ORIGINAL ARTICLE

VWF and ADAMTS13 behavior in estradiol-treated HUVEC

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Abstract

Objectives: In this study, the role of 17β-estradiol (E2) in the regulation of von Willebrand factor (VWF) and ADAMTS13 synthesis, storage, and secretion was investigated in cultured human umbilical vein endothelial cells (HUVEC). *Methods:* HUVEC were grown to 80–90% confluence and replaced with fresh medium containing E2 (1 nm) or vehicle for 24 h, after which the supernatant medium and cell lysates were collected to measure VWF and ADAMTS13. VWF was evaluated by VWF:Ag and multimeric analysis. ADAMTS13 was evaluated by SDS-PAGE. VWF and ADAMTS13 mRNA were quantified by real-time PCR after E2 or vehicle exposure for 18 h. A functional effect of ADAMTS13 on HUVEC VWF protein synthesis was further evaluated using a short hairpin RNA (shRNA) to knockdown the expression of endogenous ADAMTS13. *Results:* E2 did not increase the release or intracellular VWF levels in HUVEC. However, E2 increased the production of intracellular ADAMTS13, although there was no evidence of significant effects of their release into culture medium. Incubation of HUVEC with E2 resulted in a significantly increased expression of VWF and ADAMTS13 mRNA. ADAMTS13 gene inactivation upregulates release and intracellular VWF levels in E2-treated HUVEC. *Conclusion:* The results demonstrated that E2 may play a role in the regulation of VWF and ADAMTS13 gene expression and in its production in human endothelial cells. The mechanism of the protective effects of E2 on the cardiovascular system could be explained by the intracellular regulation of VWF produced by ADAMTS13.

Key words von Willebrand factor; ADAMTS13; 17β-estradiol; endothelial cells

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Accepted for publication 16 October 2010

doi:10.1111/j.1600-0609.2010.01545.x

von Willebrand factor (VWF) is a blood glycoprotein that is required for normal hemostasis. VWF is constitutively secreted and stored in intraendothelial Weibel–Palade granules (1). The synthesis and secretion of VWF seems to be closely regulated, so that plasma levels remain constant. ADAMTS13 physiologically reduces ultralarge-VWF multimers size into smaller forms by cleaving VWF. The plasma VWF molecules are composed of intact and degraded multimers with different molecular weights (2). Recent reports have shown that ADAMTS13 is synthesized and released from human umbilical vein endothelial cells (HUVEC) and could be detected by its enzymatic activity in cell lysates using both static and flow assays (3).

There is increasing evidence that 17β-estradiol (E2) acts directly on the endothelium and influences vascular functions and reactivity, producing changes in the production of several endothelium derived factors (4–10). E2 has been shown to upregulate eNOS mRNA expression in bovine fetal pulmonary artery endothelium and in rat and porcine aortic endothelial cells (11, 12). Also, E2 modulates caveolin-1 and endothelin-1 mRNA in bovine aortic endothelial cells and HUVEC, respectively (13, 14). There are a great number of candidate genes for E2 regulation, which are involved in coagulation and fibrinolysis, including tissue factor, fibrinogen, protein S, factor VII, factor XII, plasminogen-activator inhibitor, tissue plasminogen activator, and antithrombin III (15–18). Clinical and experimental data suggest that endothelium is a target for E2 (19).

In postmenopausal women, the plasma estrogen level is reduced to < 100 pmol/L, making estrogen the primary

and the most logical component of hormonal replacement therapy (HRT) (20). There are controversial results with respect to HRT on hemostatic factors in postmenopausal women. Rabbani *et al.* (21) suggested that oral conjugated equine estrogen (CEE) increases plasma VWF in postmenopausal women. In a randomized placebo-controlled trial, Brussaard *et al.* (22) assessed the effect of oral E2 daily, during 6 weeks on indicators of coagulation and of fibrinolysis in postmenopausal women with type 2 diabetes mellitus. They observed a significant increase in VWF after E2 treatment. However, several authors observed that the levels of FVIII and VWF were not altered by oral or transdermal HRT in postmenopausal women (23–29). As far as we know, there is no background information related to ADAMTS13, HRT, and postmenopausal women.

Considering that HUVEC expresses estrogen receptors (30, 31), we used this model to investigate whether E2 could affect the synthesis, storage, and release of VWF and ADAMTS13.

Materials and methods

Cell culture and treatments

Experiments were performed in primary HUVEC obtained with 0.1% collagenase type 1 (32) seeded onto T25 flask coated with 1% gelatin and studied at passage 4–6. HUVEC were cultured in RPMI 1640 (Gibco, Paisley, UK) medium supplemented with 2 mM L-glutamine, 2-mercaptoethanol, 0.25 mg/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin, 50 μg/mL endothelial cell growth supplement (Sigma, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; Gibco). They were incubated at 37°C, in a 5% CO_2 humidified incubator.

Estradiol (E2), histamine (H), and ICI-182780 were purchased from Sigma. Stock solutions of E2 were prepared in 99.9% ethanol and stored at −20°C.

The effects of E2 treatments on VWF and ADAMTS13 expression and secretion were studied under the following culture conditions: 80–90% confluent cultures were washed and serum starved for 1 h. Thereafter, the cells were treated simultaneously with E2 (1 nM) or vehicle (negative control, ethanol/culture medium 1 : 10 000) for the indicated time periods. ICI-182780 (Fulvestrant) is the first in a new class of novel, steroidal, 'pure' antiestrogens – the estrogen receptor down-regulators. Given that ICI-182780 leads to rapid degradation and loss of E2 receptors, it was used as E2 antagonist at a dose range of 100–1000 nM. We used histamine (100 μM) as a positive control of VWF release (33).

Preparation of cell lysates

T-75 flasks of HUVEC were washed thoroughly with phosphate-buffered saline prior to being lysed with hypotonic saline containing 6 mM *N*-ethyl maleimide (NEM, used to inhibit de-sumoylation of proteins), 1 mM Pefabloc, 5 mM EDTA, 200 kIU/mL aprotinin, 1% sodium dodecyl sulfate (SDS), and 150 mM NaCl. The cells were scraped, pelleted, and re-suspended in 1 mL of NEM/Pefabloc/EDTA/aprotinin/SDS/NaCl saline and then sonicated. Cellular debris was removed by centrifugation.

VWF antigen and activity assays

The VWF was estimated by ELISA (34) in HUVEC supernatants, and lysates were collected 24 h after treatments. The supernatants and cell lysates were dialyzed against diluted buffer at 10°C and concentrated using a Speed Vac concentrator (SAVANT, Thermo Scientific, Asheville, NC, USA) to evaluate VWF:Ag levels. The total protein concentration was quantified by Bradford technique (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a reference (35). The VWF:Ag was expressed as IU/100 mg protein. VWF multimer analysis was performed according to Ruggeri *et al.* with modifications (36). SDS-agarose gel electrophoresis was performed using 1% and 1.4% agarose running gels. After electrophoresis, the gels were washed, dried, and reacted with anti-VWF antibody (Sigma), then with anti-rabbit IgG biotin (Sigma) and then with StrepABComplex/HRP (DakoCytomation, Glostrup, Denmark). The reaction was developed with 4-chloro-1-naftol and 0.04% H_2O_2.

ADAMTS13 SDS/PAGE assay

The supernatant and cell lysates were collected 24 h after cell treatment. Supernatants and cell lysates (500 μg protein) were then subjected to SDS/PAGE (6%) and transferred by electroblot to a nitrocellulose membrane. After blocking, the membrane was incubated with anti-ADAMTS13 (ab28273-100 and ab28274-100; ABCAM, Cambridge, UK) and then with peroxidase-labeled horse anti-goat IgG (ABCAM). Chemiluminescence was developed using the ECL western blotting detection reagents (Sigma) and X-ray films. The films were scanned, and the amount of signal was quantified by densitometric analysis using the Image Master 1D software (Pharmacia). Densitometric analysis of ADAMTS13 content was expressed as pixel intensity.

RNA isolation, RT-PCR, and real-time PCR

RNA from confluent T-75 flasks of HUVEC was isolated using TRIZOL (Invitrogen, San Diego, CA, USA), chloroform extraction, and isopropanol precipitation and its

integrity were verified by 260/280 optical density ratios. The RNA was reverse transcribed into cDNAs with ImProm-II reverse transcriptase (Promega, Madison, WI, USA) using specific pairs of primers (Table 1). Real-time PCR were performed in a LightCycler (Roche Molecular Biochemicals). Detection carried out in the SYBR Green I format using the LightCycler FastStart DNA master SYBR Green I kit with the following program: 10 min 95°C to activate the polymerase followed by 45 cycles of 10 s 95°C, 11 s 60°C, and 4 s 72°C. PCR products were identified by their melting curve and by gel electrophoresis. β-Actin was the housekeeping gene for HUVEC.

ADAMTS13 gene inactivation by short hairpin RNA (shRNA) transfection

Human ADAMTS13 shRNA (29-mer) and control plasmids were purchased from Origene (Rockville, MD, USA) and used according to the manufacturer's protocols.

HUVEC were transfected with lipofectamine (Invitrogen). Preconfluent HUVEC (50–60%) grown in six-well plates were incubated in 1 mL of opti-MEM and ADAMTS13 shRNA (30 μM) or HuSH 29-mer non-effective (scrambled) pRS vector (1 μg/well) mixed with 3 μL of lipofectamine for 18 h; the mix was then replaced with normal medium. Experiments were performed 72 h after the transfection.

Statistical analysis

All experimental data were presented as mean ± standard deviation of the mean. The statistical plan included a traditional sample size calculation using classical criteria: significance, 0.001 and power, 0.8. This resulted in an estimated sample size of 6 to determine differences. Significance ($P < 0.01$) of the differences was evaluated with one-way ANOVA. Turkey method was used in multiple comparisons for *post hoc* tests.

Interassay variation, expressed as between run coefficient of variation (CV), was determined using vehicle-treated HUVEC supernatant, for ELISA assays and SDS-PAGE, ADAMTS13 construct for real-time PCR, and tested repeatedly, on different days (Table 2). Intra-assay variation (also expressed as within run CV) was determined using those samples and tested repeatedly on the same day (Table 2).

Table 2 Reproducibility of the assays

	VWF:Ag	SDS PAGE	Real-time PCR
Intraassay coefficients of variation (% CV, $n = 6$)	4.2	20	1.6
Interassay coefficients of variation (% CV, $n = 6$)	4.3	24	2.7

VWF, von Willebrand factor.

Results

Released and intracellular VWF

After 24 h of treatment, VWF concentration was measured in the supernatants of the HUVEC cultures. The concentration of VWF observed in control, corresponded both to the FBS used to sustain growth and to the VWF secreted constitutively (Fig. 1). While H (positive control) induced a significant threefold increase ($P < 0.001$) in the release of VWF, E2 did not cause any stimulation effect. When we examined intracellular VWF levels, we did not observe a significant difference between E2 and control (Fig. 2).

Released and intracellular ADAMTS13

Immunoblots were performed on HUVEC supernatant using the ADAMTS13 amino- and carboxy-terminal antibodies directed against human ADAMTS13. While HUVEC incubated with E2 showed no significant differences in supernatant ADAMTS13 levels (Figs 3a,b), intracellular levels showed a significant eightfold increase, ($P < 0.001$), with regard to control (Figs 4a,b).

VWF and ADAMTS13 mRNA levels

The mRNA for VWF and ADAMTS13 was detected in HUVEC using real-time PCR. E2 leads to a marked

Table 1 Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR primers

	Sequence	Product size (kb)
Forward primer VWF (exon 3)	5′-AGA AAC GCT CCT TCT CGA TTA TTG-3′	84
Reverse primer VWF (exon 4)	5′-TGT CAA AAA ATT CCC CAA GAT ACA-3′	
Forward primer ADAMTS13 (exon 15)	5′-CCCAACCTGACCAGTGTCTACA-′3	93
Reverse primer ADAMTS13 (exon 16)	5′-CTTCCCAGCCACGACATAGC-′3	
Forward primer β-actin (exon 2)	5′-TCACCAACTGGGACGACATG-′3	200
Reverse primer β-actin (exon 3)	5′-GTACAGGGATAGCACAGCCT-′3	

VWF, von Willebrand factor.

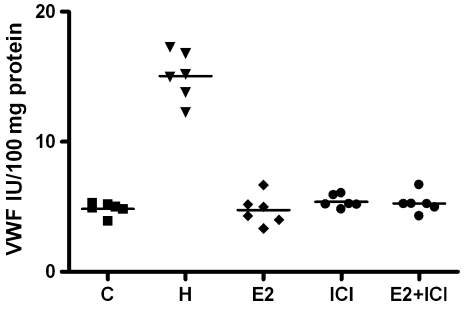

Figure 1 Human umbilical vein endothelial cells were incubated with vehicle (C), H, E2, ICI-182780 (ICI), and E2 plus ICI for 24 h. The amount of von Willebrand factor released from cells into the media was measured by ELISA (n = 6, mean ± SD).

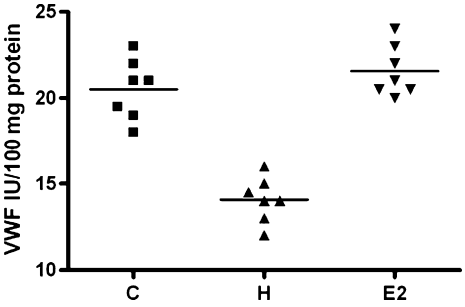

Figure 2 Human umbilical vein endothelial cells (HUVEC) were incubated with vehicle (C), H, and E2 for 24 h. The amount of intracellular von Willebrand factor was measured by ELISA (n = 6, mean ± SD).

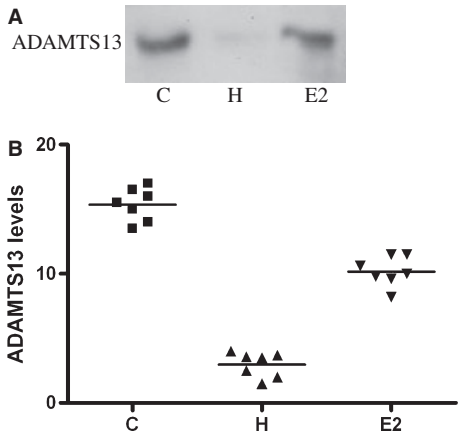

Figure 3 The culture medium was subjected to SDS-PAGE analysis under non-reducing conditions and western blotting using monoclonal mouse anti-human ADAMTS13 antibody. (A) Representative western blot of ADAMTS13. Human umbilical vein endothelial cells were incubated with vehicle (C), H, and E2 for 24 h. (B) Densitometric analysis of ADAMTS13 bands.

ninefold increase ($P < 0.001$) in the levels of VWF and ADAMTS13 mRNA, when it was incubated with HUVEC. When E2 treatment was combined with the

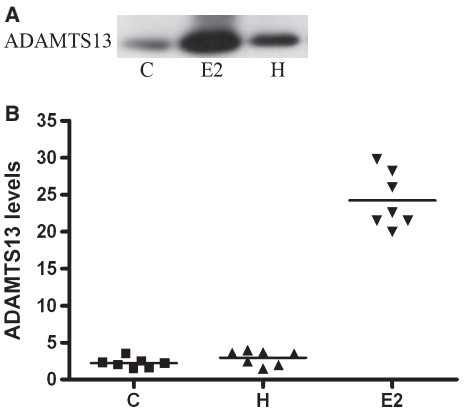

Figure 4 Human umbilical vein endothelial cells (HUVEC) protein extracts were analyzed using SDS-PAGE analysis under non-reducing conditions and western blotting using monoclonal mouse anti-human ADAMTS13 antibody. (A) Representative western blot of ADAMTS13. HUVEC were incubated with vehicle (C), H, and E2 for 24 h. (B) Densitometric analysis of ADAMTS13 bands.

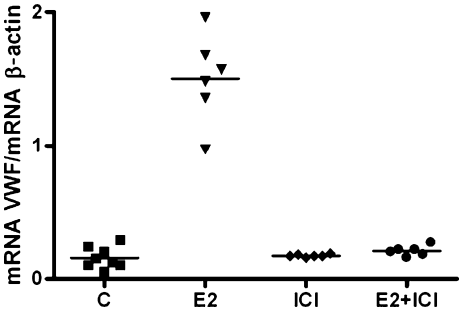

Figure 5 Real-time polymerase chain reaction analysis of von Willebrand factor mRNA expression in human umbilical vein endothelial cells (HUVEC). The amount of relative expression was normalized to that of β-actin mRNA. HUVEC were incubated with vehicle (C), H, E2, ICI-182780 (ICI), and E2 plus ICI for 18 h.

antiestrogen ICI-182780, we obtained a significant 7- and 10-fold decrease ($P < 0.001$) in the levels of VWF and ADAMTS13 mRNA, respectively (Figs 5 and 6).

Released and intracellular VWF with ADAMTS13 gene inactivation

Western blotting analysis revealed that transfection of ADAMTS13 shRNA, but not the scrambled control shRNA (pRS), attenuated ADAMTS13 expression in HUVEC (Fig. 7), without affecting the expression of VWF (Figs 8 and 9).

There were no major differences between VWF mRNA levels from HUVEC treated with E2 transfected with pRS and those transfected with ADAMTS13 shRNA (ninefold increase with regard to control; data not shown).

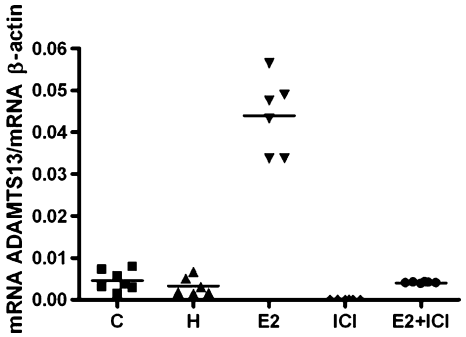

Figure 6 Real-time polymerase chain reaction analysis of ADAMTS13 mRNA expression in human umbilical vein endothelial cells (HUVEC). The amount of relative expression was normalized to that of β-actin mRNA. HUVEC were incubated with vehicle (C), H, E2, ICI-182780 (ICI), and E2 plus ICI for 18 h.

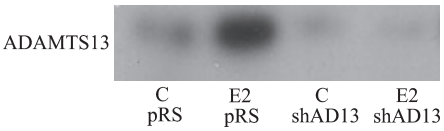

Figure 7 Human umbilical vein endothelial cells (HUVEC) protein extracts were analyzed using SDS-PAGE analysis under non-reducing conditions and western blotting using monoclonal mouse anti-human ADAMTS13 antibody. Representative western blot of ADAMTS13. HUVEC were transfected with pRS or ADAMTS13 short hairpin RNA and incubated with vehicle (C) or E2 for 24 h.

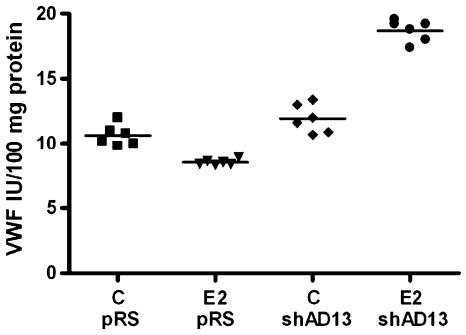

Figure 8 Human umbilical vein endothelial cells were transfected with pRS or ADAMTS13 short hairpin RNA and incubated with vehicle (C) or E2 for 24 h. The amount of von Willebrand factor released from cells into the media was measured by ELISA.

We observed a significant increase of two- and three-folds in released and intracellular VWF levels from HUVEC treated with E2 transfected with ADAMTS13 shRNA, with regard to pRS transfected (Figs 8 and 9). There was no significant difference between ADAMTS13 shRNA and pRS transfected in VWF:Ag levels from vehicle-treated HUVEC.

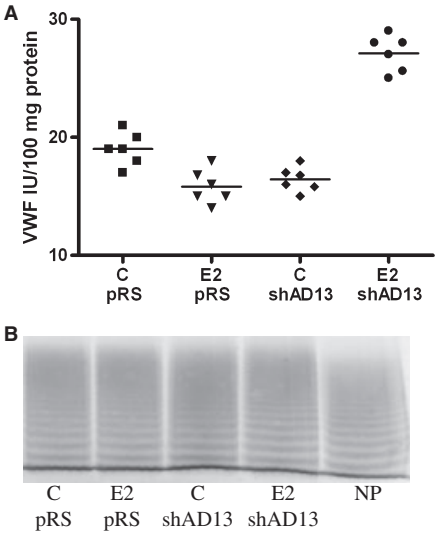

Figure 9 Human umbilical vein endothelial cells were transfected with pRS or ADAMTS13 short hairpin RNA and incubated with vehicle (C) or E2 for 24 h. (A) The amount of intracellular von Willebrand factor (VWF) was measured by ELISA. (B) Representative multimer analysis of intracellular VWF (NP, diluted normal plasma 1 : 10).

Intracellular and secreted VWF from HUVEC transfected with pRS or ADAMTS13 shRNA and treated with E2 or vehicle were analyzed for multimeric composition and compared with normal plasma (NP). Ultra-large VWF was detected in intracellular and secreted HUVEC samples, and there was no difference in the multimeric pattern between them.

Discussion

To the best of our knowledge, this is the first time that estrogens are associated with a positive regulation of VWF and ADAMTS13 transcription. Furthermore, inhibition of ADAMTS13 by gene inactivation using shRNA indicates that this enzyme probably participates in the intracellular regulation of VWF.

Yasiu *et al.* (37) demonstrated that using combined CEE at a dosage of 0.625 mg/d and medroxyprogesterone acetate at a dosage of 2.5 mg/d and that mean levels of E2, after 12 months in postmenopausal women treated every other day and every day were 14.3 and 31.4 pg/mL. In another study, oral estrogen (2 mg) was given twice a day for 21 d to women in an artificial luteal phase. At day 21, serum E2 level was 279 $\pm$ 76 pg/mL (38). These studies suggest that E2 in HRT has a plasma concentration lower than 280 pg/mL. Our results demonstrated that in HUVEC E2 (1 nM, 250 pg/mL) does not cause an increase in VWF release. This finding is in accordance with the results of Harrison *et al.* (39) who found the increase in VWF concentration

in the media from the E2-treated cultures to be statistically significant at 500, 1000, and 2000 E2 pg/mL ($P < 0.001$), but not at 250 pg/mL ($P > 0.1$). For each set of endothelial cell cultures, the increased percentage of VWF concentration, when compared with its corresponding control, was normalized to the DNA content of that control culture (39). During a clinical study of inflammatory markers in postmenopausal women, Eilersten *et al.* (40) observed increased levels of VWF in the raloxifene group and no response in E2 and norethisterone acetate (conventional and low doses) group. In a study to investigate the relationship between sex hormones and coagulation-fibrinolysis system in postmenopausal women with coronary heart disease (CHD), Yang el al (41) suggested that the decrease in E2 was negatively correlated with VWF. Our results could implicate that E2 levels in postmenopausal women treated with HRT did not produce a VWF plasma increase. Data from experimental studies or observations from the natural lack of endogenous estrogen occurring during menopause show that estrogen deprivation in women is associated with adverse effect on the cardiovascular system and with acceleration of atherosclerosis (42) In the Women's Health Initiative (WHI), the administration of CEE was associated with the risk of stroke and no effect on CHD incidence in postmenopausal women with prior hysterectomy (43); the administration of a combination of CEE with progestin was associated with an increase in C-reactive protein levels (44). The odds ratios for incident CHD were similar among HRT users and non-users in analyses stratified by underlying levels of each inflammatory biomarker (44). Finally, Saltiki *et al.* (45) suggested that to make use of the protective effect of E2, further investigations into the compound, the dosage, the duration, and the ideal and safest route of administration must be carried out.

ADAMTS13 and VWF appear to be partially colocalized to the endoplasmic reticulum, Golgi apparatus, and granules, consistent with the localization of the secretory proteins (46). The results of the present study confirmed the previous observations that ADAMTS13 is located intracellularly, and we demonstrated that E2-treated HUVEC increased the ADAMTS13 intracellular but not the released levels. Liu *et al.* (47) demonstrated that ADAMTS13 can be detected on the surface of platelets and that the expression increases upon platelet activation.

Recent studies have suggested that VWF mRNA levels are significantly increased via estrogen receptor in human osteosarcoma cells U2OS stably transfected with estrogen receptor alpha (48). Our results indicated that the expression of VWF gene can be upregulated by E2. There is no background information available about ADAMTS13 and its direct link with estradiol, in terms of either synthesis or release. Using reverse transcription polymerase chain reaction, amplification of ADAMTS13 mRNA from the liver cell line Hep3B following various stimuli, both inflammatory and immunosuppressive, suggested that any observed decrease in ADAMTS13 activity was not because of transcriptional regulation (49). More recently, however, a study using real-time PCR found that inflammatory cytokines that decreased ADAMTS13 activity, but not antigen, also decreased ADAMTS13 mRNA in both hepatic stellate cells and HUVEC (50). Our results indicated that the expression of ADAMTS13 gene can be upregulated by E2. Furthermore, the estrogen receptor antagonist ICI-182780 inhibited VWF and ADAMTS13 mRNA expression in response to E2 treatment, which suggests that E2 stimulates VWF and ADAMTS13 transcription through E2 receptor-mediated mechanisms.

Turner *et al.* (51) suggest that constitutive ADAMTS13 released from endothelial cells may contribute to the maintenance of cell surfaces free of hyperadhesive VWF multimeric strings. Also, it has been only described in HeLa cells cotransfected with ADAMTS13 and VWF that ADAMTS13 is able to degrade the VWF in the endoplasmic reticulum, considering that VWF has the enzyme cleavage site exposed (52, 53). In E2-treated HUVEC, our results showed no increase in intracellular and released VWF levels, although mRNA VWF levels increased. To determine whether the inhibition of ADAMTS13 contributes to the promotion of VWF protein synthesis, we utilized shRNA to knockdown the ADAMTS13 in HUVEC. Knockdown of ADAMTS13 resulted in a significant increase in VWF protein synthesis by E2-treated HUVEC when compared with control shRNA-treated cells. We conclude from these results that ADAMTS13 gene silencing helps to promote the synthesis of VWF from E2-treated HUVEC. Apparently, there were no qualitative defects in VWF of HUVEC transfected with pRS or shADAMTS13 and treated with E2 or vehicle, because all multimers were presented in the samples.

In summary, the present study indicates that E2 upregulates the expression of VWF and ADAMTS13 genes in HUVEC. In addition, E2 may also indirectly protect against overproduction of VWF through the increases in the production of ADAMTS13. These findings may provide useful information for the understanding of VWF/ADAMTS13 regulatory system in HRT-treated postmenopausal women.

Acknowledgements

We are grateful to SJ Torres and VA Zapata for their technical assistance and to AI Aureggi for the manuscript revision. This work was supported by CONICET, SECyT, the Alberto J. Roemmers Foundation, and the Rene Baron Foundation.

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