)

**17β Estradiol (50 nM)**
Fig 6a

**ERK**

- **GAPDH**

42/44 kD

37 Kd

**ERK contents (relative density units)**

<table>
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<tr>
<th>Time</th>
<th>ERK Contents</th>
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<tr>
<td>CTL</td>
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</tr>
<tr>
<td>5 min</td>
<td>0.6</td>
</tr>
<tr>
<td>30 min</td>
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**Progesterone (1pM)**
Fig 6b

p-ERK

ERK

**p-ERK contents (relative density units)**

<table>
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<tr>
<th></th>
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<th>30 min</th>
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<tr>
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<td>2</td>
<td>4</td>
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</table>

**Progesterone (1pM)**
Fig 6c

PKCα contents (relative density units)

Progesterone (1pM)

PKCα contents (relative density units)

CTL  5 min  30 min

PKCα  GAPDH

80 Kd  37 Kd
Progesterone (1pM)

PKCα

p-PKCα

0
0.4
0.8
1.2
1.6
2

5 min
30 min

Fig 6d
Fig 7

![Bar chart showing nmole arginine/mg protein for Control and E2+progesterone conditions. The Control group has a higher nmole arginine/mg protein compared to the E2+progesterone group, indicated by an asterisk (*) on the E2+progesterone bar.]