Thombospondin-1 Disrupts Estrogen-Induced Endothelial Cell Proliferation and Migration and Its Expression Is Suppressed by Estradiol

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Abstract

The natural hormone 17β-estradiol (17β-E2) is known to induce tumor angiogenesis in various target organs by activating positive regulators of angiogenesis. In this study, we show for the first time that in human umbilical vein endothelial cells (HUVECs), 17β-E2 transiently down-regulates the expression and secretion of a potent negative regulator of angiogenesis, thrombospondin-1 (TSP-1). This inhibitory effect of 17β-E2 is mediated through nongenomic estrogen receptor (ER)/mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) 1/2 and c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) signaling pathways, because this effect can be abolished by a pure ER antagonist (ICI 182,780) and inhibitors of downstream signaling proteins of MAPK signaling cascades, including MAPK kinase 1/2 and ERK1/2 inhibitor and JNK/SAPK inhibitor. To understand the functional role(s) of TSP-1 during estradiol-induced angiogenesis, we examined the growth and migration of endothelial cells in different experimental environments. Using a recombinant protein, we show that increments of TSP-1 protein concentration in culture medium significantly reduce the migration and proliferation of HUVECs stimulated by 17β-E2. Together, these studies suggest that TSP-1 can be considered an important negative factor in understanding the increased angiogenesis in response to estrogens.

Introduction

Angiogenesis is a multistep process, which involves cellular interactions with the extracellular matrix components, endothelial cell proliferation, migration, and differentiation into capillaries. Angiogenesis is a potential rate-limiting event in the development of tumor growth (1) and is regulated by a balance between a variety of proangiogenic proteins such as vascular endothelial growth factor (VEGF; Ref. 2), basic fibroblast growth factor (3), platelet-derived growth factor, pleiotrophin (4), and antiangiogenic proteins such as thrombospondin-1 (TSP-1; Ref. 5).

TSP-1 is the most studied and the most potent antiangiogenesis regulator, which is a homotrimeric extracellular matrix glycoprotein of 450 kDa. Numerous cell types, including platelets, endothelial cells, macrophages, fibroblasts, and vascular smooth muscle cells, secrete TSP-1. In endothelial cells, TSP-1 inhibits cell proliferation (6), motility (7), and morphogenesis (8). TSP-1 is an effector molecule for the tumor suppressor gene *p53*; loss of wild-type *p53* is associated with loss of TSP-1 expression (9) and increased transcription of VEGF and basic fibroblast growth factor binding protein expression (10). TSP-1 suppresses tumor growth in mice by inhibiting the activity of matrix metalloproteinase-9, which mediates VEGF release from the extracellular sources (11). In endothelial cells, inhibition of angiogenesis *in vivo* and *in vitro* and induction of apoptosis by TSP-1 require involvement of a transmembrane receptor CD36 and a sequential activation of Src family kinase $p59^{fyn}$, caspase-3-like proteases, and p38 mitogen-activated protein kinases (MAPK; Ref. 12). In a recent study, repression of TSP-1 via sequential activation of Ras, phosphatidylinositol-3-kinase (PI3K), Rho, ROCK, and Myc phosphorylation has been described as essential for mammary and kidney tumor angiogenesis (13).

Estrogens have been considered as potent stimulators of angiogenesis and this effect is mediated through the estrogen receptor (ER; Refs. 14, 15). Previously, our laboratory (16, 17) and others (18–20) have demonstrated stimulation of the proangiogenic factor VEGF-A in rat and human endocrine-related tumors induced by a natural estrogen, 17β-estradiol (17β-E2). In a recent study, we have described the ER-α-dependent biphasic up-regulation of VEGF-A mRNA in breast tumor cells (21). The concept of "the balance hypothesis for the angiogenic switch" (22) has proposed that the modulation of antiangiogenic factors is also a crucial event during tumor angiogenesis. However, whether estrogen modulates the antiangiogenic factors during activation of the angiogenic switch has remained elusive. In the present study, we therefore have examined modulation of expression and function of the antiangiogenic factor TSP-1 in human umbilical vein endothelial cells (HUVECs) that are exposed to 17β-E2. We demonstrate that down-regulation of TSP-1 at the transcriptional and translational (cellular and secretory) levels is a function of

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ER-α in HUVEC treated with estrogen. We also provide evidence that down-regulation of TSP-1 by 17β-E2 is nongenomic and mediated through MAPK signaling pathways. Further, we provide *in vitro* evidence that cellular migrations and proliferation of endothelial cells induced by 17β-E2 are repressed by exposure to TSP-1.

Results

17β-E2 Differentially Modulates the TSP-1 mRNA and Protein Levels in Endothelial Cells in a Time-Dependent Manner

To elucidate the effect of 17β-E2 on TSP-1 expression at mRNA and protein levels (cellular and secretory), HUVECs were treated with 17β-E2 (10 nM) for different time periods in serum-free and hormone-depleted culture conditions. Levels of TSP-1 mRNA and protein (cellular and secretory) in 17β-E2-treated or vehicle-treated endothelial cells were measured using nonradioactive Northern blot and immuno-Western blot analyses, respectively. The Northern blot analyses indicate that TSP-1 mRNA expression was significantly down-regulated after exposure to 17β-E2 for 2 h, and this impact was transient, and after 6 h of 17β-E2 exposure, the mRNA levels were gradually increased to the basal level (Fig. 1, A and B). Similar to mRNA, TSP-1 protein levels in cell lysates were significantly decreased by ~4-fold as compared with vehicle-treated controls after 2 h of 17β-E2 exposure, and a gradual return of protein to the basal level was observed after exposure to estradiol for 6–24 h (Fig. 1, C and D). Moreover, secretion of TSP-1 protein was similarly affected by estrogen as it was observed in cell lysates. When cells were exposed to estrogen for 2 h, secreted levels of TSP-1 were significantly reduced as compared with the basal level. However, the secreted level of TSP-1 was increased almost to the basal level when the cells were exposed to estrogen for ≥6 h (Fig. 1E). These findings together suggest that acute treatment with 17β-E2 suppresses expression of TSP-1 at both transcriptional and the translational levels, but prolonged exposure to estrogen produces a minimal or no effect on mRNA expression and protein synthesis but might cause an increase in stability of this mRNA and protein, which has not yet been confirmed.

Down-Regulation of TSP-1 by 17β-E2 Is Mediated Through an ER in HUVEC

To clarify whether the suppression of TSP-1 expression by 17β-E2 was mediated by an ER, HUVECs were exposed to 17β-E2 along with the pure anti-estrogen ICI 182,780 for 2 h, and TSP-1 protein levels were quantified by immuno-Western blot analyses. As shown in Fig. 2, 17β-E2 alone suppressed TSP-1 synthesis. However, this suppression was abolished by concomitant exposure to estrogen and the ER antagonist ICI 182,780. The anti-estrogen alone had no effect on expression of TSP-1 protein. These results therefore indicate that the 17β-E2-induced reduction in TSP-1 protein is ER dependent in human endothelial cells.

17β-E2-Induced Suppression of TSP-1 Expression at mRNA and Protein Levels Are Mediated Through Nongenomic MAPK and c-Jun NH_2-Terminal Signaling Pathways

The diverse actions of estrogens are arbitrated via either a classical ER-mediated genomic pathway or plasma membrane estrogen-receptor-mediated nongenomic pathway through the activation of PI3K and MAPK signaling pathways depending on the target tissues (23–31). In this study, we were interested in investigating the status of ER-α in HUVECs and the signaling pathways involved in the 17β-E2-induced suppression of TSP-1 expression in HUVECs. To determine the status of plasma membrane and nuclear ER-α in HUVECs, confocal immunofluorescent and immuno-Western blot analyses were

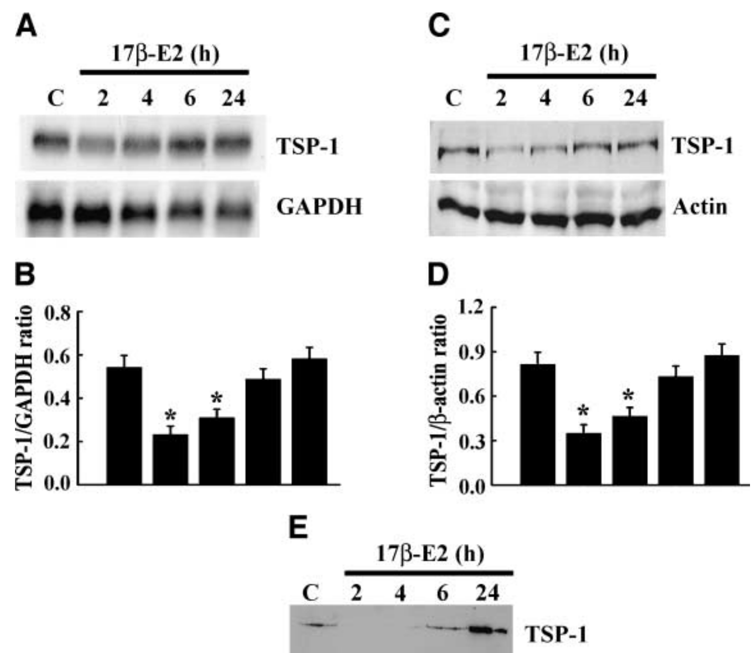

FIGURE 1. Time-dependent effects of 17β-E2 on TSP-1 mRNA and protein expression in HUVECs. Human endothelial cells were treated with 17β-E2 (10 nM) for indicated periods in serum-free and hormone-depleted culture conditions. Total RNA and proteins were extracted and assayed for TSP-1 expression by Northern and Western blot analyses, respectively. Ethanol vehicle (0.1%)-treated cultures were treated as the control. **A.** Single representative Northern blot analysis showing TSP-1 mRNA expression in 17β-E2-treated HUVECs at indicated periods. GAPDH mRNA expression is shown as the loading control. **B.** Normalized values indicate the ratios of TSP-1 and GAPDH mRNA expressions. **C.** Single representative protein blot illustrating the time course effects of 17β-E2 on the TSP-1 protein synthesis in HUVECs, and the expression level of actin in the respective sample is shown as the loading control. **D.** Normalized values indicate the ratios of TSP-1 and β-actin protein expressions. *Columns,* mean from three separate experiments; *bars,* SD. *, $P < 0.001$ *versus* control (Student's *t* test). **E.** Single representative immunoblot showing the expression levels of secreted TSP-1 in the culture medium of 17β-E2 (10 nM)-treated HUVECs at indicated time periods.

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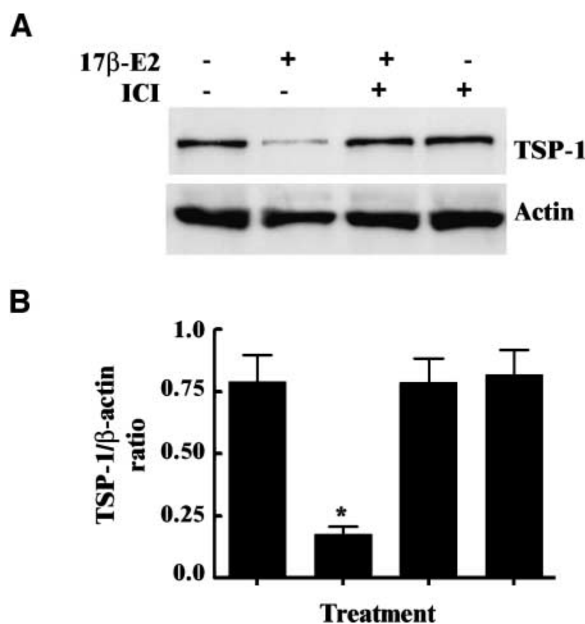

FIGURE 2. Effect of anti-estrogen ICI 182,780 on 17β-E2-induced down-regulation of TSP-1 protein expression in HUVECs. Cells were treated with 17β-E2 (10 nM) for 2 h in the presence or absence of ICI 182,780 (1 μM). Cellular proteins were extracted and analyzed for TSP-1 and β-actin protein levels by Western blot assay. In each lane, 30 μg of proteins were loaded for electrophoresis, and to confirm equal loading, the same membrane was reprobed with anti-actin mAb and detected with ECL detection kit. **A.** Single representative blot shows the TSP-1 and β-actin protein levels in 17β-E2-treated and untreated HUVECs. **B.** Normalized values indicate the ratios of TSP-1 and β-actin protein in treated and untreated control HUVECs. *Columns,* mean from three separate experiments; *bars,* SD. *, $P < 0.001$ *versus* control (Student's *t* test).

performed. As shown in Fig. 3, ER-α protein levels were identified by both confocal immunofluorescence and immuno-Western blot in the plasma and nuclear membranes. However, the expression level of ER-α was very high in the nuclear membrane as compared with plasma membrane. The result of this finding is identical with the previous studies (24, 32), and it indicates that HUVECs are having both membrane and nuclear ER-α. Next, we determined whether estrogen-induced suppression of TSP-1 expression is mediated through plasma membrane ER-α-mediated nongenomic pathways. For this study, cells were treated with wortmannin (100 nM), a PI3K inhibitor, or U0126 (10 μM), a MAPK kinase (MEK)/extracellular-regulated kinase (ERK) inhibitor, for 1 h prior to a 2 h exposure to 17β-E2 (10 nM). As shown in Figs. 4 and 5, wortmannin was unable to block the 17β-E2-induced suppression of TSP-1 mRNA or the cellular and secretory expression of TSP-1 protein. In contrast, U0126 significantly blocked the 17β-E2-induced inhibition of TSP-1 mRNA and protein (cellular and secretory) expression. However, the effect of U0126 on 17β-E2-induced TSP-1 mRNA expression was not as prominent as its effect on protein expression. These results suggest that an involvement of a MAPK pathway, including MEK1/2-ERK1/2, is required for 17β-E2-induced down-regulation of TSP-1 expression and secretion in HUVECs. For further confirmation of the estrogen action on MEK1/2 signaling, phosphorylation of the downstream signaling proteins, ERK1/2, was assessed. 17β-E2 caused an increase in phosphorylation of ERK1/2 after 1 h exposure, and it sustained for several hours (Fig. 6). However, total protein levels of ERK1/2 were unaffected by estrogen treatment. Next, we wanted to know whether additional downstream signaling enzymes in the MAPK cascade were activated by estrogen in HUVECs during the suppression of TSP-1. To test this, cells were treated with 17β-E2 for different duration. Lysates from treated and untreated cells were analyzed for the activities and expressions of c-Jun NH_2-terminal kinase (JNK; p54/p46) and p38 protein kinase was assessed by immuno-Western blot analyses using specific antibodies (Fig. 6). Gradual induction of JNK activity, as reflected by increased phosphorylation, by 17β-E2 was identified with a peak at 15 min (Fig. 6). By 120 min, JNK activities were reduced to basal levels. In contrast, Western blot analyses showed that the total protein level of JNK was unaltered by the estrogen treatment. Concentration and activity of p38 kinase were unaffected by brief exposure to estrogen (Fig. 6). The results of these studies caused us to determine whether JNK activation is associated with estrogen-induced suppression of TSP-1. Endothelial cells were exposed to a JNK inhibitor, SP600125, prior to the estrogen treatment, and TSP-1 levels were determined.

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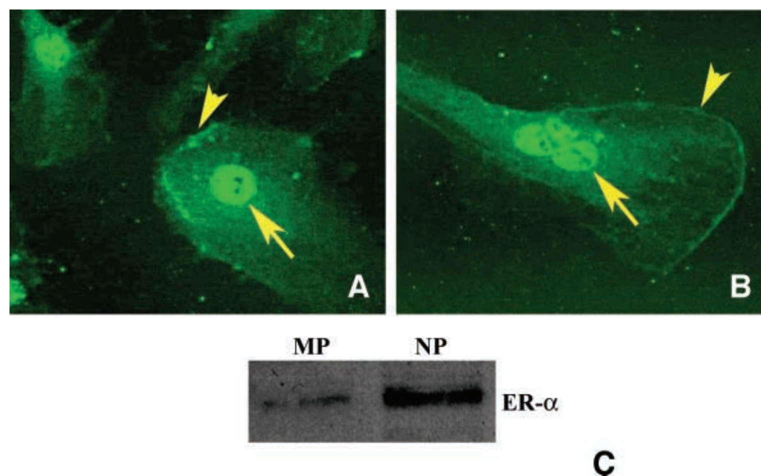

FIGURE 3. ER-α localization in the nuclear and plasma membranes of endothelial cells (**A** and **B**). Confocal immunofluorescence microscopy shows ER-α in the plasma membrane and nuclear membrane of paraformaldehyde-fixed HUVECs. **C.** Immuno-Western blot analysis of ER-α at 66 kDa in HUVEC nuclear protein (*NP*) as well as in plasma membrane protein (*MP*) extracts. *Arrows,* ER-α-immunopositive nucleus; *arrowheads,* immunopositive edges of plasma membrane. Results are representative of three independent cultures.

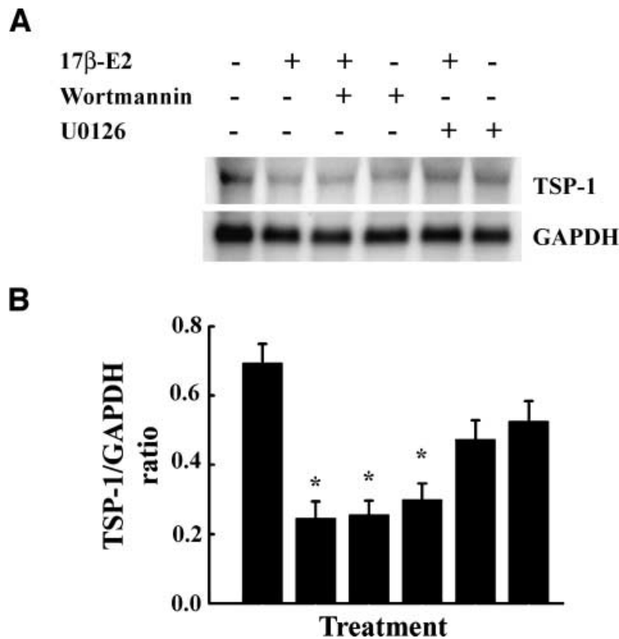

FIGURE 4. Effect of PI3K and MEK inhibitor on 17β-E2-induced down-regulation of TSP-1 mRNA expression in HUVECs. Cells were treated with wortmannin (100 nM), a PI3K inhibitor, and U0126 (10 μM), a MEK inhibitor, for 1 h followed by treatment with 17β-E2 (10 nM) for 2 h. DMSO (0.1%)-treated cultures were considered as the untreated control. Total RNA was extracted from the control and treated cultures by Trizol extraction method and TSP-1 mRNA expressions were evaluated by nonradioactive Northern blot analyses using TSP-1-specific cDNA probe. **A.** Single representative Northern blot shows 17β-E2-induced TSP-1 mRNA expression in HUVECs in the presence or absence of wortmannin or U0126. **B.** Arbitrary values indicate the ratio of TSP-1 and GAPDH mRNA expressions derived from densitometric analyses in each experiment. *Columns,* mean from three sets of experiments; *bars,* SD. *, $P < 0.001$ *versus* control (Student's *t* test).

Expectedly, acute exposure of 17β-E2 suppresses the TSP-1 synthesis in endothelial cells. However, blocking the increase in JNK activity by JNK inhibitor removed the estradiol-induced suppression of TSP-1 protein synthesis (Fig. 7). Taken together, these results indicate that in addition to ERK1/2, JNK is also involved in the regulatory process of TSP-1 synthesis. Similar effect was observed at the secretory level (data not shown).

TSP-1 Inhibits the 17β-E2-Induced Proliferation and Migration of HUVECs

For neovascularization, the proliferation of vascular endothelial cells is necessary. Previously, it has been shown that estradiol stimulates endothelial cell proliferation and migration (33), and these stimulations are mediated by a classical ER, which is abundantly expressed in endothelial cells (34, 35). On the other hand, in endothelial cells, the antiangiogenic factor TSP-1 has been shown to inhibit cellular proliferation (6) and migration (36). In our study, we investigated the effects of increased concentration of TSP-1 on 17β-E2-induced endothelial proliferation and migration. In the cell proliferation study, we measured BrdUrd incorporation as an index of cellular proliferation. As expected, 17β-E2 significantly enhanced endothelial cell proliferation by 45% over the untreated control (Fig. 8). When cells were exposed concomitantly to recombinant TSP-1 protein and estradiol, the action of estrogen on cellular proliferation was significantly reduced (Fig. 8). TSP-1 added to the culture reduced proliferation by 28% when compared with the untreated controls.

Subsequently, we investigated whether TSP-1 interferes with estradiol action on endothelial cell migration. In agreement with previous studies (33), we found that the migration of endothelial cells, as chemotaxis of attached cells through 8 μm pores in the Boyden chamber, can be enhanced after 24 h of 17β-E2 treatment. Migration was increased by 80% in estrogen-treated cells when compared with that of untreated control cells (Fig. 9). When cells were incubated with an excess of TSP-1 (10 μg/ml) in the presence 17β-E2, the increase in migration was significantly reduced by 58%. TSP-1 had only a

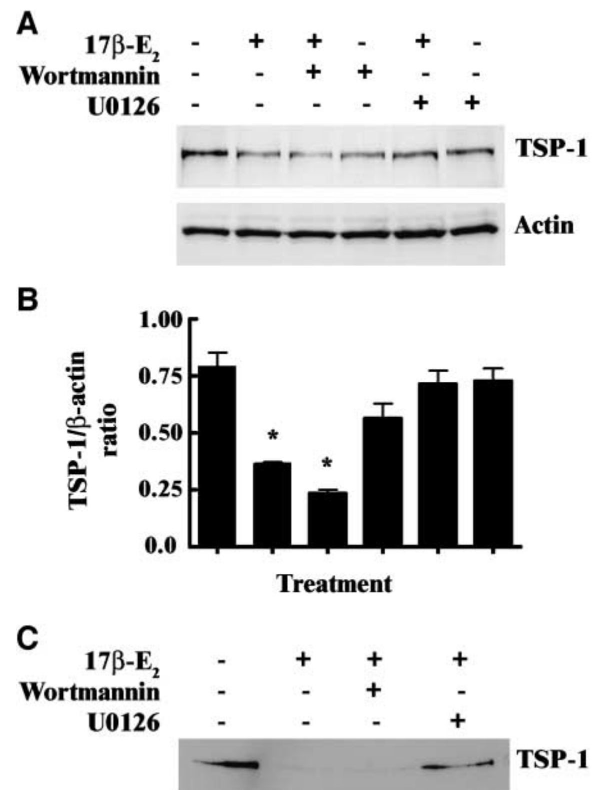

FIGURE 5. Effect of PI3K and MEK inhibitor on 17β-E2-induced down-regulation of TSP-1 protein expression in HUVECs. Cells were pretreated with wortmannin (100 nM), a PI3K inhibitor, or U0126 (10 μM), a MEK inhibitor, 1 h prior to treatment with 17β-E2 (10 nM) for 2 h in serum-free and hormone-depleted culture conditions. Total cell lysates were prepared and 30 μg/lane protein were loaded for electrophoresis. Nitrocellulose blots were probed with anti-TSP-1 mAb and the immunoreactions were detected with ECL kit. Blots were stripped and reprobed with anti-actin mAb to confirm the equal loading in each lane. **A.** Single representative blot shows TSP-1 protein levels in 17β-E2-treated HUVECs in the presence or absence of inhibitors as indicated. **B.** Normalized values indicate the ratios of TSP-1 and β-actin protein in treated and untreated control HUVECs. **C.** Single representative immunoblot shows the expression of secreted TSP-1 protein in the culture medium of untreated and treated HUVECs as indicated. *Columns,* mean from three separate experiments; *bars,* SD. *, $P < 0.001$ *versus* control (Student's *t* test).

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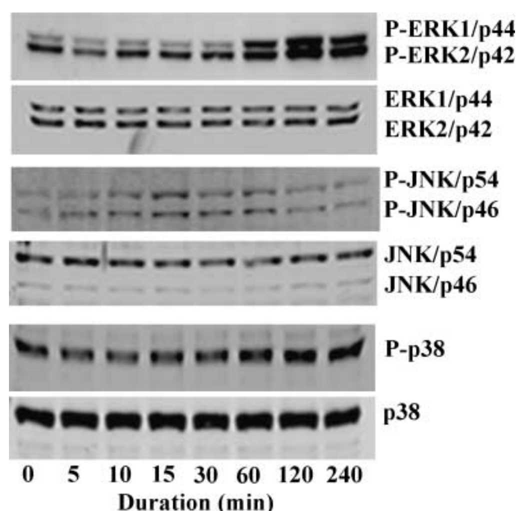

FIGURE 6. Effect of 17β-E2 on activation of the proteins of the MAPK signaling pathways. HUVECs were treated with 17β-E2 (10 nM) for the indicated periods of time; 15 μg/lane of total cell lysate protein were subjected to electrophoresis. Blots were reacted with the antibodies to phosphorylated and nonphosphorylated forms of ERK1/2, JNK (p54/p46), and p38 as indicated and detected with ECL detection kit.

slight lowered effect on migration in cells not exposed to estrogen (Fig. 9). These findings together clearly indicate that TSP-1 can inhibit 17β-E2-induced proliferation and migration of endothelial cells *in vitro*.

Discussion

Angiogenesis or neovascularization is a fundamental step in tumor progression and can happen at different stages of tumor development as a result of an ultimate imbalance in proangiogenic and antiangiogenic factors (13, 37, 38). Stimulatory effects of estrogens on tumorigenesis in rodents have been attributed to its regulation of angiogenesis by activating proangiogenic factors such as VEGF-A (15, 16, 18, 39). Inhibition of protein synthesis by the antiangiogenic molecule 2-methoxyestradiol may account for much of the latter's ability to inhibit estrogen-induced tumor growth and tumor angiogenesis (40). Subsequent studies by us and others have shown that VEGF-A expression can be up-regulated by estrogen in human breast tumor epithelial cell lines and that transcriptional regulation of this gene is mediated through a classical ER-α (19, 21, 41). Collectively, these studies indicate that steroid actions are elicited in both estrogen-responsive endothelial and breast tumor epithelial cells by positive regulators of angiogenesis.

One important issue concerning understanding estrogen-induced angiogenesis is whether regulation of antiangiogenic factors is involved in the modulation of endothelial cell proliferation, migration, and angiogenesis. We found that 17β-E2, a natural estrogen, is capable of transiently suppressing expression and secretion of an angiogenic inhibitor, TSP-1, in human endothelial cells. This provides evidence for the involvement of negative regulators during the induction of angiogenesis by estrogen. Ironically, prolonged exposure to 17β-E2 did not sustain the inhibition of TSP-1 expression and secretion in endothelial cells. Both mRNA and protein of TSP-1 increased to the basal levels in estrogenized endothelial cells by unknown mechanisms. The functional relevance of these changes in TSP-1 mRNA and protein levels after prolonged exposure of estrogen remain elusive. Presumably, TSP-1 protein is required for a later wave of angiogenesis induced by estrogen. This anticipation is likely to be coherent because TSP-1 is a multifunctional protein. For example, mice lacking TSP-1 developed hyperplasia and pneumonia in the lung with multifocal inflammatory sites (42), and up-regulation of TSP-1 is strongly associated with inflammation and several pathophysiological conditions (43, 44). In addition, TSP-1 promotes the proliferation of vascular smooth muscle cells (45), invasive properties of breast tumor cells of breast (46, 47), and cellular adhesion (48, 49). Moreover, both proliferative and inhibitory effects of TSP-1 have been documented in bovine endothelial cells and these biphasic effects are dependent on time and dosage (47).

In general, the physiological or pathophysiological functions of estrogen action are mediated via a receptor, either ER-α or ER-β (50). Several genes have been shown to be up-regulated by estrogen and their activation is mainly mediated through intracellular ERs (15, 21, 51) that interact with a specific response element (ERE) on DNA or coregulators of the general

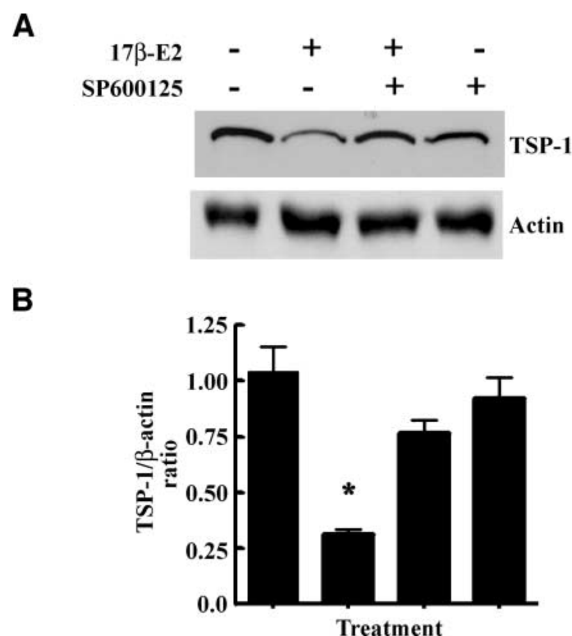

FIGURE 7. Effect of JNK inhibitor SP600125 on 17β-E2-induced down-regulation of TSP-1 protein expression in HUVECs. Cells were pretreated with SP600125 (20 μM), a specific JNK inhibitor, 1 h prior to treatment with 17β-E2 (10 nM) for 2 h in serum-free and hormone-depleted culture conditions. DMSO (0.2%)-treated cultures were considered as the untreated control. Total cell lysate proteins were prepared, and 30 μg/lane protein were loaded for electrophoresis. Nitrocellulose blots were probed with anti-TSP-1 mAb, and the immunoreactions were detected with ECL kit. Blots were stripped and reprobed with anti-actin mAb to confirm the equal loading in each lane. **A.** Single representative blot shows TSP-1 protein levels in 17β-E2-treated HUVECs in the presence or absence of JNK inhibitor (SP600125) as indicated. **B.** Normalized values indicate the ratios of TSP-1 and β-actin protein in treated and untreated control HUVECs. *Columns,* mean from three separate experiments; *bars,* SD. *, $P < 0.001$ *versus* control (Student's *t* test).

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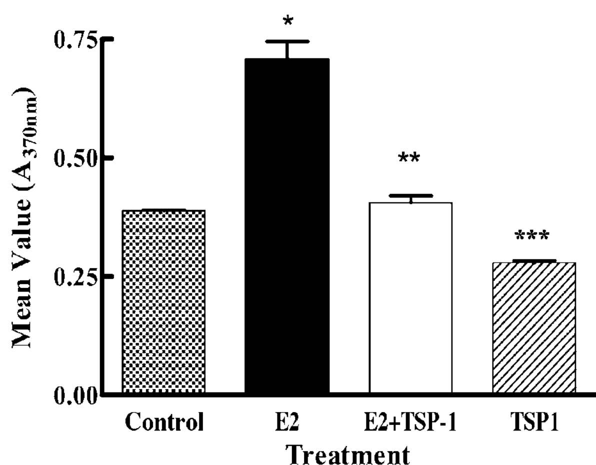

FIGURE 8. Effect of TSP-1 on 17β-E2-induced cell proliferation in human endothelial cells. Equal numbers of HUVECs were plated in each well of 96-well plate, and after attachment, the cells were serum starved and treated with recombinant TSP-1 (10 μg/ml) in the presence or absence of 17β-E2 (10 nM) for 24 h. Ethanol (0.1%)-treated cultures were considered as the control. Cellular proliferations in the control and the treated cultures were measured by colorimetric immunoassay based on BrdUrd incorporation into the cellular DNA. *E2,* 17β-E2 (10 nM). All treated and untreated control cultures were run in quadruplicates in each set of experiments. *Columns,* mean absorbance ($A_{370\ nm}$) from three separate experiments; *bars,* SD. *, $P < 0.005$ *versus* control; **, $P < 0.01$ *versus* 17β-E2-treated cells; ***, $P < 0.05$ *versus* control (Student's *t* test).

transcription machinery (51). In the present study, we determined whether analogous signaling pathways were involved in estrogen-induced suppression of TSP-1 protein synthesis. Human endothelial cells were exposed to 17β-E2 in the presence or absence of an anti-estrogen and TSP-1 protein levels were determined. The results showed that 17β-E2-induced suppression of TSP-1 protein expression could be abolished byanti-estrogen, suggesting that an ER was required for this event in human endothelial cells.

The next important issue regarding estrogen action on TSP-1 is how does the estrogen-ER signaling complex contribute. Ample evidence has established that in addition to a direct transcriptional activation effect (genomic effect), steroid hormones, including estrogen, can act via nongenomic signaling pathways (52–55). These effects are presumably mediated through a plasma membrane ER (54–59), which interacts with multiple signaling pathways including cyclic AMP (60), G-protein signaling (58), c-Src/ERK (61), PI3K, and MAPK-ERK (23–28, 59) pathways. In the present study, we employed a variety of approaches in an attempt to identify the intermediate signaling proteins associated with estrogen action on TSP-1 regulation. These included experiments demonstrating that the 17β-E2-ER complex modulates the expression of TSP-1 in endothelial cells through nongenomic pathways by activating MAPK-MEK1/2-ERK1/2 and JNK signaling pathways. To our knowledge, this is the first evidence that an estrogen-ER protein complex interacts with two different MAPK modules to down-regulate the antiangiogenic factor, TSP-1.

Despite some controversy, different studies have shown that the functional role of TSP-1 in angiogenesis is biphasic having both inhibitory and stimulatory effects depending on the environment (47). Here, we found that 17β-E2, an established inducer of angiogenesis, transiently suppressed the expression and secretion of TSP-1 in endothelial cells. Although the functional relevance of brief down-regulation of TSP-1 by estrogen is unknown, the preventive effects of exogenously added TSP-1 (10 μg/ml) on both proliferation and migration of HUVECs that was induced by 17β-E2 stimulation suggest that immediate silencing of TSP-1 activity by estradiol may contribute to the initial wave of estrogen-induced endothelial cell proliferation and migration that result in angiogenesis. Moreover, late induction of TSP-1 by estradiol or supplementation with exogenous TSP-1 after the initial effect with estradiol, which is about 2 h after the treatment, is unable to impede the effect of estrogen (data not included) further support the hypothesis.

In conclusion, the data presented here demonstrated two important findings: (*1*) 17β-E2 is capable of suppressing an antiangiogenic factor, TSP-1, transiently in endothelial cells through nongenomic ER-MAPK pathways and (*2*) the increased level of TSP-1 in culture environment impedes the estrogenic action on the proliferation and migration of human endothelial cells. Together, these findings argue for a negative role of TSP-1 in estrogen-induced regulation of cellular proliferation and migration. However, further studies are warrant to determine the significance of transient suppression of TSP-1 by 17β-E2 in estrogen-induced endothelial cell proliferation, migration, and angiogenesis.

Materials and Methods

Reagents

17β-E2, collagen, wortmannin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and anti-actin monoclonal antibody (mAb) were purchased from Sigma Chemical Co.

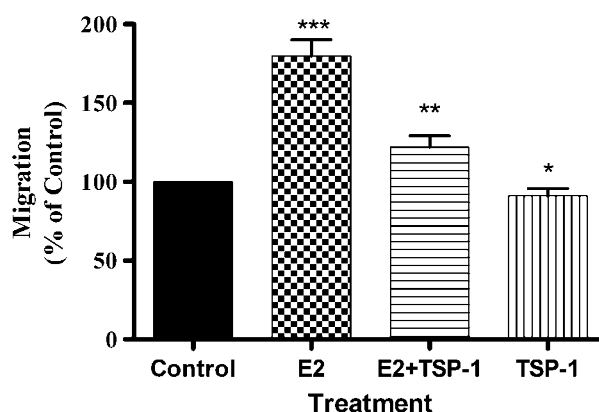

FIGURE 9. Effect of TSP-1 on the 17β-E2-induced migration of human endothelial cells. Equal numbers of serum-starved cells were plated on the filter (8 μm pore size) of cell culture insert and allowed to attach and spread for 2 h. To the lower chamber, 17β-E2 (10 nM) was added in the presence or absence of TSP-1 protein (10 μg/ml). Cellular migrations were scored at 20 random fields on the opposite side of the filter after 24 h of treatment. Ethanol (0.1%) was added to the lower chambers of the control cultures. *E2,* 17β-E2 (10 nM). All treated and untreated control cultures were run in quadruplicates in each set of experiments. *Columns,* percentage of control from three separate sets of experiments; *bars,* SD. *, $P < 0.05$ *versus* control; **, $P < 0.0065$ *versus* 17β-E2-treated cells; ***, $P < 0.0002$ *versus* control (Student's *t* test).

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(St. Louis, MO). Anti-estrogen (ICI 182,780), U0126, and recombinant human TSP gp140 were purchased from Tocris (Ellisville, MO), Promega (Madison, WI), and Protein Sciences Corp. (Meriden, CT), respectively. Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit and anti-ER antibody were purchased from Pierce Chemical Co. (Rockford, IL) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibodies to the phosphorylated and nonphosphorylated forms of the MAPK family [*i.e.*, p44/42 MAPK (ERK1 and ERK2), JNK/stress-activated protein kinase, and p38 MAPK] were purchased from Cell Signaling Technology (Beverly, MA). Anti-TSP-1 mAb (clone A6.1) and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from NeoMarkers (Freemont, CA) and Amersham Pharmacia Biotech, Inc. (Piscataway, NJ), respectively.

Cell Culture Conditions and Treatments

HUVECs were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in endothelial cell basal medium-2 supplemented with EGM 2-MV Single-Quots (Clonetics/BioWhittaker, Inc., Charlotte, NC).

The effects of 17β-E2 treatments on TSP-1 expression and secretion were studied under the following culture conditions: 70–80% confluent cultures were washed and serum starved in phenol red-free medium (mammary epithelium basal medium; Clonetics/BioWhittaker) for 1 h. Thereafter, the cells were treated with 17β-E2 (10 nM) for the indicated time periods. The effect of anti-estrogen (ICI 182,780) was studied by treating the cells at a concentration of 1 μM in 0.1% ethanol; control cultures received only the same volume of ethanol.

For MEK, PI3K, and JNK inhibitor assays, the serum-starved cells were pretreated with inhibitors 1 h before exposure to estradiol for 2 h. To determine the secretory level of TSP-1 protein, culture medium of untreated and treated cultures were collected from different experiments as indicated. Culture medium was spun at 10,000 × *g* for 10 min and stored at −70°C until used for Western blot analysis.

RNA Extraction, Nonradioactive cDNA Probe Preparation, and Northern Blot Analyses

Total cellular RNA was isolated by Trizol (Life Technologies, Inc., Grand Island, NY), separated on 1% agarose gel containing formaldehyde (2.2 M) in 4-morpholinepropanesulfonic acid buffer, and blotted on supercharge nylon membranes (Schleicher & Schuell, Keene, NH). Blots were probed with a nonradioactive digoxigenin (DIG)-labeled human TSP-1-specific cDNA probe. Hybridized membranes were incubated with anti-DIG conjugated with alkaline phosphatase (1:20,000 dilutions) for 30 min at room temperature. Finally, the membranes were incubated with CSPD and exposed to autoradiography with Hyper film (Amersham Pharmacia Biotech). To check for even loading of RNA in each lane and normalization, the blotted membranes were stripped in 0.05× SSC, 0.1% SDS buffer and reprobed with DIG-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cDNA probe.

cDNA and DIG-labeled nonradioactive probes were synthesized following the method described earlier (62). The sequences of primers are as follows: TSP-1: 5′-AAG GTG TTG TAC ATA GCA T-3′ (forward) and 5′-CCA ATG GCA ATG AGA AAA TA-3′ (reverse); GAPDH: 5′-ATG AGA AGT ATG ACA ACA GCC-3′ (forward) and 5′-TGA GTC CTT CCA CGA TAC C-3′ (reverse).

Subcellular Fractions and Immuno-Western Blot Assay

Subcellular fractions including highly purified plasma membrane and nuclear fractions and total cell lysate were extracted from HUVECs using the protocols provided by the manufacturer (Pierce Chemical) and the method described previously (16). Briefly, the cells were lysed and membrane proteins were solubilized in detergents (supplied in Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit) in two subsequent steps, and the detergent-soluble membrane fraction was separated from the cytosolic/nuclear protein-containing hydrophilic fraction through phase partitioning. Purity of plasma membrane and exclusion of other cellular fractions were confirmed by measuring plasma membrane alkaline phosphatase and reduced $NADP^+$ cytochrome *c* reductase (endoplasmic reticulum) activity (63). For the extraction of total protein, cells were washed with chilled PBS and lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS] containing PMSF (1 mM), leupeptin (1 μM), and aprotinin (1 μM) and incubated on ice for 20 min with occasional vortexing. The lysates were spun at 14,000 × *g* for 1 h at 4°C and the supernatants were collected and stored at −70°C. Protein concentrations were measured using the Coomassie blue reagent assay (Pierce Chemical). Equal volume of untreated and treated culture medium was run in 7.5% SDS-PAGE to determine the levels of secretory TSP-1 protein using Western immunoblot assay. For assessment of MAPK family proteins, cell lysates were prepared in 10 mM Tris-HCl (pH 6.8), 0.4 mM EDTA, 1% SDS, 10 mM sodium fluoride, 0.4 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin.

Equal amounts of cell lysate proteins were resolved in 7.5% SDS-PAGE, transferred onto nitrocellulose membrane, and reacted with anti-TSP-1 mAb (0.4 μg/ml) at 4°C overnight. Equal amounts of protein loaded in each lane were determined by incubating the stripped membrane with anti-actin mAb (0.2 μg/ml) for 1 h at room temperature. The antigen-antibody reactions were probed with horseradish peroxidase-conjugated goat anti-mouse IgG. Immunoreactions were detected by ECL reagent kit.

Confocal Microscopy

Immunofluorescence assay was carried out as described earlier (64). Briefly, HUVECs were plated and grown on glass coverslips until 50–60% confluence was achieved. Cells were fixed in 2% paraformaldehyde for 20 min at 37°C and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After blocking the nonspecific sites with blocking solution (Histostain kit; Zymed, San Francisco, CA), the cells were incubated with rabbit anti-ER-α (1 μg/ml) overnight at 4°C followed by incubation with anti-rabbit IgG fluorescent conjugate (Alexa Fluor 488; Molecular Probes, Eugene, OR).

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Finally, cells were washed with 1× PBS and mounted in PBS glycerin. Immunofluorescent stained cells were visualized using a Nikon (Japan) Eclipse TE-300 microscope fitted with MicroRadiance Plus confocal system (Bio-Rad, Hercules, CA) and images were analyzed by Laser Sharp 2000 software (Bio-Rad). Cells incubated without the primary antibody were treated as the negative controls.

Cell Proliferation Assay

HUVECs were plated in 96-well plates at a density of 10,000 cells/well and incubated at 37°C with 5% CO_2 overnight for attachment. In each experimental set, cells were plated in quadruplicates and were washed and incubated for 1 h prior to treatments in serum-free mammary epithelium basal medium devoid of phenol red. Cells were treated with 17β-E2 (10 nM) in the presence or absence of recombinant TSP-1 (10 μg/ml) for 24 h. Cellular proliferations were measured by colorimetric immunoassay based on BrdUrd incorporation into the cellular DNA by following the instructions recommended by the vendor (Cell Proliferation ELISA, BrdUrd Kit; Roche Molecular Biochemical, Indianapolis, IN). Briefly, cells were pulsed with BrdUrd labeling reagent for 6 h followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1:100 dilution of anti-BrdUrd-POD for 1 h at room temperature. Finally, the immunoreaction was detected by adding the substrate solution and the color developed was read at 370 nm with the help of SOFTmaxPRO. The concentration of recombinant TSP-1 protein used in this study was the same as that described for previous studies (49, 65, 66).

Cell Migration Assay

Cell migration assay was performed following methods described earlier (11) with some modifications. Semiconfluent HUVECs were serum starved for 2 h. Cells were dislodged by trypsin and replated at a concentration of 5×10^4 cells/well on the surface of an 8 μm filter of cell culture insert (Becton Dickinson, Franklin Lakes, NJ). The opposite side of the filter was coated with collagen (1 mg/ml). Cells were allowed to attach and spread for 2 h. To the lower chamber, 17β-E2 (10 nM) in the presence or absence of TSP-1 protein (10 μg/ml) was added. Cell migration was allowed to proceed for 24 h at 37°C in the presence of 5% CO_2 and the cells on the upper surface of the filter were scrapped by cotton swab. The cells on the bottom surface were fixed in methanol for 5 min at room temperature and then stained with Harris hematoxylin for 10 min. Washed and dried filters were cut from the culture inserts and mounted on glass slides with glycerol. Cells were counted in 20 random fields under 20× objective.

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