



Estrogen receptor β -dependent Notch1 activation protects vascular endothelium against tumor necrosis factor α (TNF α)-induced apoptosis

Received for publication, April 8, 2017, and in revised form, September 5, 2017. Published, Papers in Press, September 11, 2017, DOI 10.1074/jbc.M117.790121

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Edited by Joel Gottesfeld

Unlike age-matched men, premenopausal women benefit from cardiovascular protection. Estrogens protect against apoptosis of endothelial cells (ECs), one of the hallmarks of endothelial dysfunction leading to cardiovascular disorders, but the underlying molecular mechanisms remain poorly understood. The inflammatory cytokine TNF α causes EC apoptosis while dysregulating the Notch pathway, a major contributor to EC survival. We have previously reported that 17 β -estradiol (E2) treatment activates Notch signaling in ECs. Here, we sought to assess whether in TNF α -induced inflammation Notch is involved in E2-mediated protection of the endothelium. We treated human umbilical vein endothelial cells (HUVECs) with E2, TNF α , or both and found that E2 counteracts TNF α -induced apoptosis. When Notch1 was inhibited, this E2-mediated protection was not observed, whereas ectopic overexpression of Notch1 diminished TNF α -induced apoptosis. Moreover, TNF α reduced the levels of active Notch1 protein, which were partially restored by E2 treatment. Moreover, siRNA-mediated knockdown of estrogen receptor β (ER β), but not ER α , abolished the effect of E2 on apoptosis. Additionally, the E2-mediated regulation of the levels of active Notch1 was abrogated after silencing ER β . In summary, our results indicate that E2 requires active Notch1 through a mechanism involving ER β to protect the endothelium in TNF α -induced inflammation. These findings could be relevant for assessing the efficacy and applicability of menopausal hormone treatment, because they may indicate that in women with impaired Notch signaling, hormone therapy might not effectively protect the endothelium.

Despite many efforts to improve primary prevention and treatment, cardiovascular diseases remain the leading cause of death for women worldwide (1). Premenopausal women have lower risk of developing cardiovascular diseases when compared with age-matched men but this difference disappears when considering post-menopausal women (2). The different cardiovascular risk among pre- and post-menopausal women has been largely ascribed to the protective actions of estrogens against endothelial dysfunction, which represents the first step toward onset and progression of atherosclerosis and other cardiovascular diseases (3). Apoptosis of endothelial cells is one of the hallmarks of endothelial dysfunction, which also include reduced nitric oxide (NO) production, increased endothelium permeability, and adhesion of inflammatory cells (4). Estrogens reduce endothelial cell apoptosis caused by inflammatory cytokines (5, 6), oxidized low-density lipoproteins (5), hypoxia (7), or oxidative injury (8). The estrogen receptors α (ER α)² and β (ER β) (9) are both expressed in endothelial cells (10–13), but there are still conflicting findings on which type of ER mediates the protective action of 17 β -estradiol (E2) in the endothelium (14–17). Similarly, the molecular mechanisms involved in the estrogen-mediated protection have not yet been completely explained.

Among the many pathways that interact with estrogen receptors the Notch pathway has a prominent role in endothelial cells survival. In general, Notch signaling mediates the communication between adjacent cells, influencing their proliferation, migration, survival, and differentiation (18). Mammals have four Notch receptors (Notch 1–4) and five ligands (Delta-like 1, 3, 4 and Jagged-1 and 2), all single-pass transmembrane proteins located at the plasma membrane. Notch receptors are

The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Figs. S1 and S2.

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² The abbreviations used are: ER, estrogen receptor; EC, endothelial cell; E2, 17 β -estradiol; HUVEC, human umbilical vein endothelial cell; qRT, quantitative RT; PI, propidium iodide; NICD, Notch1 intracellular domain; csFBS, charcoal/dextran-treated FBS; PHTPP, 2-phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine; DAPT, *N*-[*N*-(3,5-difluorophenyl)-L-alanyl]-5-phenylglycine *t*-butyl ester; DPN, diarylpropionitrile; PPT, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-trisphenol.

synthesized as single-chain precursors and cleaved by a furin-like protease into an extracellular and a transmembrane subunit in the Golgi apparatus. These two subunits are held together on the cell membrane by non-covalent bonds. The binding of a Notch ligand triggers the removal of the extracellular subunit, a subsequent proteolytic cut by A Disintegrin And Metalloprotease (ADAM10 and/or -17) followed by an intramembranous cleavage by γ -secretase, a multisubunits membrane protease that releases the intracellular active form of Notch (NICD). In canonical Notch signaling, NICD translocates into the nucleus where it modulates transcription of several genes via binding the RBP-J κ (recombinant signal-binding protein 1 for J κ) transcription factor (19) and mediating the displacement of co-repressors and the recruitment of co-activators. The most studied Notch target genes belong to the Hairy and Enhancer of Split (*HES*) and Hairy and Enhancer of Split with YRPW motif (*HEY*) gene families, which regulate genes involved in cell proliferation and apoptosis (20). A non-canonical Notch signaling, active in the cytoplasm and linked to activation of the serine threonine kinase Akt, has been recently identified (21). In the endothelium, Notch1 and Notch4 prevent apoptosis induced by lipopolysaccharides (LPS), TNF α (22, 23), and turbulent blood flow (24, 25). Conversely, activation of Notch2 induces endothelial cells apoptosis by inhibiting the expression of survivin (26), indicating that different Notch receptors play opposite roles in the endothelium. Among Notch ligands, Jagged1, Delta-like ligand 1 (Dll1) and -4 (Dll4) are all expressed in the adult endothelium (12, 27–29). Jagged1 and Dll4 have been shown to regulate angiogenesis (30, 31) but no studies have investigated the possible different roles of Notch ligands in modulating endothelial cells apoptosis.

The Notch signaling is tightly regulated by cross-talks with other pathways (32, 33). In the endothelium, Notch activity is down-regulated by TNF α (26, 27, 30, 34), whereas, as shown by us (12) and others (35, 36), treatment with E2 activates Notch. Based on the observation that TNF α and E2 have opposite effects both endothelial cells apoptosis and Notch signaling, we sought to determine whether the activation of Notch exerted by estrogens under inflammatory conditions is involved in the protective role of the endothelium. With this aim, we investigated whether cross-talks between E2 and Notch signaling have an effect on TNF α -induced apoptosis in human umbilical vein endothelial cells (HUVECs). Furthermore, we evaluated the possible role of ER α and ER β in the E2-mediated reduction of endothelial cells apoptosis.

Results

17 β -Estradiol partially counteracts the dysregulation of the Notch pathway induced by TNF α in HUVECs

To determine whether E2 increases endothelial cells survival by counteracting the TNF α -mediated dysregulation of Notch signaling, HUVECs were hormone-deprived for 20 h before treatment with 10 ng/ml of TNF α and 1 nM E2 for 24 h and Notch1, -2, and -4 receptors and ligands Dll4 and Jagged1 protein and mRNA levels were analyzed by Western blot analysis and quantitative real-time PCR (qRT-PCR), respectively. To detect Notch1, -2, and -4, we used antibodies against the C

terminus of these receptors, which recognize the precursor (PC), transmembrane (TM), and active (NICD) forms. An antibody specific for Notch1, cleaved at valine 1744, was used to identify the active form of this receptor (N1ICD). Treatment with 5 μ M DAPT (LY-374973), an inhibitor of the γ -secretase, was included to identify the active form of the receptors. We found that TNF α treatment did not affect the precursor (N1PC, 250 kDa) or the transmembrane form of Notch1 (N1TM, 110 kDa), whereas it strongly reduced the levels of the active form (N1ICD, 100 kDa), and this effect was partially counteracted by E2 (Fig. 1A and supplemental Fig. S1). In agreement with our previous findings (12), E2 increased the level of N1ICD also in the absence of TNF α (Fig. 1A and supplemental Fig. S1). Either TNF α alone or co-treatment with E2 did not affect Notch1 mRNA levels (Fig. 1B). Treatment with DAPT completely abolished the activation of Notch1 (Fig. 1A and supplemental Fig. S1). In agreement with studies by others (26, 34), following treatment with TNF α , we observed an increase of the Notch2 protein (N2ICD, 100 kDa) and mRNA and a decrease of Notch4 protein (N4ICD, 64 kDa) and mRNA. E2 had no effect on the modifications induced by TNF α on Notch2 and -4 proteins (Fig. 1A and supplemental Fig. S1) and mRNAs (Fig. 1B). Of interest, treatment with DAPT did not interfere with the activation of Notch2 or Notch4 (Fig. 1A and supplemental Fig. S1). We also confirmed that TNF α positively modulates the Notch ligand Jagged1, both on protein and mRNA levels, whereas it decreases the levels of Dll4 protein (Fig. 1A and supplemental Fig. S1) and mRNA (Fig. 1B) (27). Co-treatment with E2 did not modify the effects of TNF α on Notch ligands protein (Fig. 1A and supplemental Fig. S1) or mRNA (Fig. 1B). Our results confirm that TNF α induces Notch2 and Jagged1 and inhibits Notch4 and Dll4 transcription, and show that E2 does not alter the TNF α -mediated transcriptional regulation of these components of the Notch pathway. Furthermore, we show that TNF α negatively affects the levels of N1ICD and that E2, at least partially, counteracts this effect. Additionally, DAPT inhibited Notch1 processing, but had no effect on Notch4 and Notch2, suggesting that, at least at the concentrations used, this γ -secretase inhibitor negatively affects Notch1-, but not Notch2- and Notch4-mediated signaling in HUVECs.

Active Notch1 is required for 17 β -estradiol-mediated protection against TNF α -induced apoptosis

To determine whether Notch signaling is required for the protective action of E2 from TNF α -induced apoptosis, we evaluated the effect of E2 treatment on both early (Annexin V-positive) and late (Annexin V/propidium iodide-positive) apoptotic HUVECs following 24 h of exposure to TNF α , in the presence or absence of DAPT. Flow cytometric analysis showed that 24 h of treatment with 10 ng/ml of TNF α increased the number of apoptotic cells, compared with control cells, and that E2 partially counteracted this effect (Fig. 2, A and B). DAPT alone did not affect HUVECs survival but the co-treatment with TNF α increased the number of apoptotic cells, in comparison with treatment with TNF α only (Fig. 2, A and B). Furthermore, in the presence of DAPT, E2 was not able to protect cells from TNF α -induced apoptosis (Fig. 2, A and B). These findings suggest that an active Notch pathway is necessary for the E2 pro-

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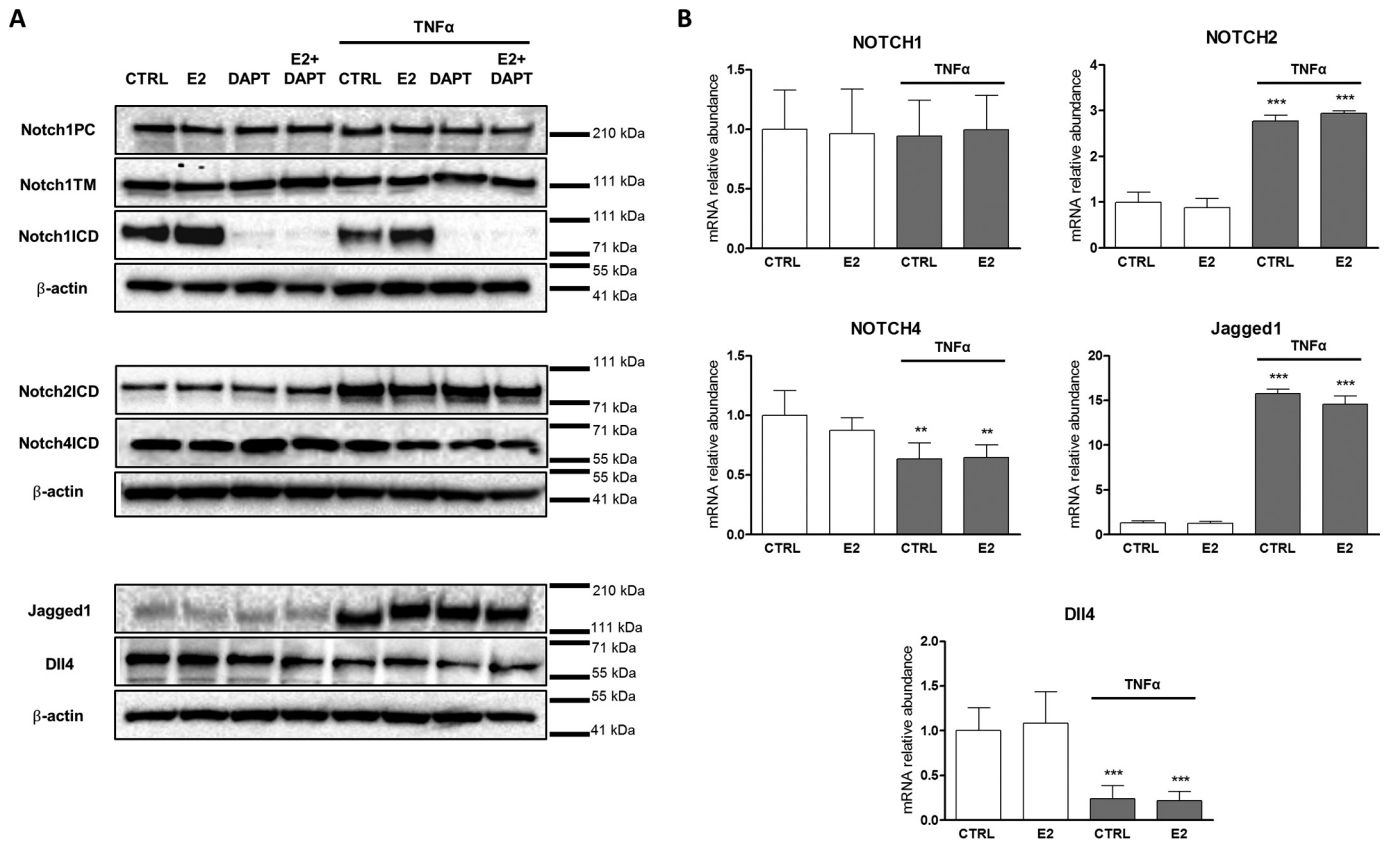


Figure 1. Effect of E2, TNF α , and DAPT treatments on Notch receptors and ligands in HUVECs. A, HUVECs were treated for 24 h with DMSO (CTRL), E2 (1 nM), TNF α (10 ng/ml), or DAPT (5 μ M), alone and in combination. Cell lysates were electrophoresed and immunoblotted with antibodies for total Notch1 (C20), cleaved Notch1 (Val-1744), active Notch2 (clone C651.6DbHN), active Notch4 (H-225), Jagged1, and Dll4 to detect the precursor (Notch1PC), the transmembrane (Notch1TM) and the active form of Notch1 (Notch1ICD), the active form of Notch2 (Notch2ICD), the active form of Notch4 (Notch4ICD), the ligands Jagged1 and Dll4. β -Actin antibody was used to ensure equal loading. Densitometry analyses are shown in supplemental Fig. S1. B, HUVECs were treated for 24 h with DMSO (CTRL), TNF α (10 ng/ml), or E2 (1 nM) alone and in combination. Total RNA was extracted and qRT-PCR analysis of Notch1, Notch2, Notch4, Jagged1, and Dll4 genes expression was performed. Relative changes in mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method using RPL13A as reference gene. Results are expressed as mean \pm S.D. of three independent experiments, each performed in triplicate. **, $p < 0.01$; ***, $p < 0.001$ (pairwise comparison between plus or minus TNF α).

tective action against TNF α -induced endothelial cells apoptosis. Because DAPT treatment only inhibited Notch1 activation (Fig. 1A and supplemental Fig. S1), we hypothesized that N1ICD might be necessary for the protective effect of E2. To test the involvement of Notch1, HUVECs were transfected with siRNA against Notch1 before treatments with E2 and/or TNF α and apoptosis was detected 24 h later. Notch1 mRNA knock-down was confirmed by Western blot and qRT-PCR analysis (Fig. 3A). Notch1 siRNA did not increase the number of apoptotic cells, compared with control siRNA, but significantly enhanced HUVECs apoptosis in the presence of TNF α (Fig. 3, B and C). Furthermore, in Notch1-silenced HUVECs, E2 was unable to protect cells from TNF α -induced apoptosis (Fig. 3, B and C). To further validate the direct implication of Notch1 in the protective action of E2 against TNF α -induced apoptosis, HUVECs were transfected with a plasmid encoding for N1ICD. Overexpression was confirmed by Western blot analysis (Fig. 4A). Upon treatment with TNF α , HUVECs overexpressing N1ICD showed fewer apoptotic cells compared with cells transfected with the empty vector (Fig. 4, B and C). Taken together, these results suggest that active Notch1 is necessary for the anti-apoptotic action of E2 in TNF α -treated HUVECs.

17 β -Estradiol protection of HUVECs against TNF α -induced apoptosis involves Notch1-mediated Akt phosphorylation

The serine-threonine kinase Akt, a major determinant of endothelial cells survival, is regulated by several factors, including E2 (37) and TNF α (38). Therefore, to identify effectors acting downstream of Notch1 in the E2-mediated protection of endothelial cells, we investigated the activation of Akt (phosphorylation at serine 473) in HUVECs treated with TNF α for 1, 2, and 24 h, with or without E2. Short-term treatments were included because phosphorylation is an early event in the pro-survival activity of Akt (39, 40). Western blot analysis showed that 1 and 2 h of treatment with TNF α activated Akt, whereas, 24 h later, Akt phosphorylation was reduced compared with untreated cells. TNF α also had a biphasic effect on N1ICD, which increased during early treatment and decreased, compared with control, after 24 h (Fig. 5, A and B). The addition of DAPT abolished Notch1 activation and strongly decreased Akt phosphorylation induced by TNF α (Fig. 5, C and D). In the presence of E2, at each time point tested, TNF α -treated cells showed a more pronounced phosphorylation of Akt and higher levels of N1ICD compared with cells treated with TNF α only (Fig. 5, A and B). Also in the presence of E2, DAPT treatment

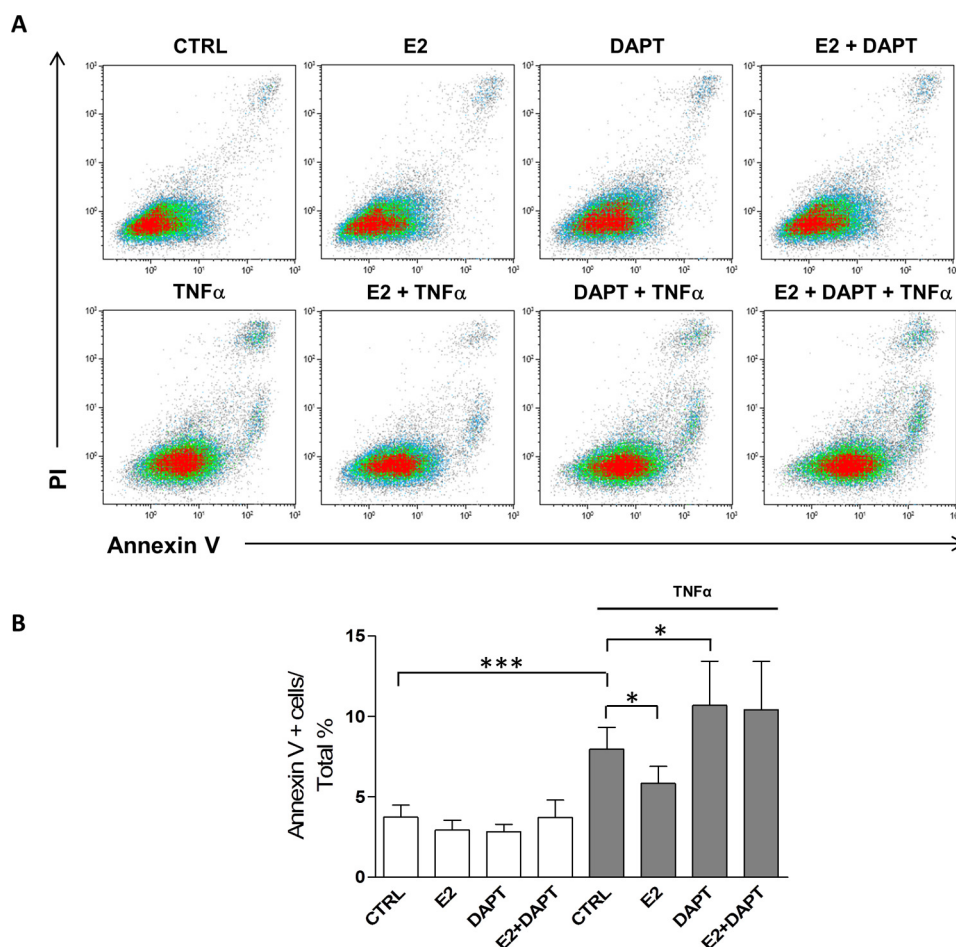


Figure 2. Effect of E2 and DAPT treatments on TNF α -induced HUVECs apoptosis. A, HUVECs treated for 24 h with DMSO (CTRL), E2 (1 nM), TNF α (10 ng/ml), or DAPT (5 μ M), alone and in combination, were stained with Annexin V and PI and cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. B, percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) is shown. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

abolished Notch1 activation and strongly reduced the effects of E2 on Akt phosphorylation, both in control and TNF α -treated cells (Fig. 5, E and F). In conclusion these experiments show that, in HUVECs, early treatment with TNF α induces phosphorylation of Akt, which requires N1ICD and is enhanced by E2. After 24 h, both pAkt and N1ICD levels decline more pronouncedly in TNF α -, compared with E2/TNF α -treated cells. These data suggest that Notch1-dependent Akt phosphorylation contributes to the pro-survival action of E2 in the presence of TNF α . To confirm the role of Akt in the E2-Notch1-mediated protection against TNF α -induced apoptosis, we assessed apoptosis in the presence of wortmannin, a specific phosphatidylinositol 3-kinase (PI3K) inhibitor. Cells were treated with E2 in the presence of wortmannin and then apoptosis was assessed. Flow cytometric analysis showed that in the presence of wortmannin, E2 was not able to protect against TNF α -induced apoptosis (Fig. 6, B and C). The wortmannin-mediated reduction of Akt phosphorylation was confirmed by Western blot (Fig. 6A).

Estrogen receptor β is required for protection against TNF α -induced apoptosis

To test the involvement of one or both isoforms of ERs in the anti-apoptotic action of E2, HUVECs were treated for 24 h with

E2 and TNF α , alone or in combination, following transfection with siRNA targeting ER α or ER β . ER α and ER β mRNA knock-down was confirmed by Western blot analysis (Fig. 7C) and apoptotic, Annexin V-positive cells were detected by flow cytometry. As shown in Fig. 7, A and B, TNF α increased the percentage of apoptotic cells, compared with untreated cells, independently of the type of transfected siRNA, whereas co-treatment with E2 protected cells transfected with scrambled siRNA and ER α siRNA, but not with ER β siRNA (Fig. 7, A and B). These findings indicate that ER β , but not ER α , is involved in E2-mediated counteraction of TNF α -induced apoptosis. Furthermore, while in the cells transfected with scrambled siRNA, E2 treatment increased the levels of N1ICD, both in the presence or absence of TNF α , in ER β siRNA-transfected cells, E2 capacity to increase N1ICD levels was abrogated (Fig. 7D and supplemental Fig. S2A). These results show that ER β is required for E2-mediated counteraction of TNF α -induced N1ICD down-regulation. Of interest, knockdown of ER β , but not of ER α , led to increased apoptosis compared with scrambled siRNA-transfected cells, even in the absence of TNF α (Fig. 7, A and B), which suggests that ER β is a pro-survival factor in HUVECs. To determine whether TNF α affects HUVECs survival by regulating the expression levels of this ER isoform, we

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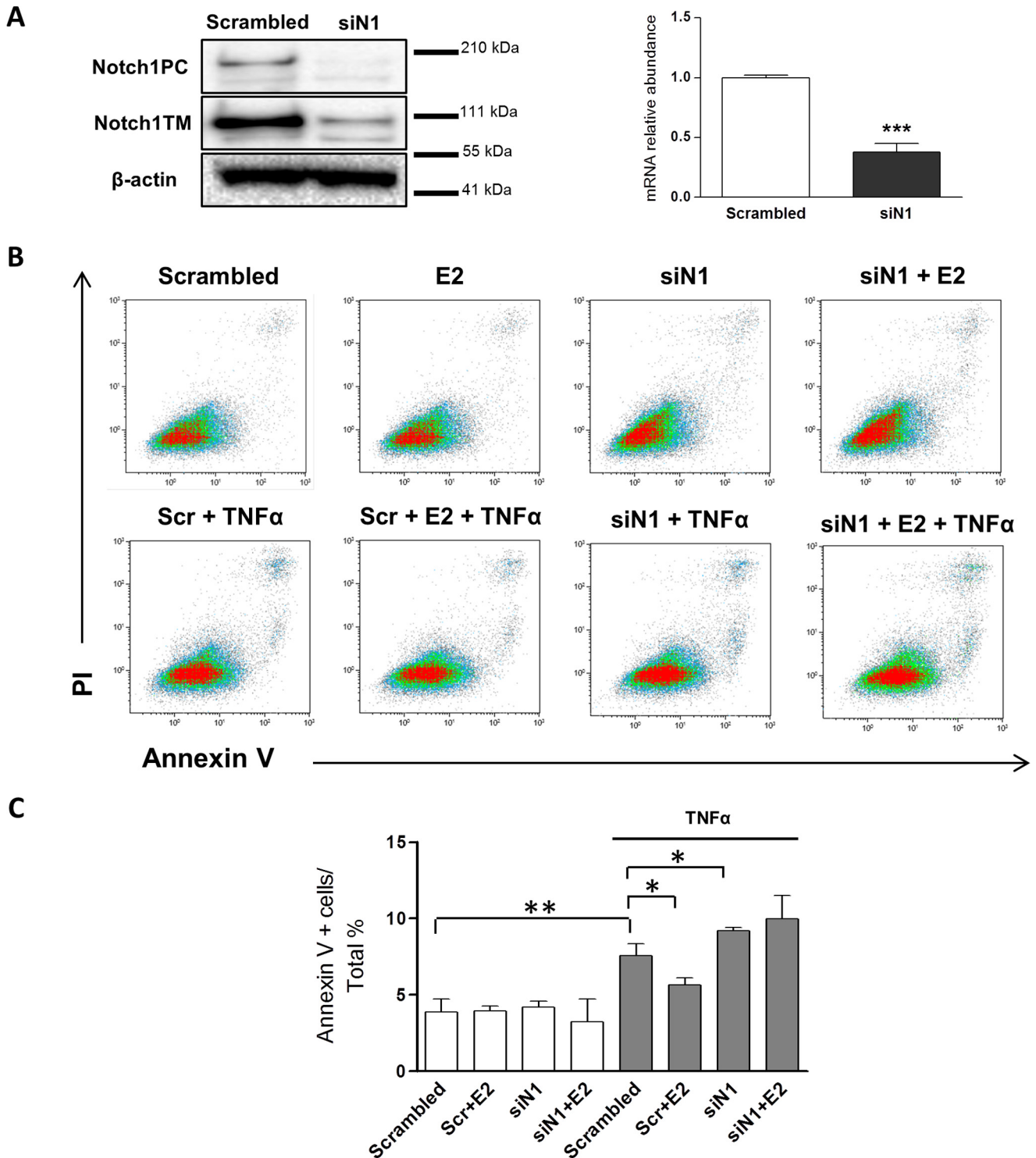


Figure 3. TNF α -induced apoptosis in Notch1-silenced HUVECs. *A*, HUVECs were treated for 48 h with siRNA against Notch1. Cell lysates were electrophoresed and immunoblotted with antibody for total Notch1 (C20). β -Actin was used to ensure equal loading. qRT-PCR analyses were performed to detect reduction of Notch1 mRNA levels in HUVECs after siRNA against Notch1 treatment for 48 h. Scrambled siRNA was used as control. Relative changes in mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method using RPL13A as reference gene. Results are expressed as mean \pm S.D. of three independent experiments, each performed in triplicate. ***, $p < 0.001$. *B*, HUVECs were transfected with siRNA against Notch1 and, 24 h later, they were treated with TNF α (10 ng/ml) and E2 (1 nM), alone, and in combination, for 24 h. Cells were stained with Annexin V and PI, then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. *C*, percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) is shown. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

assessed ER α and ER β protein levels after treatment with TNF α , alone, and in combination with E2. As shown in Fig. 7E and supplemental Fig. S2B, TNF α decreased ER β while increas-

ing ER α protein levels, compared with control, and E2 did not counteract this effect. Nevertheless, because E2 down-regulated ER α but not ER β protein, the ER α /ER β ratio increased in

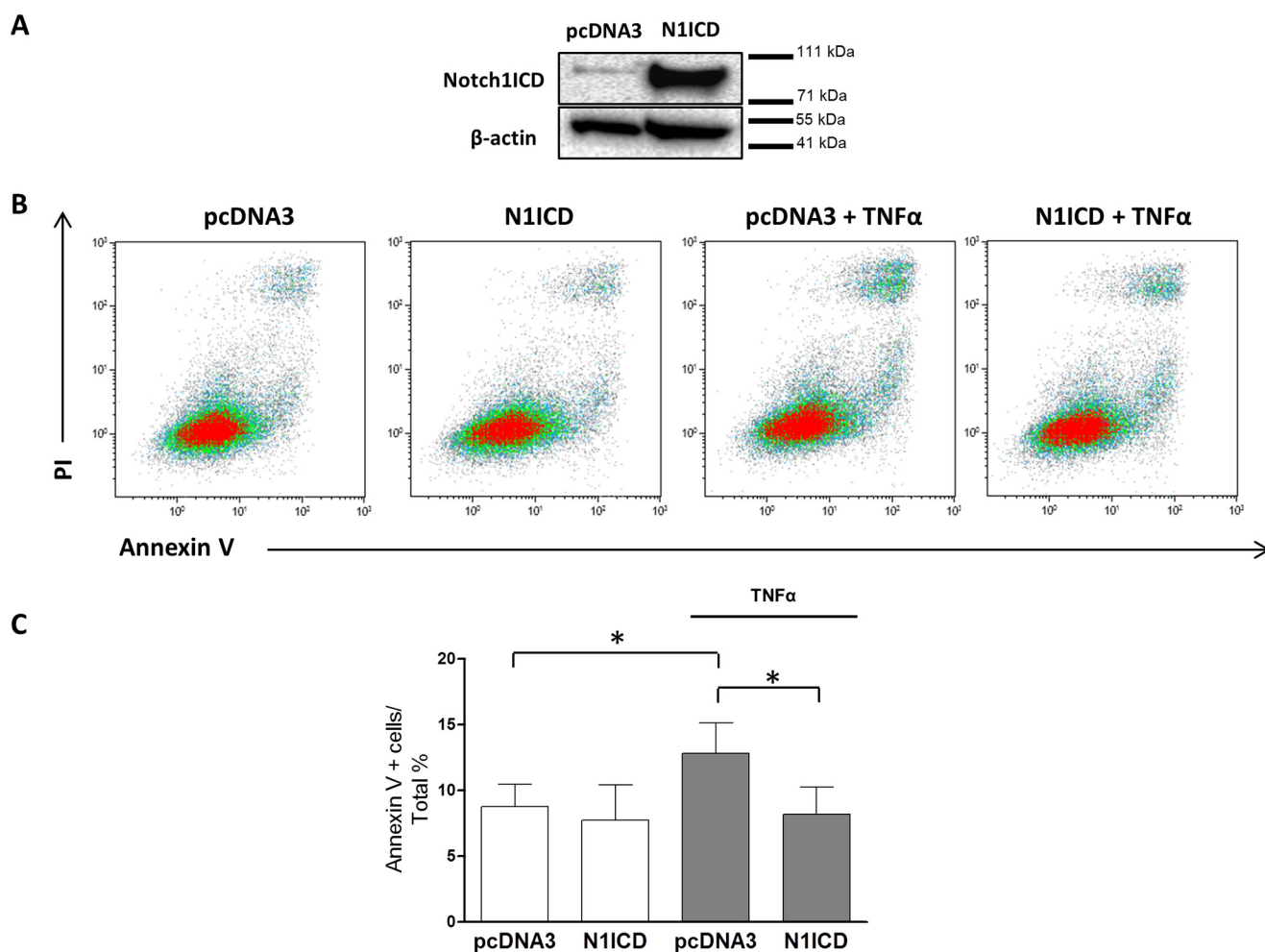


Figure 4. TNF α -induced apoptosis in Notch1 overexpressing HUVECs. *A*, HUVECs were transfected with a plasmid encoding the Notch1 intracellular domain (Notch1ICD) and, 24 h later, were treated with TNF α (10 ng/ml) for 24 h. The empty vector (pcDNA3) was used as a control. Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val-1744) antibody. β -Actin antibody was used to ensure equal loading. *B*, HUVECs transfected for 24 h were treated for 24 h with TNF α (10 ng/ml), stained with Annexin V and PI, then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. *C*, the histogram shows the percentage of apoptotic cells, represented as ratio of Annexin V-positive cells/total cells. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$.

TNF α -treated cells but not in co-treatment with E2. These data suggest that an increased ER α /ER β ratio could be linked to the reduced survival observed when HUVECs are exposed to TNF α .

The role of ER β in regulating apoptosis and N1ICD levels in the presence of TNF α was confirmed by experiments utilizing specific agonists of both ER isoforms. Specifically, treatment with a specific agonist of ER β (DPN), but not with an agonist of ER α (PPT), increased N1ICD levels (Fig. 8, *A* and *B*). Additionally, DPN but not PPT, reduced the ratio of apoptotic cells, compared with TNF α -treated cells, but not in the presence of DAPT (Fig. 8, *C–F*). To further confirm the involvement of ER β in E2-mediated protection, we used PHTPP, a specific antagonist of ER β . HUVECs were treated with PHTPP for 20 h before adding E2 and/or TNF α for 24 h. As shown in Fig. 9, *A* and *B*, E2 protected against TNF α -induced apoptosis only in the absence of PHTPP. Furthermore, in the presence of PHTPP, E2 was not able to increase the levels of N1ICD and enhance the phosphorylation of Akt (Fig. 9, *C* and *D*).

Discussion

The molecular mechanisms underlying the protective action of estrogens on the endothelium are still not completely understood. We report here that E2 partially counteracts the pro-apoptotic action of TNF α in endothelial cells by increasing the levels of intracellular Notch1. Furthermore, we provide evidence that ER β is required for the protective effect of E2 in this context. The protective role of E2 against endothelial cells apoptosis induced by TNF α , a cytokine elevated under inflammatory conditions (27) and in peri-menopausal women (41), has been documented (5, 6). Our study is consistent with the existing data on the protective role of E2 in HUVECs and reveals that active Notch signaling is necessary for this protection, because E2 is unable to decrease the apoptotic rate induced by TNF α when Notch activity is chemically or genetically inhibited. The role of Notch signaling in endothelial cell survival was reported for the first time by Quillard *et al.* (23) who showed that treatment of HUVECs with TNF α causes increased endothelial cells apoptosis together with the up-regulation of Notch2 and down-

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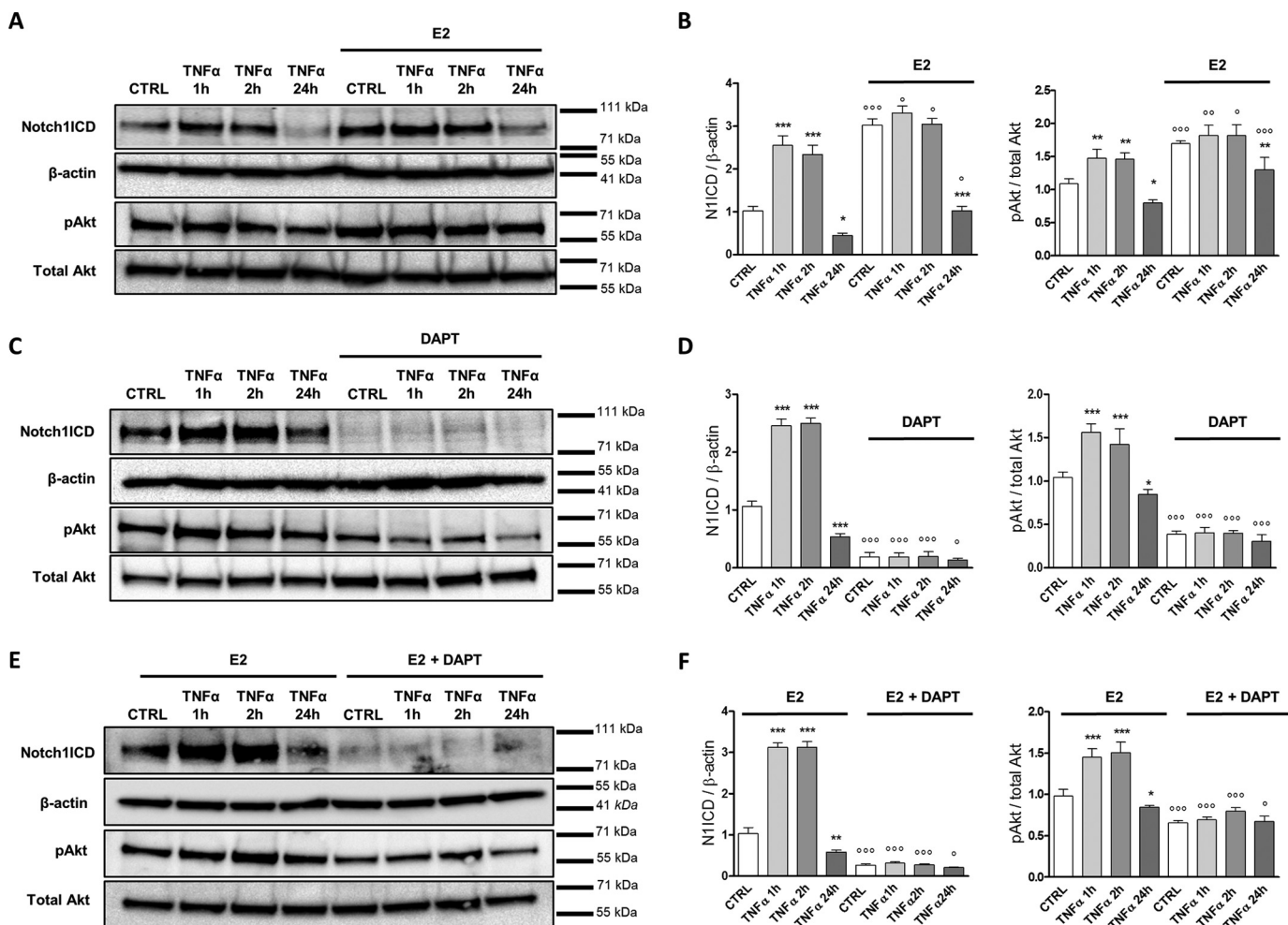


Figure 5. Role of E2 and Notch1 on TNF α regulation of Akt phosphorylation in HUVECs. A and B, Western blotting and densitometry of HUVECs treated with TNF α (10 ng/ml) for 1, 2, or 24 h in the presence of E2 (1 nM). Cells treated with DMSO were used as control (CTRL). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val-1744), pAkt (Ser-473), and total Akt antibodies. β -Actin antibody was used to ensure equal loading. B, graphs show protein levels after the indicated treatments normalized to untreated control levels, after signal comparison to β -actin expression. Phosphorylated Akt was normalized to the total Akt level. Results are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (comparison between TNF α treatment and corresponding control); °, $p < 0.05$; °°, $p < 0.01$; °°, $p < 0.001$ (pairwise comparison between plus or minus E2). C and D, Western blotting and densitometry of HUVECs treated with TNF α (10 ng/ml) for 1, 2, or 24 h in the presence of DAPT (5 μ M). Cells treated with DMSO were used as control (CTRL). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val-1744), pAkt (Ser-473), and total Akt antibodies. β -Actin antibody was used to ensure equal loading. Graphs show protein levels after the indicated treatments normalized to untreated control levels, after signal comparison to β -actin expression. Phosphorylated Akt was normalized to the total Akt level. Results are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (comparison between TNF α treatment and corresponding control); °, $p < 0.05$; °°, $p < 0.01$; °°, $p < 0.001$ (pairwise comparison between plus or minus DAPT). E and F, Western blotting and densitometry of HUVECs treated with TNF α (10 ng/ml) for 1, 2, or 24 h in the presence of E2 (1 nM) or E2 (1 nM)/DAPT (5 μ M). Cells treated with DMSO were used as control. Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val-1744), pAkt (Ser-473), and total Akt antibodies. β -Actin was used to ensure equal loading. Graphs show protein levels after the indicated treatments were normalized to untreated control levels, after signal comparison to β -actin expression. Phosphorylated Akt was normalized to the total Akt level. Results are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (comparison between TNF α treatment and corresponding control); °, $p < 0.05$; °°, $p < 0.001$ (pairwise comparison between E2 and E2 plus DAPT treatment).

regulation of Notch4 (26). We found a previously unreported reduction of the intracellular levels of Notch1 in HUVECs treated for 24 h with TNF α , which suggests that Notch1 also plays a role in endothelial cell survival under inflammatory conditions. In agreement with this hypothesis, TNF α -induced apoptosis was increased by DAPT treatment, which under our experimental conditions inhibits Notch1 but not Notch2 or Notch4 activation, and it was reduced instead by overexpression of N1ICD. Whereas the role of Notch1 in protecting endothelial cells from apoptosis has been reported in the context of ischemia (42), disturbed shear stress (25), and in the endothelium of subjects with pulmonary hypertension (43), this is the

first report showing the involvement of Notch1 in endothelial cell survival under the effect of an inflammatory cytokine.

Our study shows that E2-mediated protection against TNF α -induced apoptosis requires the up-regulation of intracellular Notch1. This conclusion is based on the observation that, in HUVECs treated with TNF α , E2 (i) does not counteract the transcriptional down-regulation of Notch4 and induction of Notch2 but partially restores the levels of N1ICD, which are decreased by TNF α and (ii) is not able to reduce the number of apoptotic cells if Notch1 mRNA is silenced. More studies are needed to clarify the molecular mechanism by which TNF α decreases the levels of active Notch1 and how E2 partially coun-

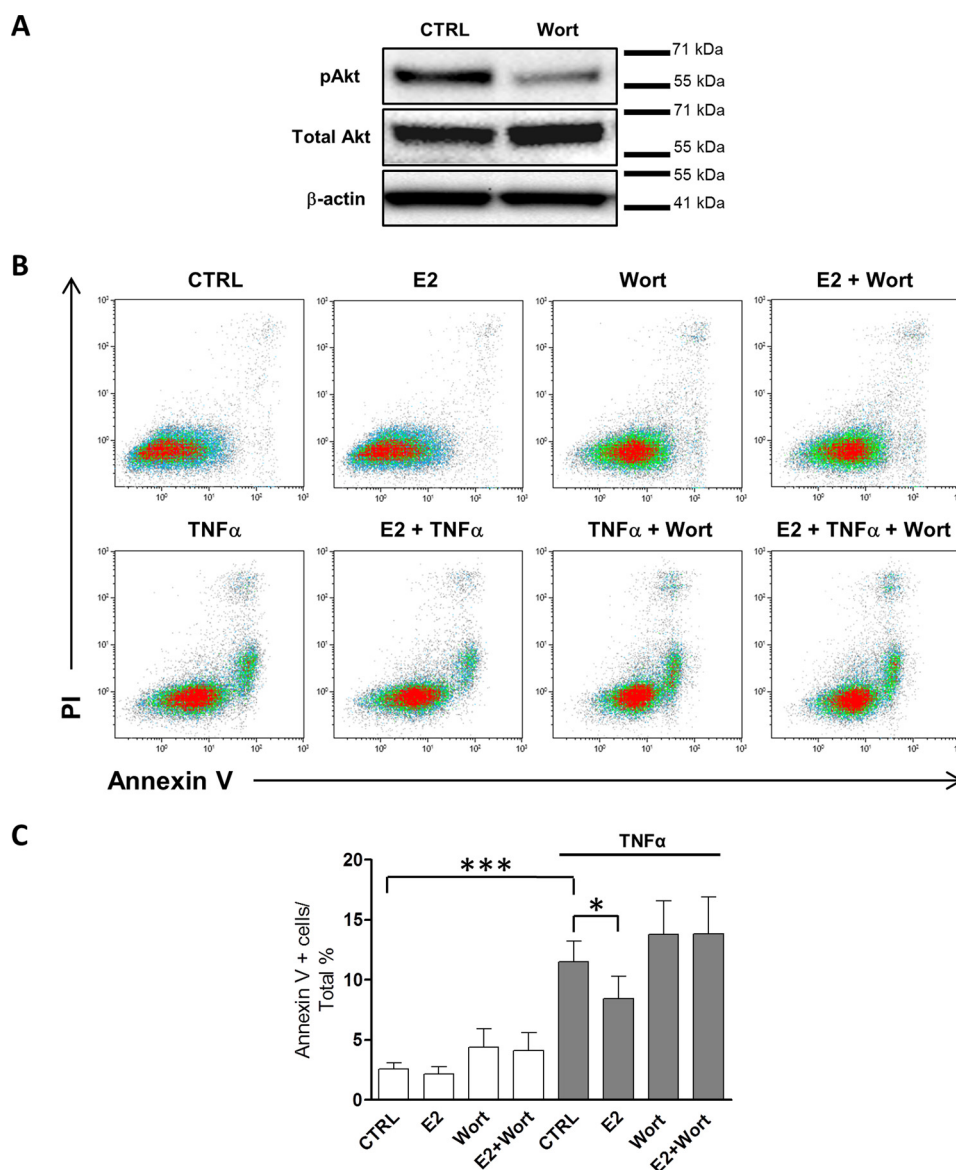


Figure 6. Effect of wortmannin on TNF α -induced apoptosis in HUVECs. *A*, HUVECs were treated for 24 h with wortmannin (100 nM). Lysates were electrophoresed and immunoblotted with pAkt (Ser-473) and total Akt antibodies. β -Actin antibody was used to ensure equal loading. *B*, HUVECs treated for 24 h with DMSO (*CTRL*), 17 β -estradiol (*E2*, 1 nM), and TNF α (10 ng/ml) in the presence or absence of wortmannin (100 nM), were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. *C*, percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) is shown. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

teracts this effect. We did not observe a transcriptional control of Notch1 promoter by TNF α : this finding is in agreement with Quillard *et al.* (23) and in contrast with Briot *et al.* (44), who reported the inhibition of Notch1 transcription in TNF α -treated human aortic endothelial cells: differences in the time points analyzed or in the source of endothelial cells (artery *versus* vein) could explain these discrepancies. Our data suggest instead that TNF α could inhibit the activation rate of the Notch1 receptor or decrease the stability of N1ICD, and, in principle, both actions could be partially counteracted by E2. TNF α treatment inhibits Dll4 and induces Jagged1 (27), with the latter being a weaker activator of Notch1 (30). These data suggest that the reduction of N1ICD in TNF α -treated cells could be a consequence of an altered Jagged1/Dll4 ratio. Nevertheless, in our study, E2 did not affect Jagged1 and Dll4

mRNAs in TNF α -treated cells, indicating that the hormone does not increase N1ICD by altering the levels of the ligands.

Consistent with an increased apoptotic rate, 24 h of treatment with TNF α caused decreased levels of phosphorylated Akt, a prosurvival factor in HUVECs. Early treatment with TNF α induced instead Akt phosphorylation, as also shown in cardiac fibroblasts (45). This dual action of TNF α on pAkt is not surprising considered the ability of this cytokine to elicit both survival and death pathways, depending on the cell context (46). At each time point investigated, the levels of active Notch1 paralleled those of pAkt and DAPT strongly inhibited the phosphorylation of Akt, suggesting that N1ICD is required for TNF α -induced phosphorylation of Akt. The addition of E2 to cells treated with TNF α for 24 h partially restored N1IC and pAkt levels, compared with treatment with TNF α only, sug-

Estrogen protection of endothelium requires Notch1 activation

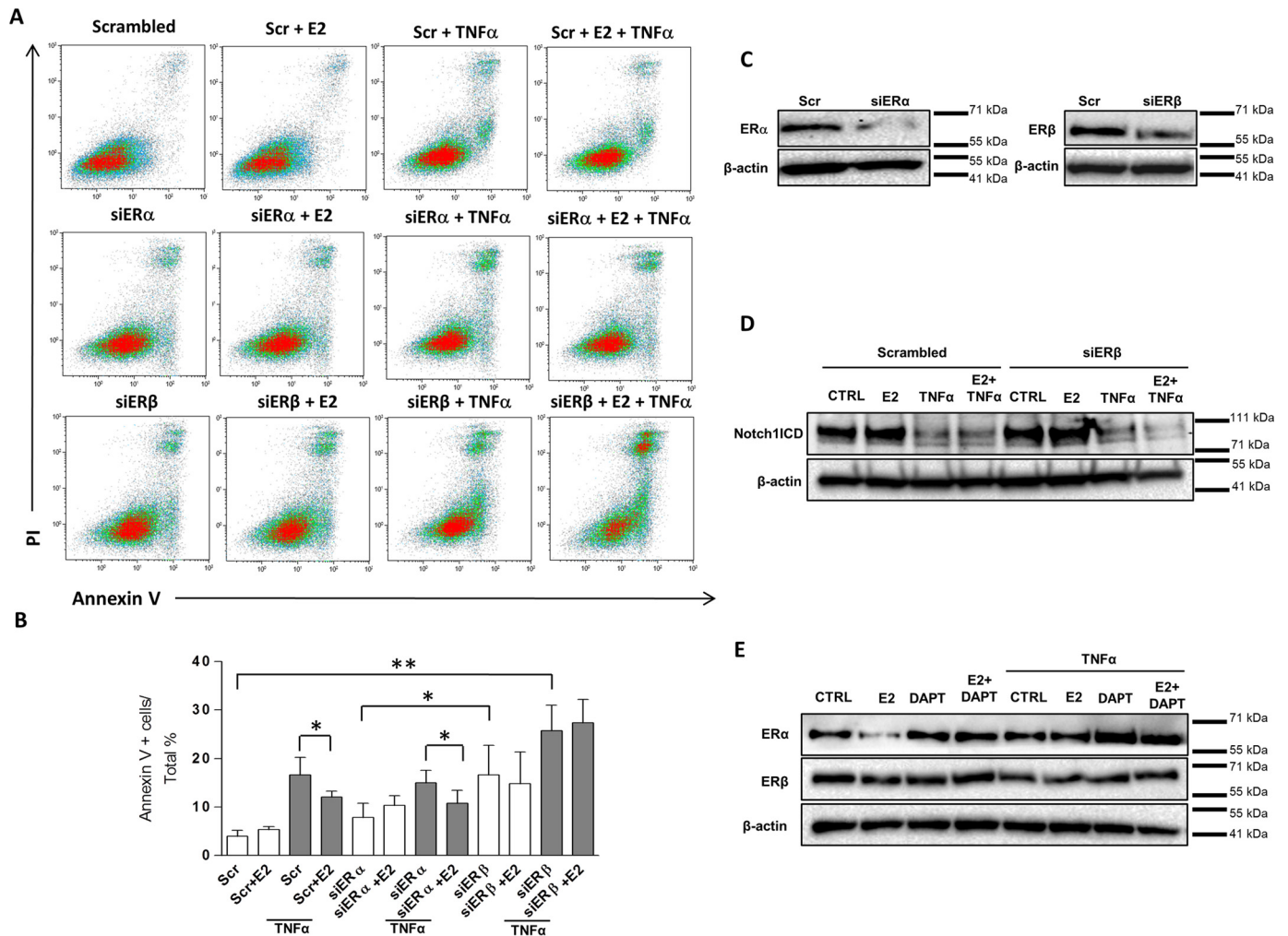


Figure 7. Role of estrogen receptor β on Notch1 activation and protection against TNF α -mediated apoptosis in HUVECs. *A*, HUVECs were transfected with siRNA against ER α and ER β and, 24 h later, they were treated with E2 (1 nM) and TNF α (10 ng/ml), alone and in combination, stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. *B*, the histogram depicts the percentage of apoptotic cells as ratio of Annexin V-positive cells/total cells. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *C*, HUVECs were transfected with siRNA against ER α or ER β for 48 h. Lysates were electrophoresed and immunoblotted with ER α and ER β antibodies, respectively. β -Actin antibody was used to ensure equal loading. *D*, Western blot analysis for cleaved Notch1 (Val-1744) in HUVECs after the transfection with siRNA against ER β and TNF α - (10 ng/ml) and 17 β -estradiol (E2, 1 nM) treatments for 24 h. β -Actin antibody was used to ensure equal loading. Densitometric analyses are shown in supplemental Fig. S2A. *E*, HUVECs were treated for 24 h with E2 (1 nM), TNF α (10 ng/ml), and DAPT (5 μ M), alone and in combination. Lysates were electrophoresed and immunoblotted with ER β or ER α antibodies. β -Actin antibody was used to ensure equal loading. Densitometric analyses are shown in supplemental Fig. S2B.

gesting that E2 might protect endothelial cells from apoptosis by increasing the levels of active Notch1, which in turn would promote the phosphorylation of Akt. In this context, the direct contribution of E2 to Akt phosphorylation (47) should also be considered. By showing that E2 activates Notch1, which in turn promotes phosphorylation of Akt, we confirmed and expanded previous work by Koga *et al.* (37) showing the crucial role of Akt in promoting survival of endothelial cells in the presence of TNF α .

More studies are needed to investigate the mechanism by which Notch1 activates Akt in TNF α -treated HUVECs. Activation of Akt by Notch1, through inhibition of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) by the Notch target gene *HES1*, has been observed in T-cell acute lymphoblastic leukemia (48), and in cervical cancer cell lines, through a non-canonical pathway (21, 49). We have previously shown that E2 treatment increased N1ICD in HUVECs but no

changes were detected in *HES1* mRNA (12) suggesting that non-canonical Notch signaling could be involved in the Notch1-mediated phosphorylation of Akt.

The protective effects of estrogens on the endothelium are mediated by both isoforms of ERs (ER α and ER β) (50). We found that E2 requires ER β , but not ER α , to reduce the TNF α -induced endothelial cells apoptosis and that ER β was required for E2-mediated up-regulation of N1ICD in TNF α -treated cells. Our studies are consistent with (i) the observation that E2 prevents early stage atherosclerosis in ER α -deficient mice (14) and (ii) a large number of studies showing distinct, even opposite, roles for ER α and ER β in regulating the biology of many cell types (51). We also found that ER β mRNA knockdown increased the apoptotic rate even in the absence of TNF α and E2, suggesting that this form of receptor contributes to endothelial cell survival according to a ligand-independent mechanism (50). Of note, TNF α down-regulated ER β , but not ER α ,

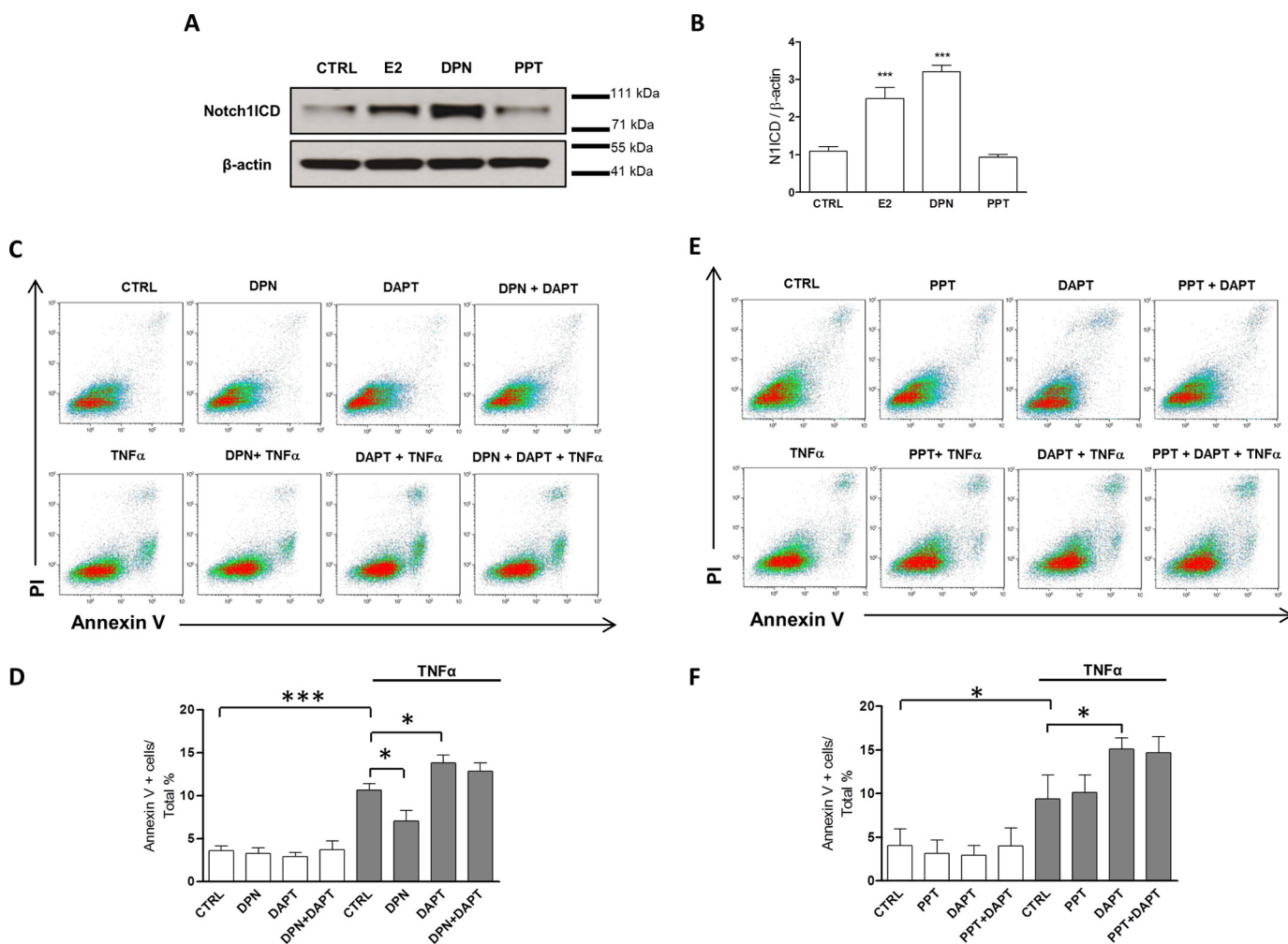


Figure 8. Effect of DPN and PPT on TNF α -induced apoptosis in HUVECs. *A*, HUVECs were treated for 24 h with 17 β -estradiol (E2, 1 nM), DPN (ER β agonist, 10 nM), and PPT (ER α agonist, 10 nM). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val-1744) antibody. β -Actin antibody was used to ensure equal loading. *B*, densitometric analysis. Graphs show protein levels after the indicated treatments normalized to untreated control levels, after signal comparison to β -actin expression. Results are expressed as mean \pm S.D. of three independent experiments. ***, $p < 0.001$ (comparison between control and agonist treatment). *C* and *D*, HUVECs treated for 24 h with DMSO (CTRL), DPN (10 nM), TNF α (10 ng/ml), DAPT (5 μ M), alone and in combination, were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. The histogram depicts the percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) following treatment as shown. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$. *E* and *F*, HUVECs treated for 24 h with DMSO (CTRL), PPT (10 nM), TNF α (10 ng/ml), DAPT (5 μ M), alone and in combination, were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. The histogram depicting the percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) following treatment is shown. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$.

leading to an increased ratio of ER α /ER β , action inhibited in the presence of E2 which, as also observed by others (52), only down-regulates ER α . Based on these results, it is tempting to speculate that TNF α may also affect endothelial cells viability by altering the ER α /ER β ratio.

In conclusion, we have shown that E2 requires active Notch1 to protect endothelial cells against TNF α -induced apoptosis, through a mechanism that depends on ER β and Akt upstream and downstream of Notch1, respectively. These findings could be relevant when assessing efficacy and applicability of menopausal hormone treatment because they suggest that in subjects with an impaired Notch1 signaling E2-based hormone therapy may be unable to prevent endothelial dysfunction and, therefore, to reduce the progression of cardiovascular diseases. Impairment of endothelial Notch signaling has been reported under dyslipidaemic conditions (44) and heart failure patients (53), and it could also be a side effect of natural (54, 55) and

synthetic anti-cancer drugs (56) that target the Notch pathway. Hence, Notch1 could constitute a therapeutic target to inhibit endothelial cells apoptosis and reduce endothelium dysfunction in these subjects. Furthermore, knowing that the protective action of estrogens in the endothelium is mediated by ER β could lead to development of selective estrogen receptors modulators specifically targeting ER β , which unlike ER α has been shown to inhibit the proliferation of breast cancer cells (57). Whether estrogen-modulated anti-inflammation effects will have therapeutic potential remains unknown at this time; however, determining the exact mechanisms involved in this newly revealed action of estrogen, and the precise role of this mechanism in the regulation of cardiovascular function, could lead to the development of novel pharmacological therapies for CVD not only in postmenopausal women but also in men, in whose endothelium, as was observed in mice (58), biologically active levels of testosterone-derived estradiol could be present.

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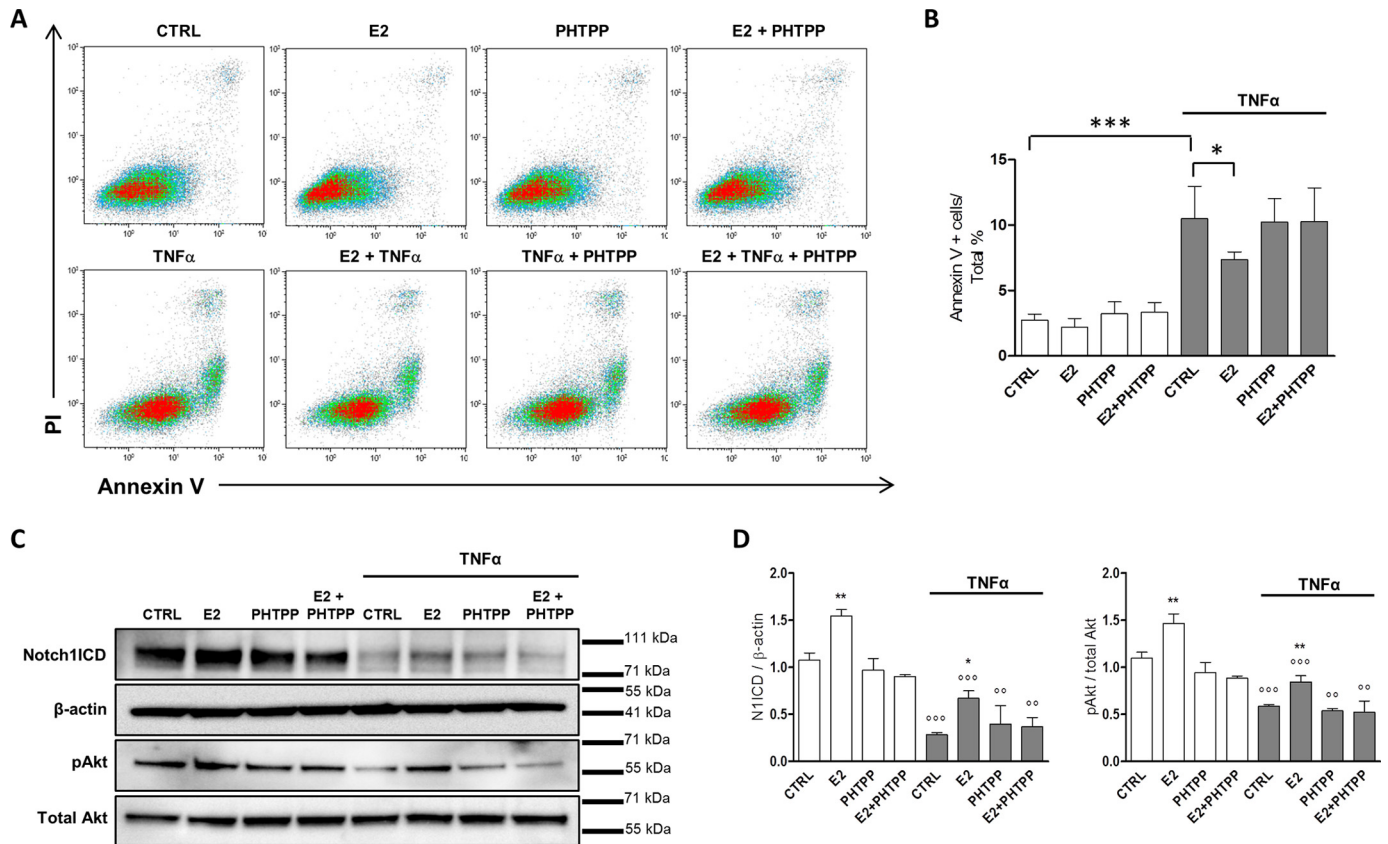


Figure 9. Effect of PHTPP on TNF α -induced apoptosis, Notch1 activation, and Akt phosphorylation in HUVECs. *A*, HUVECs treated for 24 h with DMSO (CTRL), E2 (1 nM), PHTPP (1 μ M), and TNF α (10 ng/ml) alone and in combination, were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. *B*, percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) following treatment is shown. Data are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$. *C*, Western blotting analysis of HUVECs treated with DMSO (CTRL), 17 β -estradiol (E2, 1 nM), PHTPP (1 μ M), and TNF α (10 ng/ml). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val-1744), pAkt (Ser-473), and total Akt antibodies. β -Actin antibody was used to ensure equal loading. *D*, densitometric analysis. Graphs show protein levels after the indicated treatments were normalized to untreated control levels, after signal comparison to β -actin expression. Phosphorylated Akt was normalized to the total Akt level. Results are expressed as mean \pm S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (comparison between treatments and corresponding control); °°, $p < 0.01$; °°°, $p < 0.001$ (pairwise comparison between plus or minus TNF α treatment).

Experimental procedures

Materials

Antibodies used were goat polyclonal to Notch1 (C-20, catalog number sc-6014), rabbit polyclonal to Notch4 (H-225, catalog number sc-5594), rabbit polyclonal to Jagged1 (H-114, catalog number sc-8303), and rabbit polyclonal to estrogen receptor α (MC-20, sc-542) from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal to Dll4 (catalog number ab7280) antibodies were from Abcam (Cambridge, UK). Rabbit polyclonal to estrogen receptor β antibodies (catalog number 5513), rabbit monoclonal to cleaved Notch1 valine 1744 (catalog number 4147), rabbit monoclonal to pAkt (Ser-473 D9E, catalog number 4060), and rabbit polyclonal to Akt (catalog number 9272) were from Cell Signaling Technology (Beverly, MA). Rat monoclonal antibody to Notch2 (clone C651.6DbHN) was purchased from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). The mouse monoclonal antibody to β -actin was from Sigma (AC-15, catalog number A1978). EGM-2 bullet-kit and fetal bovine serum (FBS) were from Lonza (Basel, Switzerland). Lipofectamine 3000, Oligofectamine, Opti-MEM reduced-serum medium, M200 medium, charcoal/dextran-treated FBS

(csFBS), running and transfer buffers, Annexin V-FITC, propidium iodide (PI), SuperScript III reverse transcriptase, random hexamers, dNTPs, and RNaseOut were from Life Technologies (Carlsbad, CA). siRNA against Notch1, ER α , and ER β were from Santa Cruz Biotechnology. ECL Plus Western blotting Detection Reagents were from PerkinElmer Life Sciences (Waltham, MA). RNeasy Mini Kit was from Qiagen (Hilden, Germany). PerfeCta SYBR Green SuperMix with ROX kit were from Quanta Biosciences (Gaithersburg, MD). Primers for RT-PCR were purchased from IDT (Coralville, IA). Wortmannin, DPN, PHTPP (ER β antagonist), PPT, and other materials were purchased from Sigma. All chemicals and solvents were of the highest analytical grade.

Cell culture

HUVECs pools, purchased from Life Technologies, were plated on 1.5% gelatin-coated tissue culture dishes and maintained in phenol red-free basal medium M200 (Life Technologies) containing 2% FBS and growth factors (EGM-2, Life Technologies) at 37 $^{\circ}$ C with 5% CO $_2$. Cells from passages 2 to 7 were actively proliferating (70–90% confluent) when samples were harvested and analyzed. HUVECs were hormone-deprived

using csFBS for 20 h before 24 h treatment with 17 β -estradiol (E2), TNF α , DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester), and wortmannin. Cells were pretreated with PHTPP in hormone-deprived medium for 20 h before 24-h treatments. 17 β -Estradiol (Sigma) solubilized in DMSO was used. TNF α was solubilized in Milli-Q H₂O. Notch activity was inhibited by treatment with the γ -secretase complex inhibitor DAPT (LY-374973, Sigma). DAPT was dissolved in DMSO to a stock concentration of 5 mM and diluted to a final concentration of 5 μ M in culture medium just prior to use. PHTPP and wortmannin were dissolved in DMSO.

Western blot

Western blot analysis were carried out to detect expression of Notch1, Notch2, Notch4, Jagged1, Dll4, ER α , ER β , p-Akt, total Akt, and β -actin by using the corresponding antibodies as indicated above. Cells were lysed in RIPA buffer (0.05% sodium deoxycholate was freshly added) containing 10 μ g/ml of aprotinin, 10 μ g/ml of leupeptin, 10 μ g/ml of pepstatin A, 1 mM PMSF, and 1 mM sodium orthovanadate on ice for 30 min. Protein concentration of each lysate was quantified by using Pierce BCA Protein Assay Kit (Thermo Scientific, Wilmington, DE). Protein samples were denatured by incubation at 70 $^{\circ}$ C for 10 min in sample buffer (Life Technologies) containing 0.5 M DTT. The same amount of total protein (10 μ g) was loaded in each lane, then proteins were separated on 7% NuPAGE gels (Life Technologies). Proteins were transferred to a PVDF membrane at 30 V for 150 min. Nonspecific binding was blocked by incubating membranes with Tris-buffered saline (TBS), 0.1% Tween, pH 7.6, containing 5% nonfat dry milk or 5% BSA (based on antibodies datasheet) for 1 h at room temperature. PVDF membranes were incubated overnight at 4 $^{\circ}$ C with primary antibodies, washed 4 times in TBS, 0.1% Tween, and incubated for 1 h at room temperature with secondary peroxidase-conjugated antibodies in TBS, 0.1% Tween containing 5% nonfat dry milk or 5% BSA, depending on the antibodies datasheet. Membranes were washed 4 times in TBS, 0.1% Tween and developed using Western Lightning ECL Pro (PerkinElmer Life Sciences). Images of the blots were obtained by exposing them to Chemidoc. Protein immunoreactive bands were analyzed by using the ImageLab analysis software (BioRad). Total transferred proteins were evaluated by Ponceau S staining (Sigma). Because β -actin levels were not affected by any treatment it was used as internal reference for Western blots quantification.

RNA extraction

HUVECs, grown for 20 h in deprivation medium, consisting of phenol red-free M200 medium containing growth factors EGM-2 and 2% csFBS, were exposed to DMSO, E2 (1 nM), and TNF α (10 ng/ml) in deprivation medium for 24 h. Total RNA was extracted using a commercially available kit (Qiagen) and quantified with Nanodrop (Thermo Scientific).

Real-time PCR

500 ng of total RNA were reverse transcribed in a volume of 25 μ l using 250 units of SuperScript III reverse transcriptase and 50 ng of random hexamers. Reaction conditions were as

suggested by the manufacturer. 2 μ l of the cDNA mixture was used for real-time PCR experiments. Real-time PCR was conducted on an Applied Biosystems 7500 Fast Real-Time PCR System using PerfeCta SYBR Green SuperMix with ROX kit (Quanta Biosciences, Beverly, MA) according to the manufacturer's protocol in a final volume of 25 μ l. The primer concentration was 500 nM. The following primers were used: *NOTCH1*, forward, 5'-GTCAACGCCGTAGATGACC-3', reverse, 5'-TTGTTAGCCCCGTTCTTCAG-3'; *NOTCH2*, forward, 5'-CAGATGCGAGTGTGTCCCAGGCT-3', reverse, 5'-TACC-CCGAGTGCCTGGTGGGC-3'; *NOTCH4*, forward, 5'-CAA-CTGCCTCTGTCTGATG-3', reverse, 5'-GCTCTGCCTC-ACACTCTG-3'; Jagged1 (*JAG1*), forward 5'-GACTCATCAG-CCGTGTCTCA-3', reverse, 5'-TGGGGAACACTCACACT-CAA-3'; *DLL4*, forward, 5'-CTGTGCCAACGGGGGACAGTG-3', reverse, 5'-GTGGGCGCAAGGGTTACGGG-3'; ER α (*ESR1*), forward, 5'-TATGTGTCCAGCCACCAACC-3', reverse, 5'-TCGGTCTTTTCGTATCCCACC-3'; ER β (*ESR2*), forward, 5'-AGATTCCCGCTTTGTGGAG-3', reverse, 5'-GAGCAAAGATGAGCTTGCCG-3'; *RPL13A*, forward, 5'-GGAGGTGCAGGTCCTGGTGCTT-3', reverse, 5'-CGTAC-GACCACCACCTTCCGG-3'. Changes in gene expression were calculated by the $2^{-\Delta\Delta C_t}$ formula using *RPL13A* as reference gene.

Short Interfering siRNA transfection and transfection with plasmids

HUVECs were grown in 6-well plates and transfected using Oligofectamine (Life Technologies), with scrambled (control) or Notch1 siRNA, ER α siRNA, and ER β siRNA (Santa Cruz Biotechnologies). Final concentrations used were: 50 nM for Notch1siRNA, 100 nM for ER α siRNA, 200 nM for ER β siRNA, scrambled siRNA (50, 100, or 200 nM, respectively). The siRNAs used are a pool of three target-specific 19–25-nucleotide siRNAs. For overexpression studies HUVECs were grown in 6-well plates and transfected with 1 μ g of pcDNA3 vector encoding human Notch1ICD or the empty vector (a gift from Prof. L. Miele), using Lipofectamine 3000 (Life Technologies). Cell treatments began 24 h after transfection and assays were performed 48 h after transfection.

Apoptosis detection

HUVECs were hormone-deprived using phenol red-free M200 medium containing growth factors EGM-2 and 2% csFBS for 20 h before the 24 h treatment with E2 (1 nM), TNF α (10 ng/ml), and DAPT (5 μ M). Apoptosis was assessed with the Annexin V-FITC binding assay. HUVECs were grown for 24 h in the presence of treatments then cells were collected and stained with Annexin V-FITC (Life Technologies) (100 ng/ml) and propidium iodide (Sigma) (10 μ g/ml) diluted in 1 \times binding buffer (10 mM Hepes, pH 7.4, 5 mM KCl, 150 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂) at room temperature for 20 min. Flow cytometric analysis was performed with BD FACSCalibur (BD Biosciences, San Jose, CA), for each sample, 35,000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA).

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Statistical analysis

Results are expressed as mean \pm S.D. of at least three independent experiments. For comparisons between two groups, two-tailed unpaired Student's *t* tests were used. When more than two groups were compared, one-way analysis of variance was used (Student-Newman-Keuls method for multiple comparisons).

Author contributions—F. F., F. V. D. S., C. C., G. A., and M. P. performed experiments. F. F., F. V. D. S., A. P., and P. R. analyzed the results and wrote the paper. P. R., L. M., and R. F. supervised the project and provided critical revision of data. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgment—We thank Dr. Fabio Casciano (Laboratory for Technologies of Advanced Therapies, University of Ferrara) for technical support during the analysis of the apoptosis by flow cytometry.

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J. Biol. Chem. 2017, 292:18178-18191.

doi: 10.1074/jbc.M117.790121 originally published online September 11, 2017

Access the most updated version of this article at doi: [10.1074/jbc.M117.790121](https://doi.org/10.1074/jbc.M117.790121)

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