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COORDINATORE Prof. Francesco Di Virgilio

Estrogen-mediated protection of the vascular endothelium requires Notch1

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Dottorando
Dott.ssa Francesca Fortini

Tutor
Prof. Roberto Ferrari

Co-Tutor
Dott.ssa Paola Rizzo

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ABSTRACT

Premenopausal women benefit from cardiovascular protection compared to age-matched men. Estrogens exert a protective action against endothelial cells (ECs) apoptosis, one of the hallmarks of endothelial dysfunction leading to cardiovascular disorders, but the molecular mechanisms underlying this effect remain poorly understood. The inflammatory cytokine tumor necrosis factor α (TNFα) causes ECs apoptosis while dysregulating the Notch pathway, a major regulator of ECs survival. We have previously reported that treatment with 17β-estradiol (E2) activates Notch signaling in ECs. Based on the observation that TNFα and E2 have opposite effects both on ECs apoptosis and on Notch signaling, the aim of this study was to determine whether, under inflammatory conditions, Notch is involved in E2-mediated protection against endothelial cells apoptosis. With this aim, we evaluated also the possible role of estrogen receptor (ER) α and/or β in the E2-mediated action. Human umbilical vein endothelial cells (HUVECs) were treated with E2 and/or TNFα and the effects on apoptosis and on the Notch pathway were investigated. We found that TNFα-induced apoptosis was counteracted by E2. When Notch1 was inhibited, the E2-mediated protection was not observed, whereas Notch1 ectopic overexpression diminished TNFα-induced apoptosis. In addition, TNFα reduced the levels of active Notch1 protein, which were partially restored by E2 treatment. Furthermore, we show that TNFα-mediated Akt phosphorylation is Notch1-dependent and E2 enhances this effect. Moreover, treatment with PHTPP (ERβ-antagonist) or with siRNA against ERβ or with PPT (ERα-agonist) abolished E2's effects on apoptosis and on active Notch1. Conversely, treatment with siRNA against ERα or with DPN (ERβ-agonist) did not inhibit the E2-mediated effects on apoptosis and on active Notch1. In summary, the data reported indicate that E2, through a mechanism involving ERβ, requires active Notch1 to protect the vascular endothelium. These findings could be relevant when assessing efficacy and applicability of menopausal hormone treatment, since they indicate that in subjects with impaired Notch signaling, due to pathological conditions, hormone therapy might not provide an effective protection to the vascular endothelium.

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1. INTRODUCTION

1.1 Role of the estrogens in the cardiovascular system

Despite many efforts to improve primary prevention and treatment, cardiovascular disease remains the leading cause of death in the world for both women and men. It has been found that in pre-menopausal women the incidence of cardiovascular disease, such as coronary heart disease, heart failure, and, atherosclerosis is lower compared to post-menopausal women and age-matched men (1,2). The gender-associated differences in the development of cardiovascular disease have been widely described both in human and animal models, and the impaired estrogen signaling has been viewed as a main causal factor (3,4). However, the mechanisms by which estrogens and estrogen receptors (ERs) regulate women's cardiovascular function remain largely unknown; and there is still confusion about the best therapeutic approaches to improve cardiovascular health in menopausal women (5).

Estrogens are highly conserved steroid hormones produced in all vertebrates and some invertebrates (6). Four main forms of estrogen are found in mammals: estrone, 17β-estradiol (E2), 17α-estradiol and estriol but, E2 seems to play a major role in the cardiovascular protective action (6). In healthy pre-menopausal women, over 90% of estradiol is produced by the ovaries where the precursor, androstenedione, is metabolized to estrone and then converted to estradiol (7). The conversion of androgens to estrone and then estradiol is mediated by aromatase, an enzyme belonging to the cytochrome P450 family (8). The aromatase is widely distributed in gonadal and extra-gonadal tissues, including the bone, brain, muscle, adipose tissue, blood vessels (9) and, several lines of evidence indicate that aromatase is also expressed in the heart (10,11).

The concentration range of estradiol varies between women and men, and it is affected by menopausal status and the menstrual cycle (6):

- Men: 20-55 pg/ml (12)
- Early follicular phase of menstruation: 21-72 pg/ml (13)
- Late follicular phase of menstruation: 53-312 pg/ml (13)
- Luteinizing hormone peak in menstruation: 131-388 pg/ml (13)
- Early luteal phase of menstruation: 48-154 pg/ml (13)
- Mid-luteal phase of menstruation: 72-207 pg/ml (13)
- Late-luteal phase of menstruation: 27-214 pg/ml (13)
- Menopause: 30 pg/ml (14).

Several clinical studies have shown that estrogens are directly involved in the protective actions against cardiovascular injury. In response to aortic stenosis (15-17), men have more maladaptative cardiac remodeling compared to the pre-menopausal women. Moreover, transcriptome characterization reveals that fibrosis and inflammation-related genes and pathways are upregulated in men but not in women (17) (18). Pre-menopausal women also withstand ischemia/reperfusion (I/R) injury better than men (19), and they are less subjected to develop myocardial infarction (MI) than age-matched men (20). Women with a non-ischemic etiology of heart failure show higher ejection fraction and better survival than men with non-ischemic heart failure (21). In line with these clinical data, sex differences have also been found in animal models of human cardiovascular disease. In mouse and rat models of I/R, females show significant higher post-ischemic recovery of left ventricular (LV) function and smaller infarct size compare to males (22). Additionally, there are studies *in vivo* that show the protective effects of estradiol in animal models of transverse aortic constriction (TAC) (23) and ischemia/reperfusion injury (24). However, estrogens play an important role in the protection against cardiovascular disease also in men. In fact, men with abnormal low (< 12.90 pg/mL) or high (> 37.40 pg/mL) estrogen level show the highest death rates from congestive heart failure (25). These data suggest that

estrogen modulates cardiovascular disease development and outcome in both sexes and it should be considered in the prognosis and treatment of cardiovascular disease. In addition to the estrogens, other sex hormones, such as progesterone and testosterone, are important contributors to sex differences (26), but their role is still poorly investigated.

A complete understanding of the molecular pathways modulated by steroid hormones could lead to improved novel approaches to re-establish post-menopausal hormonal balance and to reduce the risk of cardiovascular disease.

1.1.1 Hormone Replacement Therapy (HRT)

Estrogen has beneficial effects on the cardiovascular system, such as on vascular remodeling, endothelial relaxation, development of hypertrophy and cardioprotection. This evidence has formed the basis for the use of estrogen therapy and estrogen with progestin therapy to prevent cardiovascular disease in post-menopausal women. However, the relationship between hormone replacement therapy (HRT), both based on estrogen alone or combined with a progestin, and the prevention of cardiovascular disease, in particular coronary heart disease (CHD), is more complex than was initially thought.

More than ten years ago, two large prospective clinical trials, the Women's Health Initiative (WHI I and II, funded by the National Institute of Health in the USA) and the Heart and Estrogen/progestin Replacement Study (HERS), have tried to objectify the benefits and risks of hormone therapy (estrogen alone or with progestin), by determining the incidence of heart disease, breast and colorectal cancer, and fractures in post-menopausal women under hormone therapy. The WHI enrolled a cohort of mostly healthy, ethnically diverse women, spanning a large range of age, from 50 to 79 years at baseline. The primary outcome for the WHI trial of estrogen plus progestin was designed as CHD; the secondary

outcomes were: other cardiovascular diseases, stroke, endometrial cancer, colorectal cancer, other cancers, hip fracture, and other fractures (27). The WHI showed that estrogen plus progestin does not confer benefit for preventing CHD among women with a uterus. This result is in accordance with HERS findings among women with clinically apparent CHD (28), with the Estrogen Replacement for Atherosclerosis trial, in which estrogen plus progestin did not inhibit progression of atherosclerosis (29), and with a trial in women with unstable angina, in which was not observed any reduction in ischemic events (30). Moreover, the WHI results showed that the excess risk of stroke in the estrogen plus progestin group was not present in the first year but appeared during the second year and persisted through the fifth year (27). The findings in WHI for stroke are consistent with those of HERS (31) and of the Women's Estrogen and Stroke Trial of estradiol (without progestin) in women with prior stroke, which found no effect of estrogen on recurrent strokes overall, but some increase in the first six months (32). Therefore, it appears that estrogen plus progestin increases the risk of stroke in apparently healthy women. In accordance with HERS and several other observational studies, the WHI results showed an increase of venous thromboembolism after hormone therapy use (33,34). Moreover, the WHI confirmed that combined estrogen plus progestin increases the risk of incident breast cancer. Interestingly, the risk of breast cancer emerged several years after randomization, and this is consistent with the result obtained from HERS study. Conversely, the risk of endometrial cancer and colorectal cancer was reduced after hormone therapy (27). Furthermore, for the first time, the WHI showed the ability of post-menopausal hormones therapy to prevent fractures at the hip, vertebrae and other sites (27).

To explain the non-protective effect of hormone therapy against cardiovascular disease, there is one popular hypothesis, called the *timing hypothesis*, which theorizes that estradiol has harmful vascular effects in elderly women in contrast to neutral or beneficial effects in younger women (35). In accordance with the *timing hypothesis*, Glisic et al. have studied the plaque composition and they have seen

that the risk of stroke increases with increasing levels of estradiol in women far from menopause (36). Moreover, in line with the *timing hypothesis*, the lack of association between estradiol and risk of stroke in postmenopausal women without carotid atherosclerosis indicates that endogenous estradiol might have deleterious effects only in women with underlying atherosclerosis, hence many years after the onset of menopause (36). Based on this data, HRT should be taken with caution among postmenopausal women who already have diagnosis of carotid atherosclerosis and are further from menopause. A recent randomized study that administrated HRT to women (42-58 years old) within 3-36 months following the onset of menopause, supports the *timing hypothesis*, as certain risk factors for cardiovascular disease were reduced (37). Similarly, another randomized study in women (45-58 years old) showed a significantly reduced risk of mortality, heart failure, or myocardial infarction if HRT was initiated early after menopause (38).

In addition to the *timing hypothesis*, other limitation of the previous WHI study could be that it tested only one drug regimen in post-menopausal women. Moreover, the results do not necessary apply to other dosages, to different time of administration and to other kind of administration (oral versus transdermal). The drug formulation is also a critical point, in fact, it has been shown that the medroxyprogesterone acetate, that is commonly used in combination with estrogen, has adverse action on the endothelial function (39). The switch from protective to harmful estradiol effect could be because of changes in estrogen receptor signaling (40) or it is a consequence of age-related hyper-inflammatory state (41). Thus, further studies are needed to examine the expression of ERs in older women and to understand if the efficacy and applicability of HRT changes with age. Without a better understanding of the complexity of estrogen-ER signaling and the crosstalk with other pathways in the heart and in the vasculature, it will be difficult to unravel the mechanisms by which estrogen might be involved in protection in pre-menopausal women and why it fails to protect post-

menopausal women. This lack of understanding can also complicate the design and interpretation of clinical trials.

1.2 Estrogen Receptors: structure, function and localization

The physiological effects of estrogens are mediated by estrogen binding to ERs. The ERs were among the first of nuclear receptor superfamilies to be cloned, second only to the glucocorticoid receptor (GR) superfamily, cloned in 1985 (42-44). The cellular effects of estrogen are mainly mediated by two ERs: i) ESR1 is the gene that encodes ERα (595 amino acids), localized on chromosome 4 and, it was identified in 1958 (45); ii) ESR2 is the gene that encodes ERβ (530 amino acids), localized on chromosome 6 and, it was first identified in the rat prostate and ovary in 1996 (46). ERs have several different splice variants, and each one exhibits distinct tissue-specific expression and function. Like the other members of classical nuclear receptors, also ERα and ERβ are composed of six conserved functional domains, from A to F. A/B represent the N-terminal domain (NTD), which contains the activating transcription function 1 (AF-1), being responsible for ligand independent transcription activation. The highly conserved domain C is responsible for DNA binding via two zinc finger structures. D is the hinge region, responsible for recruiting and binding of co-modulators, while E and F contain the ligand-binding domain (LBD), which, together with activating transcription function 2 (AF-2), is the ligand dependent activator of transcription (Fig. 1). AF-1 and AF-2 are sites of protein-protein interaction between co-regulators. The D domain is an unstructured hinge and allows for flexibility between DNA and ligand binding domains. The E domain contains the dimerization surface region and the F domain is thought to be involved in protein stability. Apart from the DNA-binding domain C, which is 97 % similar, and the ligand-binding domain, which is 60 % similar, the homology between ERα and ERβ is low (≈ 17 %), and it is useful to facilitate the development of receptor-specific selective ligands.

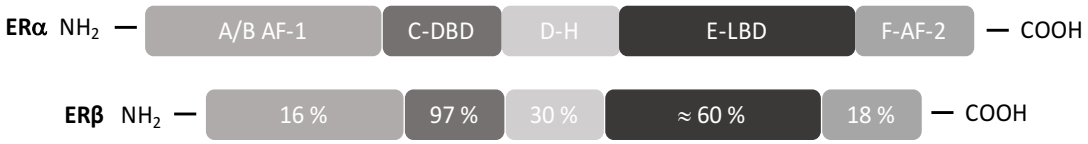


Figure 1. Structural description and percent sequence homology of human ERα and ERβ. Figure shows comparison of human ERα (595 aa) and a shorter ERβ (530 aa). These receptors are evolutionarily conserved and have five distinct structural and functional domains: DNA-binding domain (DBD; C domain), hinge domain (D), ligand-binding domain (LBD; E/F domain), and two transcriptional activation function domains AF-1 (in A/B domain) and AF-2 (in F domain). The binding of ligand (estrogen) to E domain results in conformational changes in the receptor (homo/hetero dimerization). The receptor dimer than translocates into the nucleus with the help of D domain. This D domain is also important for post-translational modifications of receptor by acetylation, lipophilic moieties, and ubiquitination. The C domain then recognizes and binds to estrogen-response element (ERE) in DNA.

ERα and ERβ are localized both in associations with the plasma membrane, in the cytosol, in the nucleus (47), and also in the mitochondria membrane (48). Estrogens can have a *genomic* effect: they diffuse through the plasma membrane and form complexes with cytosolic and nuclear ERs that alter gene expression by: i) directly binding to DNA; ii) indirectly binding to DNA (through other transcription factors) or iii) ligand-independent binding (Fig. 2). The nuclear ERs act as ligand-gated transcription factor. ER monomers in the cytosol form protein complex with chaperone heat-shock proteins. Ligand-mediated activation of the ER determines a conformational change in the receptor and promotes the dissociation of the ER monomers from the complex with chaperone heat-shock proteins and their subsequent dimerization with other free ER monomers. Dimers can be in the form of ERα-ERα or ERβ-ERβ homodimers or in the form of ERα-ERβ heterodimers. ER dimers enter into the nucleus, where they bind to consensus ERE (estrogen-response elements) sites on the DNA with the help of co-activators or co-repressors and drive the expression of target genes (1). ER dimers can also bind to the DNA indirectly, via transcription factors, such as activator protein-1 (AP1) and specificity protein-1 (Sp1). Furthermore, ER can be phosphorylated allowing it to bind to ERE or to transcription factors and to modulate gene transcription in the absence of ligand binding. The expression of a wide range of genes can be

induced or inhibited, depending on: the cell type, the presence of different transcription co-factors, the type and concentration of the ligand and the type of ER dimers (49). The ligand-mediated activation of the receptors (ERs), localized on the mitochondria can trigger transcriptional changes in mitochondrial genes (50). In addition to ligand-induced activation of ERs, it has been described also ligand-independent pathway. Growth factor signaling leads to activation of kinases that may phosphorylate and thereby activate ERs in the absence of ligand (Fig. 2) (51).

A rapid response of estrogen was first reported in 1960s, when Pietras and Szego showed that estrogens increased the cyclic adenosine monophosphate (cAMP) concentration within few minutes (52), and they paved the way for studies on the *non-genomic* response of estrogens. It is now established that estrogen can have rapid intracellular effects, which occur independently of protein synthesis or gene activation. These acute effects of estrogen are transmitted by signaling pathways through activation of ERs localized at the plasma membrane, in particular in caveolae and lipid rafts. In this case, ERs can activate the phosphoinosite 3-kinase (PI3K) signaling pathway, which activates other pathways, such as the Ca^{2+}, cyclic adenosine monophosphate (cAMP) and nitric oxide (NO) pathways, leading to the activation of tyrosine kinase receptor, such as insulin-like growth factor receptor (IGF), epidermal growth factor receptor (EGF) and protein kinase B (PKB) (53) (Fig. 2).

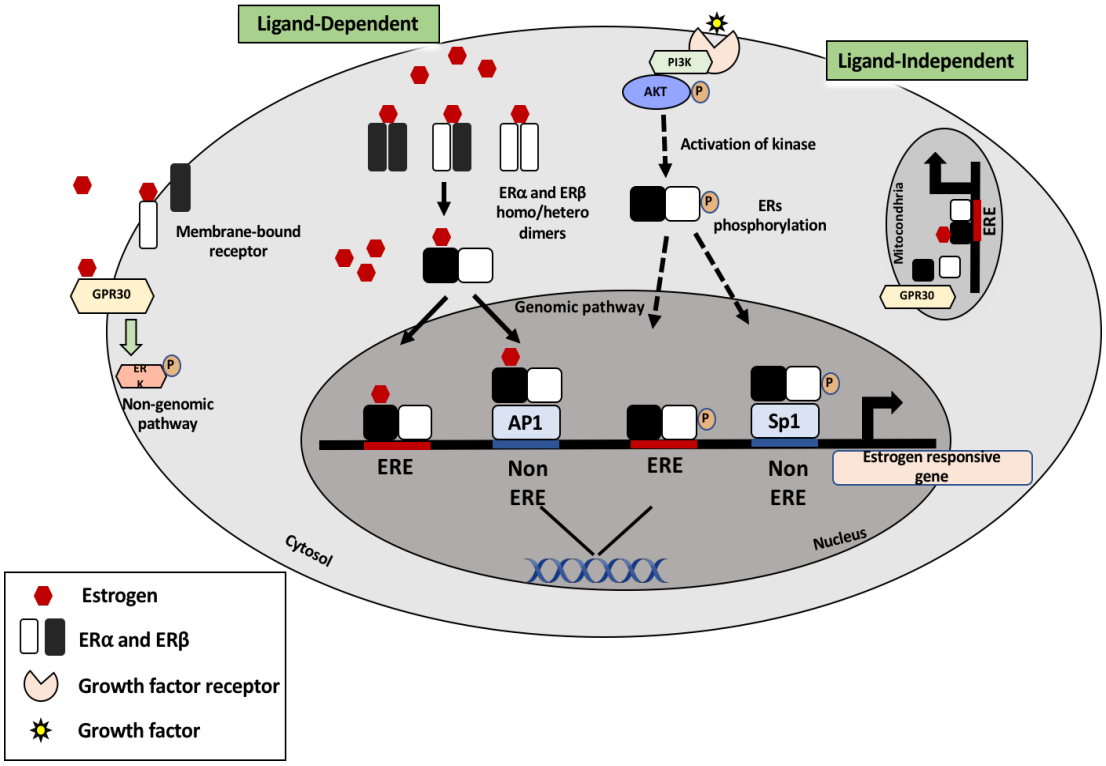


Figure 2. Schematic representation of estrogen receptor ligand-dependent and ligand-independent signaling. ER-mediated signaling occurs in a ligand-dependent (solid arrows) and ligand-independent (dashed arrows). The ligand-dependent pathway is triggered by binding of either endogenous hormone or a synthetic compound to the ligand-binding domain of ERs in the cytosol. Different ligands induce unique conformational changes of ERs, and receptor dimerization (homodimers: ERα:ERα or ERβ:ERβ or heterodimer: ERα:ERβ), which then translocate into nucleus and bind to specific EREs (consisting of a 5-bp palindrome with a 3-bp spacer; GGTCAnnnTGACC) in the regulatory regions of estrogen responsive genes. This is also called "classical" signaling pathway. In "tethered" signaling pathway, ligand-activated ERs interact with other transcription factor complexes and bind to non-EREs by binding to other transcription factors and not directly to ERE. In third ligand-dependent "non-genomic" pathway, ligand binding to plasma membrane-bound ERs via palmitoylation on cysteine 447 results in activation of cytoplasmic signaling pathways. In ligand-independent signaling pathway, there is phosphorylation/activation of ERs by other active signaling cascades in a cell. This activation results in both direct ERE and non-ERE dependent genomic actions. Estrogens also bind to ERs localized on the mitochondrial membrane, thus modulating mitochondrial genes expression and improving mitochondrial function. Abbreviation: Akt, protein kinase B; AP-1, activator protein 1; Sp1, specifity protein 1; ERE, estrogen-response element; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GRP30, an orphan G-protein coupled receptor 30; PI3K, phosphatidylinositol-4, 5-bisphosphate 3-kinase; P, indicates phosphorylation.

The two ERs are differently distributed in many organs (liver, brain and, kidneys), including bones, muscles and cardiovascular (heart and vasculature) and

reproductive system (54), where they have different expression patterns and functions. The ERs are expressed in the neonatal and adult heart and they are present in both genders. In the heart, expression of both ERs has been detected in cardiomyocytes, endothelial cells, vascular smooth muscle cells (VSMCs), and cardiac fibroblasts (55).

In addition to the classical ERα and ERβ, a third membrane bound ER has recently been identified, G protein-coupled estrogen receptor 1 (GPER1 or GPR30). GPR30 can localize both in plasma membrane and in specific intracellular sites, as endoplasmic reticulum, Golgi apparatus and mitochondria (56). It has been implicated in a rapid, *non-genomic*, estrogenic signaling (57) but, its function has been still studied. GPR30 is expressed in endothelial cells, cardiomyocytes, vascular smooth muscle cells, and evidence suggests that it mediates many of the estradiol protective effects on the vasculature (58). In addition, GPR30 seems to mediate cardioprotection following ischemic and hypertensive injury (59,60). Bopassa et al. have shown that activation of GPR30 reduces infart size in the isolated perfused male mice heart, by inhibiting opening of the mitochondrial permebility transition pore (mPTP) (61). Together these results suggest that GPR30 could be involved in the cardioprotection mechanisms.

1.2.1 ERα versus ERβ

Understanding the individual role of ERα and ERβ in the cardiovascular system has become increasingly complex because the expression and localization of ERs are tissue- and disease-dependent. Studies using mice that lack functional ERs suggest that the presence of one or both ERs is required to protect against heart damage induced by myocardial infarct or I/R injury (59). To further complicate the analysis of ERs-regulated genes, ERα and ERβ can regulate distinct genes in a time, sex, disease and tissue-dependent manner. Using ovariectomized (OVX) mice, lacking both ERα and ERβ, which were treated with estrogens for one week,

O'Lone et al. showed that, in the mouse aorta, ERα and ERβ regulate gene expression in opposite direction (62). In particular, estrogen activation of ERα up-regulated expression of genes that were down-regulated by ERβ activation. In particular, ERα up-regulated genes related to extracellular matrix synthesis, electron transport in the mitochondria and reactive oxygen species pathways (62), whereas ERβ activation down-regulated them. In contrast to the previous study, a gene array study in OVX female mice heart showed an opposite effect of ERβ, that primarily up-regulated gene expression (63). These conflicting data might be because of differences in the type of cells (aorta versus ventricular myocytes) or because of different time and different concentration of estrogen treatment. Studies in vitro have also showed the opposite action of the ERs. In endothelial cells, ERα up-regulates endothelial nitric oxide synthase (eNOS), whereas in cardiac muscle, ERβ, and not ERα, mediates the up-regulation of eNOS (64,65). In vascular smooth muscle cells (VSMCs), ERβ increases, while ERα decreases the levels of eNOS (66). Furthermore, it is thought that the different effects of ERα and ERβ on gene expression could be due to tissue and temporal variation in the protein level of these receptors and to the presence of different co-activators and co-repressors.

The investigation of differential effects and roles of ERα and ERβ is facilitated by commercially available ERs-agonists and -antagonists: ICI 182.780 is a selective estrogen receptor downregulator (SERD), 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) is an ERα-specific antagonist (67), 4,4',4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) is an ERα-specific agonist (67), 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP) is an ERβ-specific antagonist and 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN) is an ERβ specific agonist (68).

The pharmacological aim in the development of new SERMs has been to elicit specific positive effects on certain targeted tissues such as bones, heart and brain with neutral or antagonist effects of other tissues, such as breast and

endometrium, where long-term estrogen stimulation may be harmful (69). Currently, two SERMs are approved by the Food and Drug Administration (FDA) for clinical treatment: tamoxifen and raloxifene.

Tamoxifen is a SERM with predominant estrogen antagonist effect in the breast, and estrogen agonist activity in the bone (70), cardiovascular system (71), uterus (72), liver (72), and vagina (73). Tamoxifen is used for the treatment of breast cancer, but because of his agonist action in the uterus, prolonged treatment increases the risk for endometrial cancer (74,75). However, clinical observations have shown that treatment with tamoxifen reduces the incidence of ischemic heart disease and coronary atherosclerosis and decreases total serum cholesterol and (high-density lipoprotein-C) HDL-C, and it has also a vascular relaxation effect (76).

Like tamoxifen, raloxifene is an ER antagonist in breast and an agonist in bone, but it does not exert ER agonist properties in the uterus (74,75). Furthermore, raloxifene has actions similar to estrogen on the cardiovascular system, in terms of reduction of the concentration of serum cholesterol and low-density lipoprotein (LDL) and improvement of endothelial function by the induction of vasodilation and through nitric oxide (NO) synthesis in the endothelial cells (77). These cardiovascular protective properties of raloxifene provided the basis for the Raloxifene Use for The Heart (RUTH) study. The RUTH was a randomized trial, that studied raloxifene ability to decrease the incidence of myocardial infarction, coronary disease and acute coronary syndrome. In RUTH, it was observed a reduced risk of invasive breast cancer, but no effects on prevention of coronary heart disease (78). Hence, in term of cardiovascular protection, we could say that tamoxifen is better than raloxifene.

Knowing that the estrogen's protective action is mediated by ERα and/or ERβ could lead to development of SERMs, that specifically target the single receptor, thus reducing the unwanted side effects. Further studies are needed to investigate

its use for hormone therapy and to better characterize the factors that contribute to tissue-specific ERs regulation of gene expression and protein levels in the cardiovascular system (56).

1.3 Endothelial function

The endothelium is an highly selective barrier and metabolically active organ and it plays an important role in several features of vascular physiology, regulating vasoconstriction/vasodilatation, thrombosis/fibrinolysis, inflammation, and angiogenesis (79). Indeed, the functional endothelium is a fundamental element of vascular health. Endothelial monolayer separates blood, where lipoproteins and cellular participants (monocytes and lymphocytes) of atherosclerotic processes normally occur, from the arterial wall. Because of its position, the vascular endothelium is subjected to different kinds of signal both chemicals (growth factors, cytokines, hormones, circulating lipids, ROS) and mechanicals (hemodynamic forces), which they can have pro- or anti-atherogenic effects. Alterations of endothelial homeostatic mechanisms, which are known as endothelial dysfunction and, precede the loss of endothelial integrity and denudation of the arterial wall, are encountered in pathological states favoring cardiovascular disease, such as atherosclerosis, hypertension, and hypercholesterolemia (80) (81). Endothelial dysfunction has been recognized as a prognostic parameter of progression of vascular diseases, that could be reversible, at least partially, through risk factors modifications (82-84).

1.3.1 Role of estrogen in the vascular endothelium

There is a strong evidence of an association between endothelial dysfunction and reduced endogenous production of estrogens, after natural or surgical menopause

in women (85). *In vitro* studies, repeatedly, demonstrated that 17β-estradiol (E2), 17β-estradiol metabolites and, synthetic estrogens improve vascular function, by acting on endothelial cells and vascular smooth muscle cells (VSMCs), and reduce the incidence of atherosclerosis (6). Both estrogen receptors, ERα and ERβ, seem to be involved in this protective effect (1). In the vascular endothelium, one of the best-described protective action estrogen-mediated is the endothelial nitric oxide synthase (eNOS) activation and nitric oxide (NO) production, which depends on both *genomic* (expression of eNOS) and *non-genomic* action (activation of phosphatidylinositol 3-kinase and protein kinase Akt, phosphorylation of eNOS) (86,87). eNOS stimulates proliferation and migration of endothelial cells and promotes re-endothelialization, thus mediating protection in case of vascular injury (88). NO has a protective effect on the vasculature also because it is a potent vasodilator (89), it reduces inflammation (90), platelet aggregation and adhesion, and it inhibits the proliferation of VSMCs (91). Some studies show that estrogen modulation of NO production is mediated by ERα (86,92), whereas others show the involvement of ERβ (93,94). On the endothelium, estrogens limit the expression of molecules that are involved in monocyte and neutrophil adhesion to the endothelial monolayer (95,96), thereby preventing the production of cytokines and the migration of leukocytes to the sub-endothelial space (97). Furthermore, it has been shown that E2 plays a role in angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF), an important angiogenic factor (98). Furthermore, in endothelial cells, E2 treatment positively modulates the production of endothelium-derived relaxing factors, such as prostacyclin (PGI2) or the endothelium-derived hyperpolarizing factor (EDHFs) (77). PGI2 increases synthesis of cyclooxygenase 1 and/or 2 (COX1 (PTGS1) and COX2 (PTGS2) and prostaglandin synthase (PGH2), which has a vasodilatory effect. Both of ERs seems to be involved in E2-dependent PGI2 production (99). In addition to the release of vasorelaxants, estrogens also prevent the activation of endothelium, which is a hallmark of endothelial dysfunction. E2 and its metabolites reduce the cell-surface expression of Spred-1 and vascular cell adhesion molecule-1 (VCAM-1) via miR-126-3p (100), intercellular cell adhesion (ICAM-1), E-selectin, P-

selectin, CD40, CD40L (95) in endothelial cells exposed to pro-atherogenic factors, such as TNFα, IL-1β, lipopolysaccharide (LPS), IFNγ or lysophosphatidylcholine (LPC) (80). In addition to beneficial effects on endothelial function, estrogen induces increase in junctional protein levels, leading an improvement in vascular structural integrity and barrier function (101), which can reduce its permeability to pro-atherogenic factors, such as native and oxidized LDLs (102). Furthermore, estrogens promote endothelial cells proliferation and survival. In fact, endothelial cells apoptosis induced by TNFα, H_2O_2 or, oxidized LDL is significantly inhibited in the presence of estrogens, by a mechanism that involve activation of protein kinases MAPK and Akt, increased expression of anti-apoptotic proteins Bcl-2 and Bcl-XL, and disabling of the pro-apoptotic protein Bad (103-107). In addition, acting through ERs, estrogens treatment reduces the NADPH oxidase activity and thereby the mitochondrial production of reactive oxygen species (ROS) (108), as well as ROS-induced apoptosis by interfering with cytochrome c release from mitochondria (109,110). Finally, E2 increases the activity of hTERT, the telomerase catalytic subunit, that has a pivotal role in the determination of cell lifespan (111). Several protective effects of estrogens observed *in vitro*, in particular in endothelial cells, could be also recapitulated in animal models in the context of atherosclerotic disease. The main estrogen-mediated effects in the vascular endothelium are synthetized in Fig. 3.

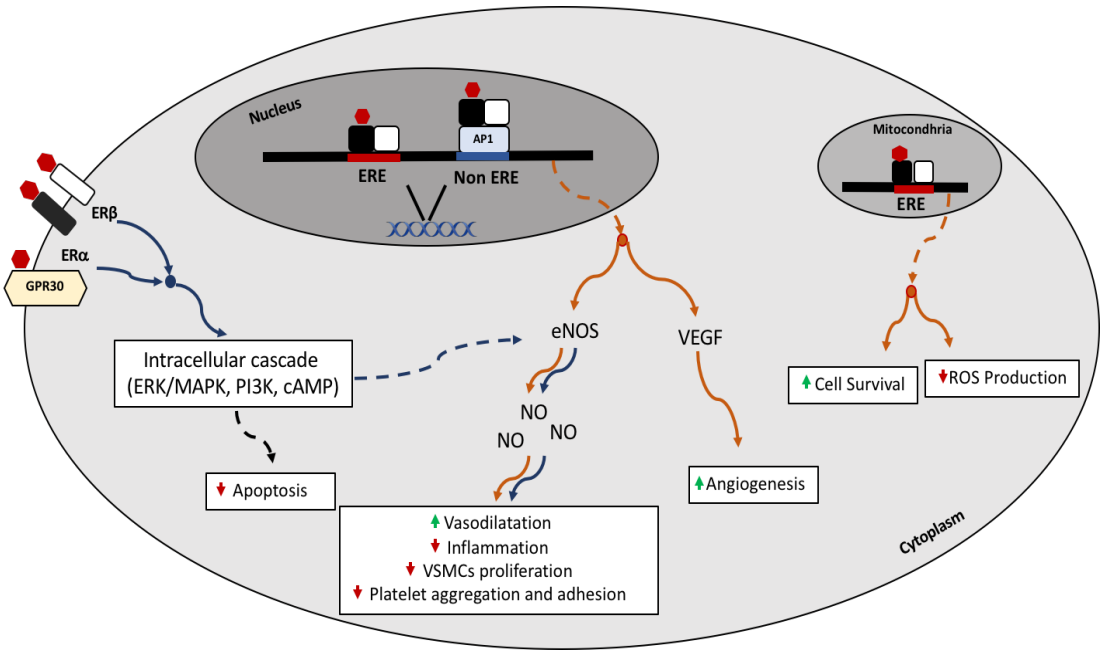


Figure 3. Main estrogen effects in the vascular endothelium. In *genomic* regulation, estrogen regulates transcription of target genes, including VEGF, a pro-angiogenic factor and eNOS, that induces vasodilation, reduces inflammation, vascular smooth muscle cells (VSMCs) proliferation, and platelet aggregation and adhesion. In *non-genomic* regulation, binding of E2 to ERs and GPR30, localized at the plasma membrane, leads to activation of MAPK/ERK/PI3K/cAMP, which induce gene expression including eNOS and a cascade signaling that leads to inhibition of apoptosis. E2 also binds to ERs localized on the mitochondrial membrane improving mitochondrial function by decreasing ROS production and increasing cell survival. *Genomic* pathway is shown in orange arrows, whereas *non-genomic* pathway is shown in blue arrows. Dashed arrows indicate transcription activity.

1.3.2 The role of the Notch pathway in the vascular endothelium

The Notch signaling pathway, originally discovered in Drosophila, is an highly conserved pathway that plays a central role in several cellular processes, such as proliferation, stem cells maintenance, and differentiation during both embryonic and adult development (112). It is a short-range communication system between two adjacent cells, based on a ligand-activated receptor. The Notch receptors contain an extracellular domain that includes multiple epidermal growth factor (EGF-like) repeats, essential for ligand binding. The intracellular portion is necessary for signal transduction and it contains RBP-Jk association module

(RAM) domain, a nuclear localization signal (NLS), a seven ankyrin repeat (ANK) domain, and a transactivation domain (PET) (113) (Fig. 4). The ligands for Notch receptors are varied, and mammals have five ligands (Delta-like (DLL) 1, 3, 4 and Jagged 1, 2), that have multiple EGF-like repeats in their extracellular domains. The Notch ligands contain an N-terminal sequence that along with the DSL (Delta/Serrate/Lag2) motif and the first two EGF-like repeats are required for ligand-receptor binding. In contrast to the DLL ligands, the Jagged ligands have almost twice the number of EGF repeats and also contain an additional cysteine-rich region. The intracellular portion is similar between ligands and some ligands, but not all, contain multiple lysine residues and C-terminal PDZ (PSD-95/Dlg/ZO-1) domain (Fig. 4).

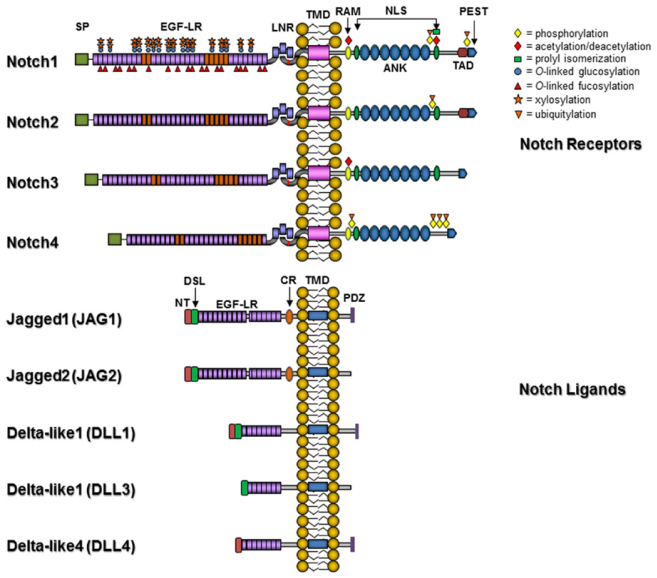


Figure 4. Notch receptors and ligands. SP, signal peptide; EGF-LR, epidermal growth factor-like repeats; LNR, Lin-Notch repeat; TMD, transmembrane domain; RAM, RBP-Jκ association module; NLS, nuclear localization signal; ANK, ankyrin/CDC10 repeat; TAD, transactivation domain; PEST, proline/glutamic acid/serine/threonine-rich motif; PDZ, PSD-95/Dlg/ZO-1 domain; CR, cysteine-rich domain; DSL, Delta/Serrate/Lag2 domain; NT, N-terminal domain. Posttranslational modifications are indicated by symbols: yellow diamonds, phosphorylation; red diamonds, acetylation/deacetylation sites; green square, prolyl isomerization site; blue circle, O-linked glucosylation; red triangle, O-linked fucosylation; orange star, xylosylation; and inverted orange triangle, ubiquitylation (113).

Notch receptors and ligands are both transmembrane proteins located on the surface of the cells. Notch receptors are synthesized as single-chain precursors and cleaved into an extracellular and a trans-membrane subunit in the Golgi apparatus. These two subunits are held together on the cell membrane by non-covalent bonds. Ligand binding allows the first proteolytic cut of Notch trans-membrane domain by a surface protease, A Disintegrin And Metalloprotease (ADAM10 or ADAM17), which removes the extracellular portion of Notch and creates a membrane tethered intermediate that is a substrate for γ-secretase, an intramembranous aspartyl-protease complex. This last cut releases the active form of Notch (Notch intracellular domain, NICD) which translocates into the nucleus, where it binds the transcriptional factor CBF-1/Suppressor of hairless/ Lag-1 (CSL), also known as recombination signal sequence binding protein J-kappa (RBP-Jk or RBPJ). NICD binding displaces a co-repressor complex (Co-R), and promotes the recruitment of co-activator molecules (Co-A) and the transcription of Notch target genes such as Hairy/enhancer of split (Hes), Hes-related proteins (Hey), and Notch-regulated ankyrin repeat protein (Nrarp). These factors regulate further downstream genes involved in the regulation of cell cycle (114), apoptosis (115) and stemness (116) (Fig. 5). Recent studies have shown that the number of transcriptional target genes is even higher than originally thought (117). The latter is commonly defined *canonical* Notch signaling pathway. During the past years, data have been accumulating on the *non-canonical* Notch signaling, which is independent of CSL, and it allows the interaction with Wnt/β-catenin, mTORC2/Akt and IKKα/β pathways at the cytoplasmatic level. *Non-canonical* Notch signaling is also associated with mitochondria, where it has been shown that Notch/PINK1 (PTEN-induced kinase 1) interaction influences mitochondrial function and activates mTORC2/Akt pathway (118-120), promoting a pro-survival pathway (Fig. 5).

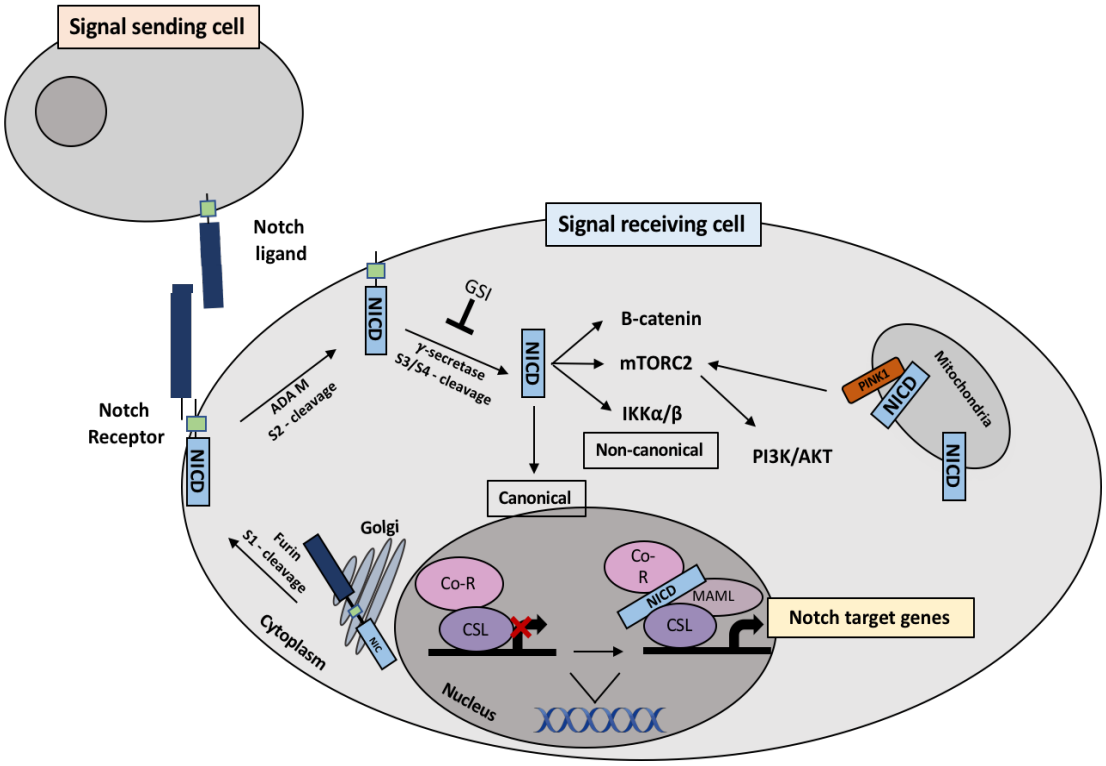


Figure 5. The Notch signaling pathway. The mature receptor is produced after proteolytic cleavage by furin at site 1 (S1). It is then targeted to the cell surface as a heterodimer that is held together by non-covalent interactions. The Notch receptor is activated by binding to a ligand presented by neighbouring cell, which induces a cleavage at site S2 mediated by ADAM family proteinases followed by a cleavage at S3 and S4 within the trans-membrane domain mediated by the γ-secretase complex. These proteolytic cleavages allow the Notch intracellular domain (NICD) to translocate into the nucleus. In the nucleus, NICD is associates with a transcription factor, RBP-Jκ (CSL), and activates transcription from the RBP-Jκ DNA-binding site. In the absence of NICD, CSL may associate with co-repressor (Co-R) proteins to repress transcription of some target genes. The non-canonical Notch signaling is independent of CSL and allows for interaction with Wnt/β-catenin, mTORC2 and IKKα/β pathway at the cytoplasmic level. Non-canonical Notch signaling is also associated with mitochondria, where Notch/PINK1 interaction influences mitochondrial function and activates mTORC2/Akt pathway. CSL indicates CBF-1/RBP-Jκ/Suppressor of hairless/Lag-1; Co-R, co-repressor; Co-A, co-activator; MAML, mastermind-like; ADAM, a disintegrin and metalloprotease; GSI, γ-secretase inhibitor; PINK1, PTEN-induced kinase 1.

The vascular endothelium expresses three receptors: Notch 1, 2, 4 (121,122), and four ligands: Delta-like 1, 4 (Dll1, Dll4) and Jagged 1, 2 (Jag1, Jag2) (123,124). In the endothelium, the Notch signaling influences a wide range of cellular processes and it is essential both during embryonic development and the adult life, in which it

regulates vascular endothelial functions by crosstalk with pathways modulated by inflammatory cytokines, such as tumor necrosis factor α (TNFα) (125) and interleukin 1β (IL1β) (126), or by pro-angiogenic factors, such as the vascular epidermal growth factor (VEGF) (127). Systemic loss of-function mice models for receptors Notch1, Notch2 and, ligands Jagged1, Jagged2, and Delta-like 4 determines an impaired vascular development, that often causes embryonic or neonatal lethality (128). The role of Notch has been well characterized in the modulation of sprouting angiogenesis. It has been shown that inhibition of Dll4-Notch1 axis induced sprouting of endothelial cells, whereas Notch activation determined a *stalk cell* phenotype, endothelial cells present at the basis of the new sprout (123,129). It has also been shown that in HUVECs, sera from advanced heart failure patients dysregulate Notch signaling, reducing the active form of Notch4 (N4IC) and Jagged1, and promoting sprouting angiogenesis (130).

Endothelial cell function and integrity is modulated by several factors. Hemodynamic shear stress (SS), a tangential frictional force of the flowing blood on the endothelium, plays a critical role in maintaining endothelium functions (131). Several studies show that the crosstalk between Notch and SS is involved in vascular development and post-natal life (132-134). In particular, recently, Polacheck et al., using an engineered model of perfused microvessels, have observed that SS triggers Dll-4-dependent proteolytic activation of Notch, that directly regulates vascular barrier function through a *non-canonical* Notch signaling, driving adherents junction assembly (135). Notch1 is crucial for the athero-protective signal SS-mediated: heterozygous nonsense mutations in Notch1, in human endothelial cells, makes cells not responsive to SS and causes induction of latent pro-osteogenic and pro-inflammatory genes (136), and inhibits SS-dependent pathways implicated in aortic valve calcification (137).

Endothelial cells integrity is influenced also by inflammation, which, among other things, causes apoptosis of endothelial cells, which is one the hallmarks of endothelial dysfunction (138). TNFα is a pleiotropic inflammatory cytokine, that is

able to induce endothelial cells apoptosis (130) and, thus it involved in the initiation and progression of vascular disorders (139). Quillard et al. (125) was the first to demonstrate that TNFα treatment triggers a selective expression pattern of Notch receptors in the endothelium, associated with a reduced Notch transcriptional activity. In particular, TNFα down-regulates Notch4 expression while up-regulates Notch2. Since Notch2 is a weaker transcriptional activator compared to Notch4, this switch could explain the overall decrease in Notch activity as measured by Hey-2 and Hes-1 mRNA levels (125). Further knockdown of Notch4 and Hes-1 by small interfering RNA (siRNA) promotes ECs apoptosis, inhibiting the repair of endothelial injury (140). Furthermore, Quillard et al. showed that Notch2 promoted apoptosis by causing the down-regulation of several cell-death-related transcripts through a mechanism involving down-regulation of the survivin, a key anti-apoptotic regulator. Of note, Notch2 silencing or survivin overexpression could rescue endothelial cells from TNFα and Notch2-mediated apoptosis, respectively (122). In these studies, Notch down-regulation caused induction of two soluble cell adhesion molecules associated with cardiovascular disease, vascular adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM). Mackenzie et al.'s studies demonstrated that constitutively active Notch4 protects endothelial cells from lipopolysaccharide (LPS)-induced apoptosis, inhibiting the c-Jun N-terminal kinase (JNK)-dependent pro-apoptotic pathway in an RBP-Jκ-dependent manner and, inducing an anti-apoptotic pathway through an RBP-Jκ-independent up-regulation of Bcl-2, an anti-apoptotic protein (141). The role of Notch in regulating endothelial apoptosis has been further investigated in retinal endothelial cells, in which Notch protects cells against apoptosis induced by the aberrant blood flow (142).

Recent works have confirmed the anti-inflammatory and protective role of Notch in the vascular endothelium. Wang et al. have shown that Notch1 activation in bone marrow-derived endothelial cells blocks the synthesis of miR155, a miRNA involved in eNOS down-regulation and NF-kB activation through the down-regulation of kB-Ras1 (143). Briot et al. showed that endothelial Notch1 signaling

is repressed by pro-atherogenic stimuli, such as inflammatory lipids and pro-inflammatory cytokines and this reduction increases the expression of inflammatory molecules and binding of monocytes (144). Moreover, in a mouse model of atherosclerosis, Schober et al. (145) have shown that microRNA-126-5p is able to activate Notch1, suppressing Delta-like 1 homolog (DLK1, a Notch inhibitor), and it promotes endothelial proliferation and reduces plaques formation. More recently, Mack et al. have shown that Notch1 is atheroprotective and it is required in adult arteries to initiate appropriate biological responses required for vascular homeostasis (146).

In contrast with these results, there are *in vitro* and *in vivo* studies, suggesting that Notch causes endothelial dysfunction. Verginelli et al. have shown that in human endothelium, treatment with an inflammatory cytokine, interleukin 1β (IL-1β), up-regulates the endothelial adhesion molecule VCAM1, which, in turn, leads the activation of Jagged1-Notch1 signaling (126). Furthermore, in human endothelial cell line (EA.hy926) treated with high glucose, the activation of Notch1, Hes1, and caspase-3, accelerate cell apoptosis (147). A recent work in human endothelial cells of carcinomas and melanomas has shown that sustained Notch1 activity induces the endothelial cells senescence, the expression of chemokines and of endothelial adhesion molecule VCAM (148). Qin et al. have shown that in ApoE -/- mice fed a high-fat diet, LSS (Low Shear Stress) activates Notch1 signaling and, genetic or pharmacological inhibition of Notch1 reduces plaque formation and inflammatory response by a NF-κB-mediated mechanism (149). Using a mouse model of pulmonary arterial hypertension, Li et al. showed that activation of nuclear factor kappa B (NF-kB) up-regulates the expression of Notch3, caspase-3 and Bax, and it down-regulates the expression of Bcl-2, an anti-apoptotic gene, in lung microvascular endothelial cells, which leads to endothelial cell apoptosis and endothelial-mesenchymal transition (EndMT) (150). Nus et al. (151) reported that endothelial specific depletion of the Notch effector RBP-J results in attenuated atheroma plaque severity in the aortic arch and decreased adhesion molecule expression, leukocytes recruitment, and macrophages accumulation in the sinus of

ApoE2/2 mice, thereby preventing endothelial dysfunction and vascular inflammation. In this model, endothelial Notch inactivation was associated to a decrease NFkB-dependent gene expression and impairment of the pro-inflammatory response. These contradictory findings could be due to: 1) differences in the mouse models of atherosclerosis used, which are characterized by differences in the onset and progression of atherosclerosis, as well as in the character and intensity of the disease (152); 2) different origin of endothelial cells; 3) different type of damage (TNFα or IL-1β, high glucose, LSS); 4) taking under consideration only one of the two modality of Notch signaling (*canonical* vs *non-canonical*); 5) studies based on overexpression or on endogenous Notch.

1.4 Crosstalk between Notch and estrogen in the endothelium

Several groups, including ours, reported that treatment with E2 activates Notch signaling in HUVECs (99,153,154). Specifically, Caliceti et al. have shown that E2 (1 nM) treatment increases the active form of Notch1 and Notch4 at protein level but they do not observe changes in the expression levels of the genes for these Notch receptors or their ligands, suggesting that E2 has effect on mRNA translation or on the processing of the protein. Furthermore, treatment with the ERs downregulator, ICI 182.780, inhibited the activation of Notch1, suggesting a role for the ERs in this context. In this study, only a small induction of Notch target genes Hey2 was observed following E2 treatment, suggesting either an involvement of *non-canonical* Notch signaling or that other target genes are affected by the treatment. In contrast with these results, Soares et al. had previously reported an increase in expression levels of Notch1 and Jagged1 mRNA and induction of RBP-J transcriptional activity in E2 (1 nM)-treated HUVECs (154). Sobrino et al. also reported induction of Notch signaling by E2 (1 nM) in HUVECs, as indicated by increased levels of mRNA for Notch4, Furin,

Jagged2 and radical Fringe, as detected by microarray analyses (99). Different cell culture conditions and different methods utilized to study Notch activation, qRT-PCR versus standard RT-PCR (154) or microarrays (99), could explain the opposite results obtained. Caliceti et al. (153) also found that E2 activates VEGFA-Dll4-Notch1 axis and modulates vascular branching when Notch signaling is inhibited by a γ-secretase inhibitor (DAPT). Furthermore, it has been shown that 17β-estradiol modulates the Notch pathway in others cell types, such as hormone responsive breast cancer cells (E2 1 nM) (155), cells of the endometrium (E2 36 nM) (156), hippocampal neurons (E2 10 nM) (157), and in the dentate gyrus (E2 100 nM) (158). These last studies show that 17β-estradiol inhibits the activation of Notch. A possible explanation of the dual role of E2, both inhibitor and activator of Notch, may be due to: i) different expression of two estrogen receptors (ERs), ERα or ERβ, in different cells: breast cancer cells and hippocampal neurons mainly express ERα and endothelial cells ERβ; ii) the two ERs have different and sometimes opposite biological effects (159), thus we can speculate that they could also have opposite activities on Notch processing; iii) different concentrations of estrogen used, as low levels have been shown to have opposite effects to high level.

AIM OF THE STUDY

As previously discussed, estrogens protect endothelial cells against TNFα-induced apoptosis, and Notch activity is down-regulated by TNFα (122,123,140,160), whereas, treatment with E2 activates Notch (99,153,154).

Based on the observation that TNFα and E2 have opposite effects both on endothelial cells apoptosis and on Notch signaling, the aim of this study was to determine whether, under inflammatory conditions, Notch is involved in E2-mediated protection against endothelial cells apoptosis. With this aim, we evaluated the possible role of estrogen receptor α (ERα) and estrogen receptor β (ERβ) in the E2-mediated reduction of endothelial cells apoptosis induced by TNFα.

2. Materials and Methods

2.1 Materials

Antibodies goat polyclonal to Notch1 (C-20, Cat. sc-6014), rabbit polyclonal to Notch4 (H-225, Cat. sc-5594), rabbit polyclonal to Jagged1 (H-114, Cat. sc-8303) and rabbit polyclonal to Estrogen receptor α (MC-20, sc-542) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies rabbit polyclonal to Dll4 (Cat. ab7280) were from Abcam (Cambridge, UK). Antibodies rabbit polyclonal to Estrogen receptor β (Cat. #5513), rabbit monoclonal to cleaved Notch1 Valine 1744 (Cat. #4147), rabbit monoclonal to pAkt (Ser^{473} D9E, Cat. #4060) and rabbit polyclonal to Akt (Cat. #9272) were from Cell Signaling Technology (Beverly, MA, USA). Antibody rat monoclonal to Notch2 (clone C651.6DbHN) was purchased from Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, USA). Antibody mouse monoclonal to β-actin was from Sigma-Aldrich (AC-15, Cat. A1978) (St. Louis, MS, USA). 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, SuperScript III reverse transcriptase, Random Hexamers, dNTPs, RNaseOut were from Life Technologies (Carlsbad, CA, USA). SiRNA against Notch1, ERα and ERβ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL Plus Western Blotting Detection Reagents was from PerkinElmer (Waltham, MA, USA). RNeasy Mini Kit was from Qiagen (Hilden, Germany). PerfeCta SYBR Green SuperMix with ROX kit were from Quanta Biosciences (Gaithersburg, MD, US). Primers for RT-PCR were purchased from IDT (Coralville, IA, USA). Wortmannin, ICI 187.780, PHTPP (ER β antagonist) and other materials were purchased from Sigma-Aldrich. All chemicals and solvents were of the highest analytical grade.

2.2 Methods

2.2.1 Cell cultures

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, Carlsbad, CA, USA) containing 2% FBS and growth factors (EGM-2, Life Technologies, Carlsbad, CA, USA) at 37°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 charcoal/dextran-treated FBS (csFBS) for 20 hours before 24 hours treatment with 17β-estradiol (E2), TNFα, DAPT, ICI 187.780, and wortmannin. Cells were pre-treated with PHTPP in hormone-deprived medium for 20 hours before 24 hours treatments. 17β-estradiol (Sigma Aldrich, Saint Louis, MS) solubilized in DMSO was used. TNFα was solubilized in Milli-Q H_2O. Notch activity was inhibited by treatment with γ-secretase complex inhibitor DAPT (LY-374973, Sigma Aldrich, Saint Louis, MO, USA). DAPT was dissolved in DMSO to a stock concentration of 5 mM and diluted to a final concentration of 5lllllllllllllllllllllllll µM in culture medium just prior to use. PHTPP, wortmannin and, ICI 187.780 were dissolved in DMSO.

2.2.2 Western blot

Western blot analysis was carried out to detect expression of Notch1, Notch2, Notch4, Jagged1, Dll4, ERα, ERβ, p-Akt, total Akt and β-actin by using the corresponding antibodies indicated in the Materials section. Cells were lysed in RIPA buffer (0.05% sodium-deoxycholate was freshly added) containing 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM PMSF and 1 mM sodium orthovanadate on ice for 30 minutes. Protein concentration of each lysate was quantified by using Pierce BCA Protein Assay Kit (Thermo Scientific, Wilmington, USA). Protein samples were denatured by incubation at 70°C for 10

minutes in sample buffer (Life Technologies, Carlsbad, CA, USA) containing 0.5 M DTT. The same amount of total protein (10 μg) was loaded in each lane, then proteins were separated on 7% NuPAGE gels (Life Technologies, Carlsbad, CA. USA). Proteins were transferred to a PVDF membrane at 30 V for 150 min. Non-specific binding was blocked by incubating membranes with Tris-buffered saline (TBS)/Tween 0.1%, pH 7.6, containing 5% nonfat dry milk or 5% BSA (based on antibodies datasheet) for 1 hour at room temperature. PVDF membranes were incubated overnight at 4°C with primary antibodies, washed 4 times in TBS/Tween 0.1%, and incubated for 1 hour at room temperature with secondary peroxidase-conjugated antibodies in TBS/Tween 0.1% containing 5% nonfat dry milk or 5% BSA, depending on antibodies datasheet. Membranes were washed 4 times in TBS/Tween 0.1% and developed using Western Lightning ECL Pro (PerkinElmer, Waltham, MA). Images of the blots were obtained by exposing them to Chemidoc (Bio Rad, Hercules, CA). Protein immunoreactive bands were analyzed by using the ImageLab analysis software (Bio Rad, Hercules, CA). β-actin was used for normalization in the quantitative assessment of western blots after verifying β-actin levels are not affected by any treatment in comparison to total protein evaluated by staining with Ponceau Red.

2.2.3 RNA extraction

HUVECs, grown for 20 hours 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), TNFα (10 ng/ml) in deprivation medium for 24 hours. Total RNA was extracted using a commercially available kit (Qiagen, Hilden, Germany) and quantified with Nanodrop (Thermo Scientific, Wilmington, USA). Protocol for RNA extraction is summarized below:

1. Completely aspirate the cell-culture medium and disrupt the cells by adding 350μl of Buffer RLT to the six-wells plates. Collect the lysate using a scraper. Pipet

the lysate into a 2ml tube. Vortex or pipet to mix, and ensure that no cell clumps are visible before proceeding to step 2.

2. Pipet the lysate directly into a QIAshredder spin column placed in a 2ml collection tube, and centrifuge for 2 min at full speed.

3. Add 1 volume (350µl) of 70% ethanol to the homogenized lysate, and mix well by pipetting.

4. Transfer up to 700µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2ml collection tube. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through and reuse the collection tube in step 5.

5. Add 700µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through and reuse the collection tube in step 6.

6. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through and reuse the collection tube in step 7.

7. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the spin column membrane.

8. Place the RNeasy spin column in a new 2ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

9. Place the RNeasy spin column in a new 1.5ml collection tube. Leave the tube open under chemical hood for 5 min to remove all traces of ethanol.

10. Add 30µl RNase-free water directly to the spin column membrane. Close the lid gently, wait 3-5 min and centrifuge for 1 min at full speed to elute the RNA and the place the samples on ice.

2.2.4 Reverse Transcription and Real-Time PCR

500 ng of total RNA were reverse transcribed in a volume of 25µl using 250 units of SuperScript III reverse transcriptase and 50 ng of random hexamers. Reaction conditions were as suggested by the manufacturer. Here briefly report the protocol:

1. Mix and briefly centrifuge each component before use.
2. Combine the following components in a 0.2- or 0.5-ml RNAse free tube:

Random Primer (50ng/ul)	RNA (500ng)	dNTPs	DEPC- water	TOTAL
2,5 µl	4 µl	1,25 µl	8,5 µl	16,25 µl

3. Incubate at 65°C for 5 min, then place on ice for at least 1 min.
4. Prepare the following cDNA Synthesis Mix:

5x First Strand Buffer	0,1M DTT	Rnase OUT	SuperScript III RT	TOTAL
5 µl	1,25 µl	1,25 µl	1,25 µl	8,75 µl

5. Add 8.75 µl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.

- 10 min at 25°C
- 50 min at 55°C
- 15 min at 85°C → chill on ice.

2µl of the cDNA mixture were used for Real-time PCR experiments. Real-time PCR reactions were conducted on an Applied Biosystems 7500 Fast Real-Time PCR System using PerfeCta SYBR Green SuperMix with ROX kit (Quanta Biosciences, MD, US) according to the manufacturer's protocol in a final volume of 25µl. 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'-TACCCCGAGTGCCTGGTGGGC-3'; Notch4: forward 5'-CAACTGCCTCTGTCCTGATG-3', reverse 5'-GCTCTGCCTCACACTCTG-3'; Jagged1: forward5'-GACTCATCAGCCGTGTCTCA-3', reverse 5'-TGGGGAACACTCACACTCAA-3'; Dll4: forward 5'-CTGTGCCAACGGGGGACAGTG-3', reverse 5'-GTGGGCGCAAGGGTTACGGG-3'; ERα: forward 5'-TATGTGTCCAGCCACCAACC-3', reverse 5'-TCGGTCTTTTCGTATCCCACC-3'; ERβ: forward 5'-AGATTCCCGGCTTTGTGGAG-3', reverse 5'-GAGCAAAGATGAGCTTGCCG-3'; RPL13A: forward 5'-GGAGGTGCAGGTCCTGGTGCTT-3', reverse 5'-CGTACGACCACCACCTTCCGG-3'.

Changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ formula using RPL13A as reference gene. For the statistical analysis of real-time results, we have used the Ct of the single samples and, we have evaluated the ΔCt and $2^{-\Delta Ct}$. We calculated the ΔCt and then the $2^{-\Delta Ct}$ mean plus/minus standard deviation (SD) for each sample. Finally, to calculate the fold change, we calculated the ratio between the mean values of $2^{-\Delta Ct}$ and of SD respect to control sample.

2.2.5 Short Interfering siRNA transfection and transfection with plasmids

HUVECs were grown in six-wells plates and transfected using Oligofectamine (Life Technologies, Carlsbad, CA), with scrambled (control) or Notch1 siRNA, ERα siRNA and ERβ siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Final concentrations used were: 50 nM for Notch1siRNA, 100 nM for ERα siRNA, 200 nM for ERβ siRNA, scrambled siRNA (50, 100 or 200 nM, respectively). The siRNAs used are a pool of three target-specific 19-25 nt siRNAs. For over-expression studies HUVECs were grown in six-wells plates and transfected with 1

μg of pcDNA3 vector encoding human Notch1ICD or the empty vector (a gift from Prof. L. Miele), using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA). Cell treatments began 24 hours after transfection and assays were performed 48 hours after transfection.

2.2.6 Apoptosis detection

HUVECs were hormone-deprived using phenol red-free M200 medium containing growth factors EGM-2 and 2% csFBS for 20 hours before the 24 hours treatment with E2 (1 nM), TNFα (10 ng/ml) and DAPT (5 μM). Apoptosis was assessed with the Annexin V-FITC binding assay. HUVECs were grown for 24 hours in the presence of treatments then cells were collected and stained with annexin V-FITC (Life Technologies, Carlsbad, CA) (100 ng/ml) and propidium iodide (Sigma-Aldrich, Saint Louis, MO) (10 μg/ml) diluited in 1X binding buffer (10 mM Hepes pH 7.4, 5 mM KCl, 150 mm NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$) at room temperature for 20 minutes. Flow cytometric analysis was performed with BD FACSCalibur (Becton-Dickinson Biosciences, San Jose, CA), for each sample, 35000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA).

2.2.7 Statistical analysis

Results are expressed as mean ± SD 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 ANOVA was used (Student-Newman-Keuls method for multiple comparisons). The equal variance was tested with the Browne-Forsythe's test.

3. RESULTS

3.1. Set up treatment conditions

To investigate how different concentrations of TNFα modulate Notch1 receptor, we treated endothelial cells with growing concentrations of TNFα: 10 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml for 24 hours and then, we determined the effect on active Notch1 protein level. We used an antibody specific for Notch1, cleaved at Valine 1744 to identify the active form of this receptor (N1ICD). We found that Notch1 is modulated in a dose-dependent manner starting from 100 pg/mL, with the most effective inhibition being 10 ng/ml (Fig. 6A). To determine the concentration of DAPT, an inhibitor of γ-secretase complex, which cleaves Notch to release the transcriptionally active Notch intracellular domain (NICD), we treated cells with growing concentration of DAPT: 0,5 μM, 1 μM, 5 μM for 24 hours and then we evaluated the effect on active Notch1 protein level. We found that the concentration that completely inhibits Notch1 activation was 5 μM (Fig. 6B). For E2 treatment we have performed the experiments at 1 nM, the concentration used by Caliceti et al. (153), corresponding to 273 pg/ml, similar to the physiological concentration of estrogen in pre-menopausal women in the follicular phase.

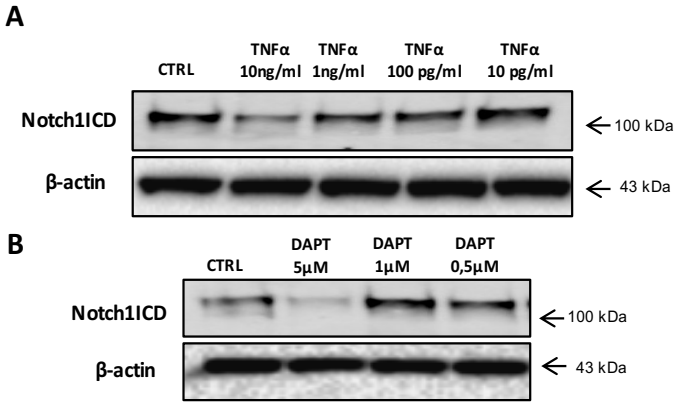
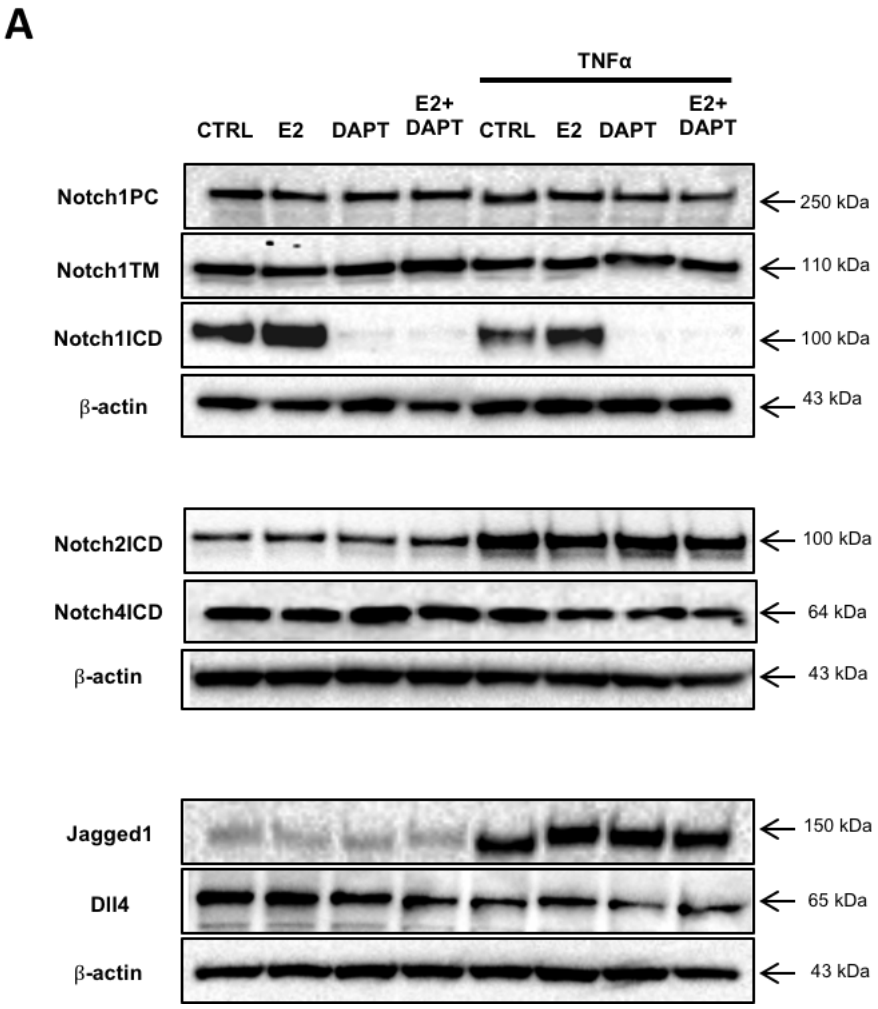


Figure 6. Set up treatment conditions. (A and B) HUVECs were treated for 24 hours with DMSO (CTRL), TNFα (10, 100 pg/ml, 1, 10 ng/ml) and DAPT (0,5, 1 and 5 μM). Cell lysates were electrophoresed and immunoblotted with antibodies for cleaved Notch1 (Val1744). β-actin antibody was used to ensure equal loading.

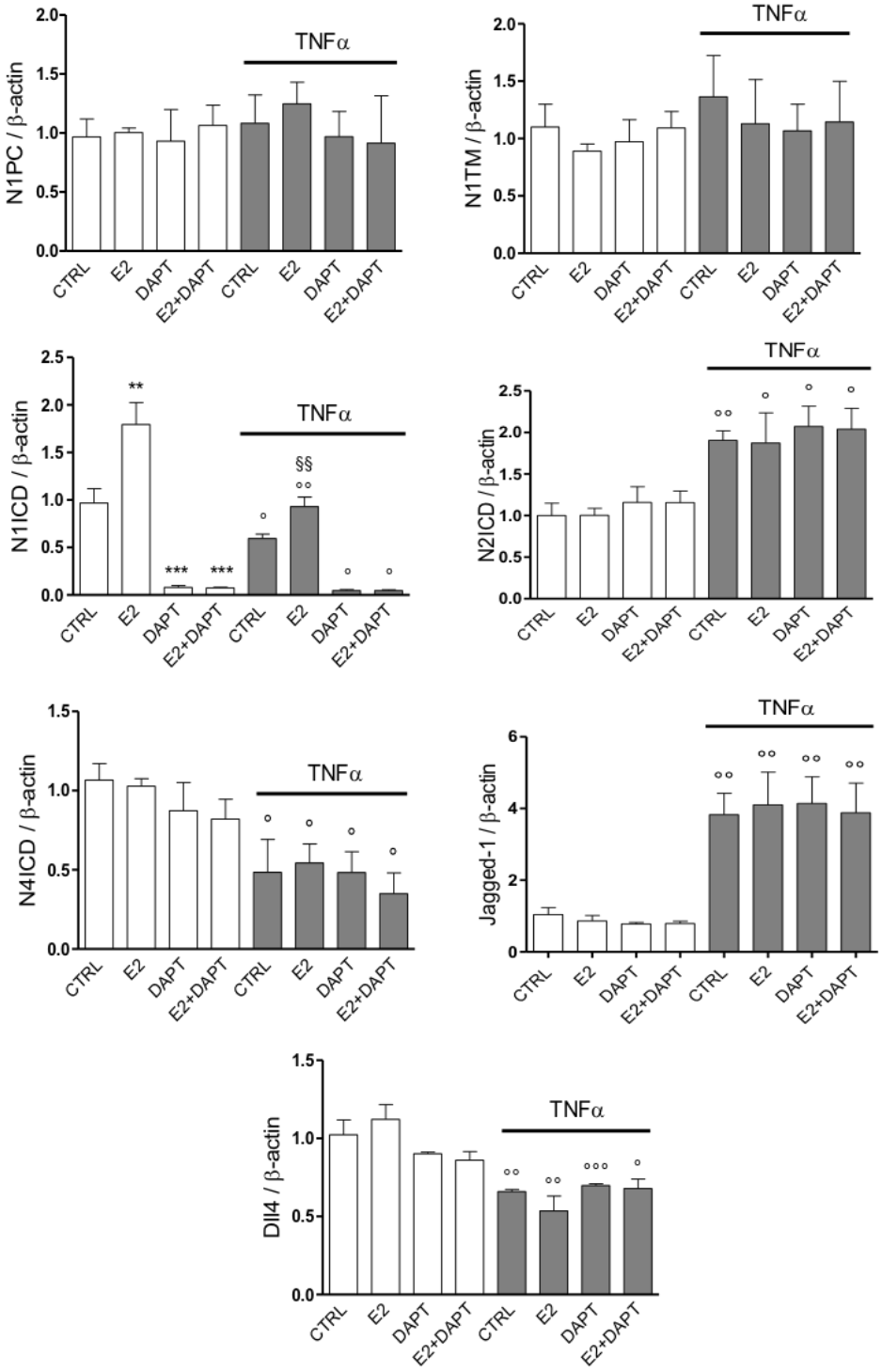
3.2 17β-estradiol partially counteracts the dysregulation of the Notch pathway induced by TNFα in HUVECs

To determine whether E2 increases endothelial cells survival by counteracting the TNFα-mediated dysregulation of Notch signaling, HUVECs were hormone-deprived for 20 hours before treatment with 10 ng/ml TNFα and 1 nM E2 for 24 hours and Notch1, 2 and 4 receptors and ligands Dll4 and Jagged1 protein and mRNA levels were analyzed by western blot 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 μM DAPT (LY-374973), an inhibitor of the γ-secretase, was included to identify the active form of the receptors. We found that TNFα 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. 7A and B). In agreement with

our previous findings (153), E2 increased the level of N1ICD also in the absence of TNFα (Fig. 7A and B). Either TNFα alone or co-treatment with E2 did not affect Notch1 mRNA levels (Fig. 7C). Treatment with DAPT completely abolished the activation of Notch1, but it did not interfere with the activation of Notch2 or Notch4 (Fig. 7A and B). In agreement with studies by others (95,140), following treatment with TNFα, 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 TNFα on Notch2, 4 proteins (Fig. 7A and B) and mRNAs (Fig. 7C). We also confirmed that TNFα positively modulates the Notch ligand Jagged1, both on protein and mRNA levels, whilst it decreases the levels of Dll4 protein (Fig. 7A and B) and mRNA (Fig. 7C) (160). Co-treatment with E2 did not modify the effects of TNFα on Notch ligands protein (Fig 7A and B) or mRNA (Fig. 7C). Our results confirm that TNFα induces Notch2 and Jagged1 and inhibits Notch4 and Dll4 transcription, and show that E2 does not alter the TNFα-mediated transcriptional regulation of these components of the Notch pathway. Furthermore, we show that TNFα 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 Notch2 and Notch4. This could be due to: 1) the DAPT concentration; 2) presence of truncated form of NOTCH2 and Notch4, that are constitutively active, as in breast cancer line (161); 3) binding of DAPT to a specific γ-secretase conformer (162), which preferentially cleaves Notch1 and not Notch2 and Notch4; 4) DAPT binding site has a functional domain within the γ-secretase presenilin C-terminal fragment, that is distinct from the catalytic site or the substrate binding site (163) and it inhibits preferentially Notch1 cleavage, and not Notch2 and Notch4.


A
TNFα
CTRL
E2
DAPT
E2+ DAPT
CTRL
E2
DAPT
E2+ DAPT
Notch1PC
Notch1TM
Notch1ICD
β-actin
250 kDa
110 kDa
100 kDa
43 kDa
Notch2ICD
Notch4ICD
β-actin
100 kDa
64 kDa
43 kDa
Jagged1
Dll4
β-actin
150 kDa
65 kDa
43 kDa

B


N1PC / β-actin
N1TM / β-actin
N1ICD / β-actin
N2ICD / β-actin
N4ICD / β-actin
Jagged-1 / β-actin
Dll4 / β-actin
TNFα
CTRL
E2
DAPT
E2+DAPT

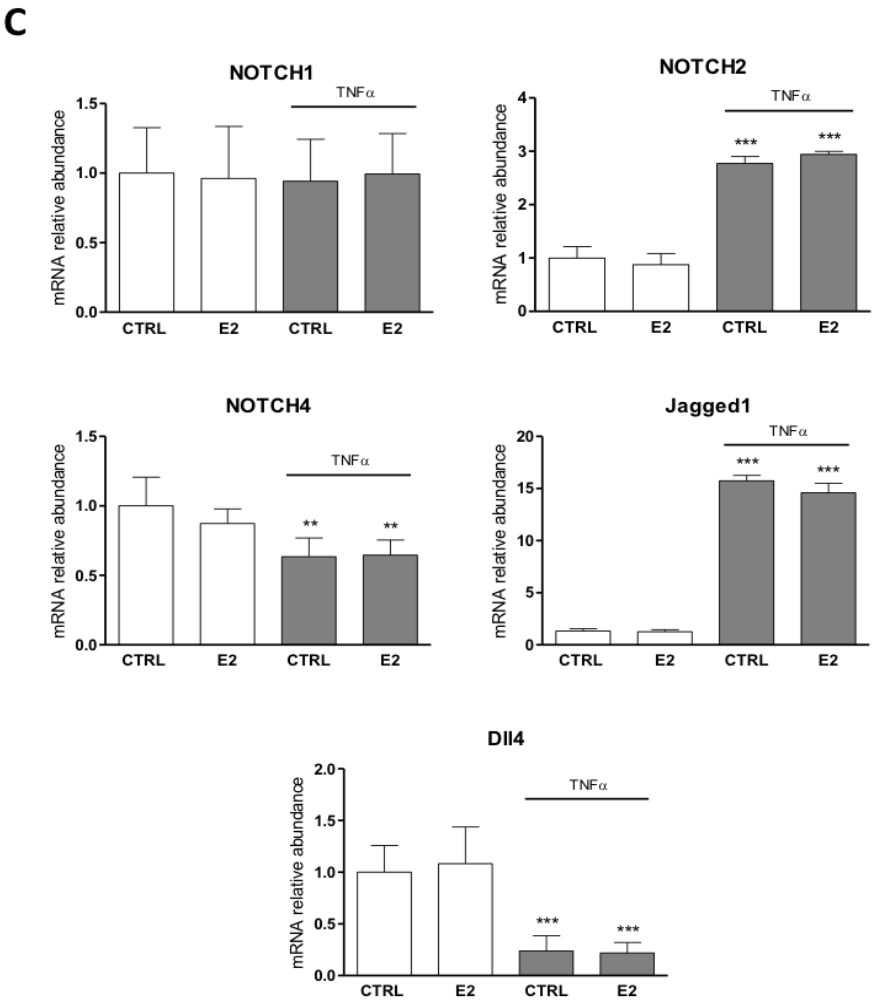


FIGURE 7. Effect of 17β-estradiol (E2), TNFα and DAPT treatments on Notch receptors and ligands in HUVECs. (A) HUVECs were treated for 24 hours with DMSO (CTRL), E2 (1 nM), TNFα (10 ng/ml) or DAPT (5 μM), alone and in combination. Cell lysates were electrophoresed and immunoblotted with antibodies for total Notch1 (C20), cleaved Notch1 (Val1744), 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. (B) Densitometric analyses of western blot assay. Graphs show protein levels after indicated treatment normalized to untreated control levels, after signal comparison to β-actin expression, included as a loading control. Results are expressed as mean ± SD of three independent experiments. ***P < 0.001 (comparison between corresponding control and treatments); °P < 0.05, °°P < 0.01, °°°P < 0.001 (pairwise comparison between plus or minus TNFα treatment). (C) HUVECs were treated for 24 hours with DMSO (CTRL), TNFα (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 ± SD of three independent experiments, each performed in triplicate. **P < 0.01, ***P < 0.001 (pairwise comparison between plus or minus TNFα).

3.3 Active Notch1 is required for 17β-estradiol-mediated protection against TNFα-induced apoptosis

To determine whether Notch signaling is required for the protective action of E2 against TNFα-induced apoptosis, we evaluated the effect of E2 treatment on early (Annexin V–positive) and late apoptotic/necrotic (Annexin V/Propidium iodide-positive) HUVECs, following 24 hours of exposure to TNFα, in the presence or absence of DAPT. Flow cytometric analysis showed that 24 hours of treatment with 10 ng/ml TNFα increased the number of apoptotic cells, compared to control cells, and that E2 partially counteracted this effect (Fig. 8A and B). DAPT alone did not affect HUVECs survival but the co-treatment with TNFα increased the number of apoptotic cells, in comparison with treatment with TNFα only (Fig. 8A and B). Furthermore, in the presence of DAPT, E2 was not able to protect cells from TNFα-induced apoptosis (Fig. 8A and B). These findings suggest that an active Notch pathway is both necessary and sufficient for E2-mediated anti-apoptosis in response to TNFα.

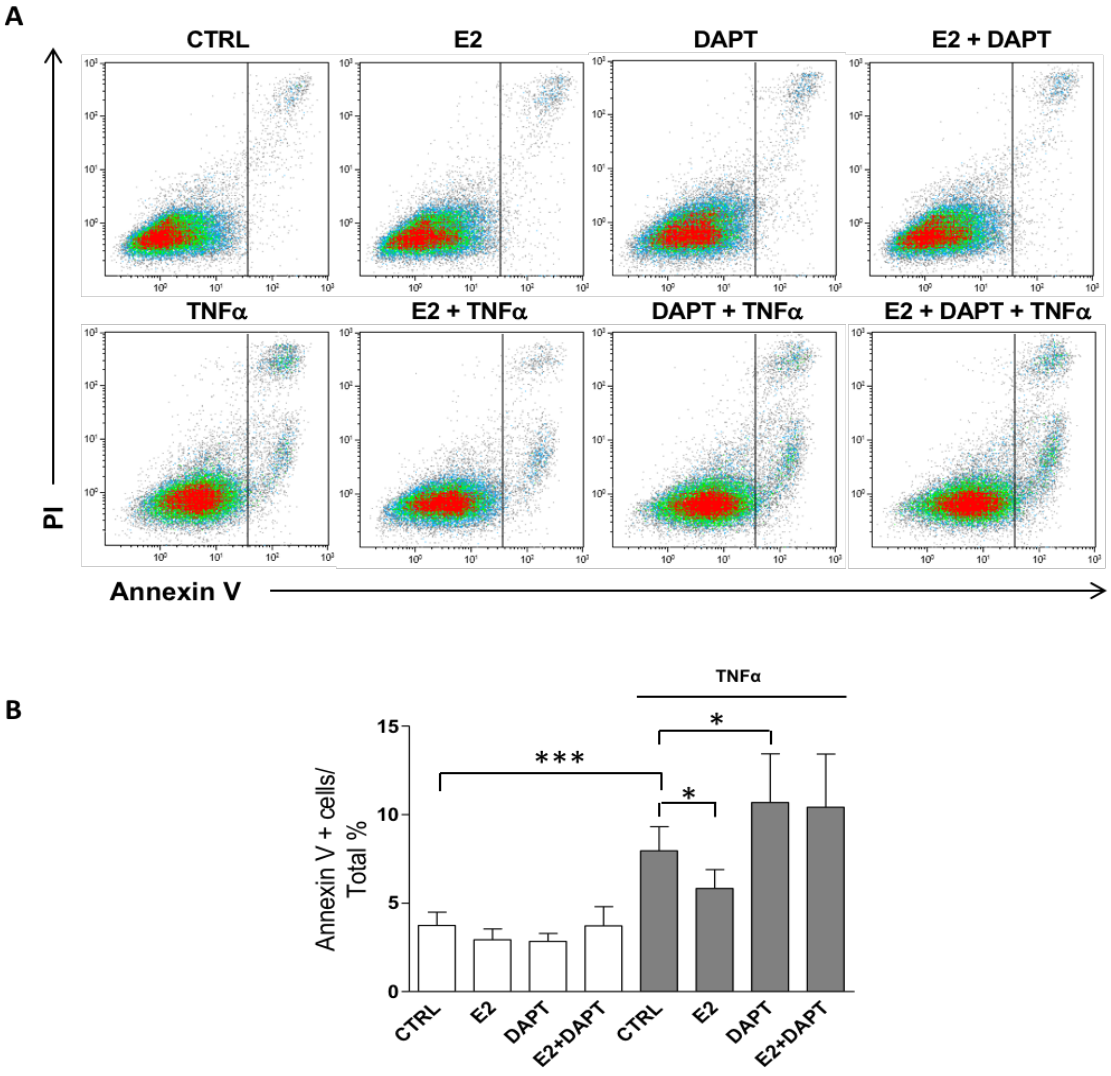


FIGURE 8. **Effect of 17β-estradiol (E2) and DAPT treatments on TNFα-induced HUVECs apoptosis.** (A) HUVECs treated for 24 hours with DMSO (CTRL), E2 (1 nM), TNFα (10 ng/ml) or DAPT (5 μM), alone and in combination, were stained with Annexin V and PI and cytometric analysis was performed. Representative 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 ± SD of three independent experiments. *P < 0.05, ***P < 0.001.

Since DAPT treatment only inhibited Notch1 activation (Fig. 7A and B), 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 TNFα and apoptosis was detected 24 hours later. Notch1 mRNA knockdown was confirmed by western blot and qRT-PCR analysis (Fig. 9A and B).

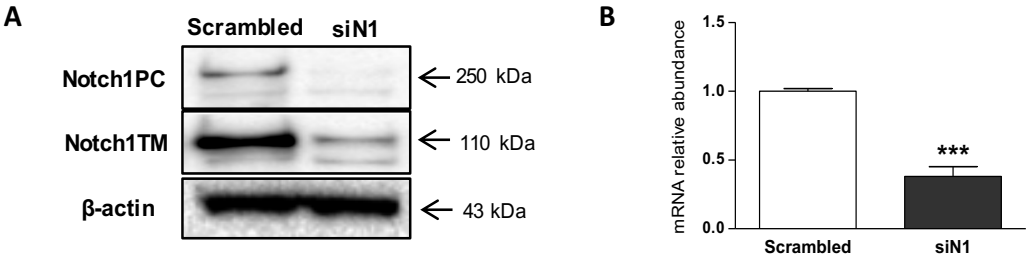


FIGURE 9. Notch1-silenced HUVECs. (A) HUVECs were treated for 48 hours with siRNA against Notch1. Cell lysates were electrophoresed and immunoblotted with antibody for total Notch1 (C20). β-actin was used to ensure equal loading. (B) qRT-PCR analyses were performed to detect reduction of Notch1 mRNA levels in HUVECs after siRNA against Notch1 treatment for 48 hours. 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 ± SD of three independent experiments, each performed in triplicate. ***P < 0.001.

Notch1 siRNA did not increase the number of apoptotic cells, compared to control siRNA, but significantly enhanced HUVECs apoptosis in the presence of TNFα (Fig. 10A and B). Furthermore, in Notch1-silenced HUVECs, E2 was unable to protect cells from TNFα-induced apoptosis (Fig. 10A and B).

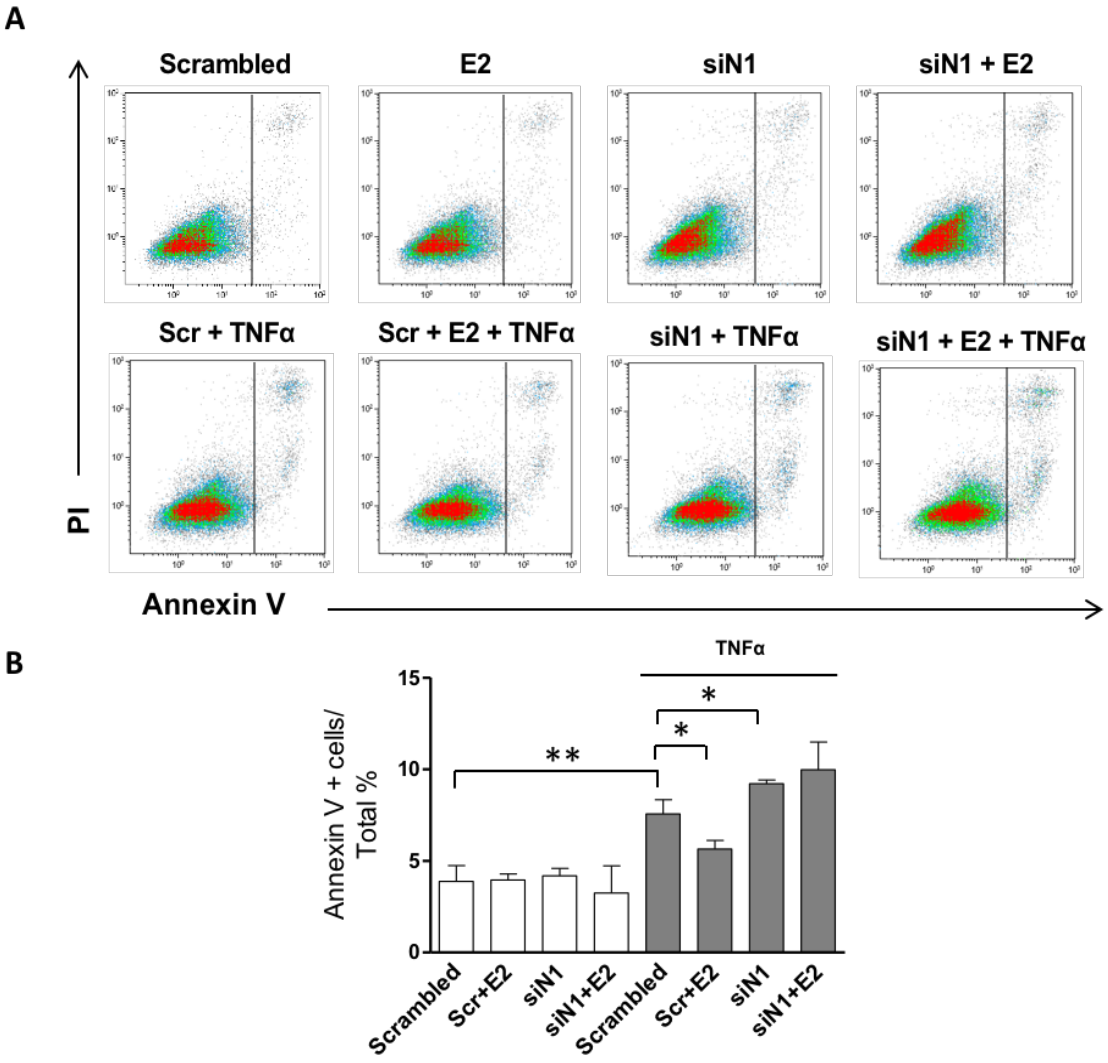


FIGURE 10. TNFα-induced apoptosis in Notch1-silenced HUVECs. (A) HUVECs were transfected with siRNA against Notch1 and, 24 hours later, they were treated with TNFα (10 ng/ml) and 17β-estradiol (E2, 1 nM), alone and in combination, for 24 hours. Cells were stained with Annexin V and PI, then cytometric 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) is shown. Data are expressed as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

To further validate the direct implication of Notch1 in the protective action of E2 against TNFα-induced apoptosis, HUVECs were transfected with a plasmid encoding for N1ICD. Overexpression was confirmed by western blot analysis (Fig. 11A). Upon treatment with TNFα, HUVECs overexpressing N1ICD showed fewer

apoptotic cells compared to cells transfected with the empty vector (Fig. 11B and C). Taken together, these results suggest that active Notch1 is necessary for the anti-apoptotic action of E2 in TNFα-treated HUVECs.

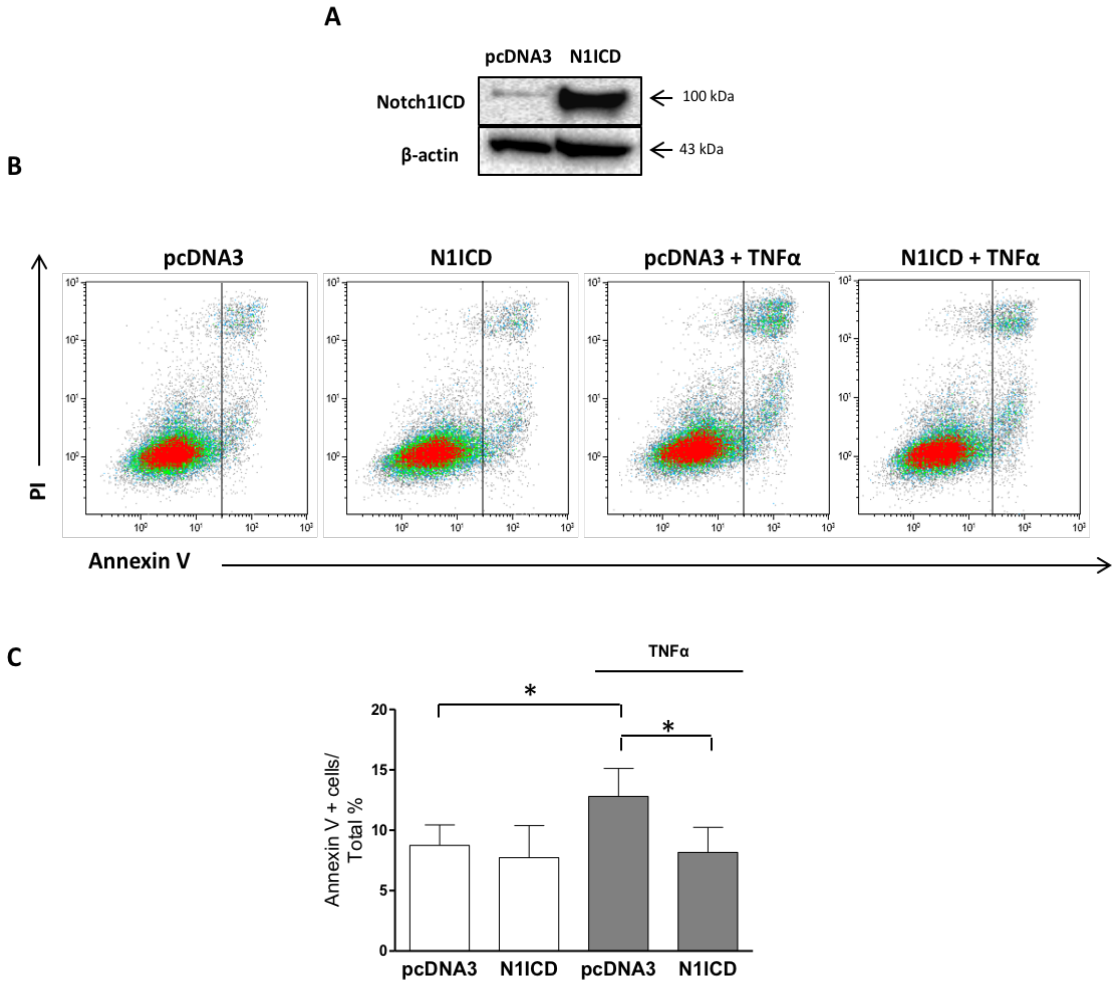
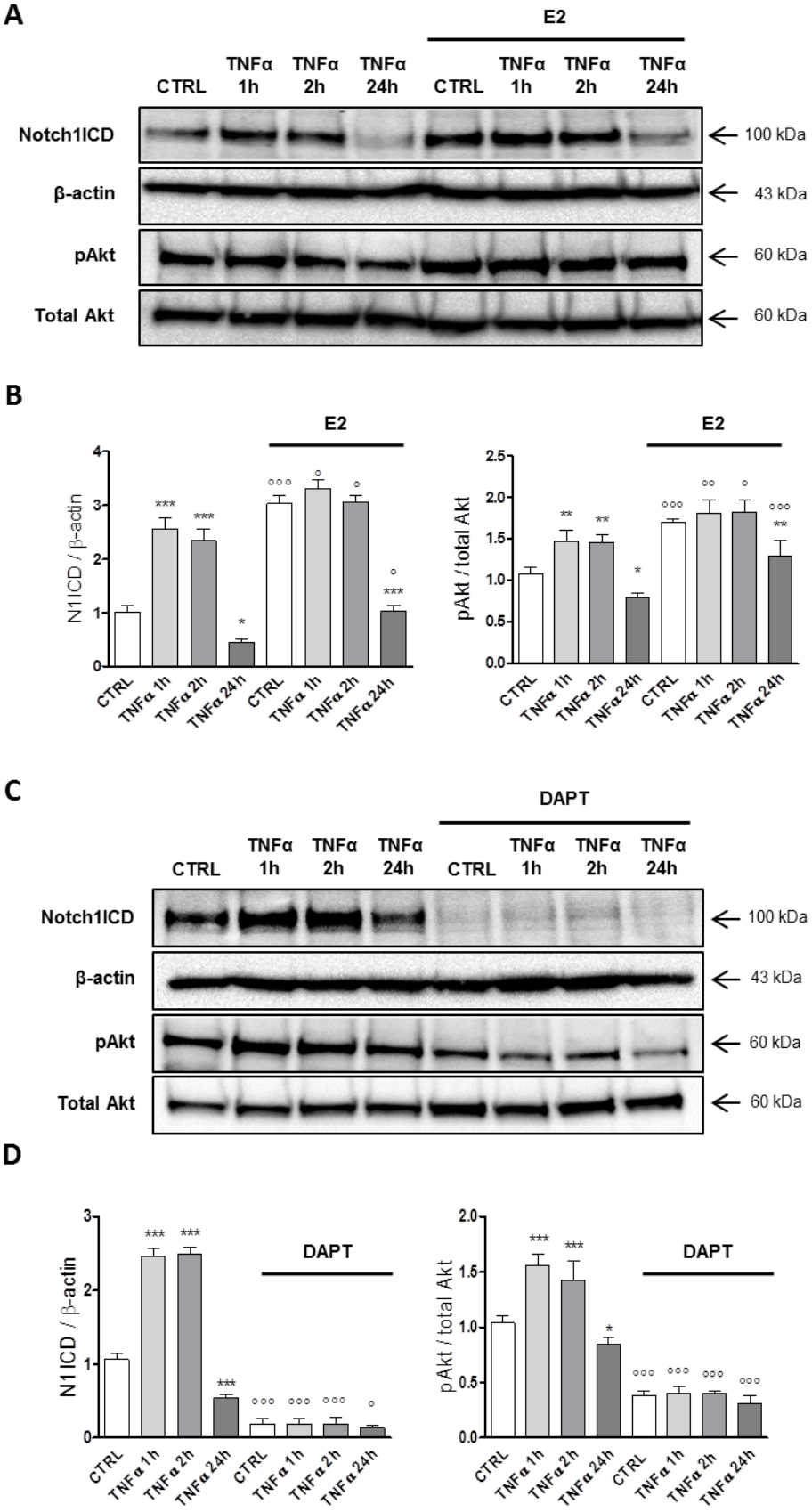


FIGURE 11. TNFα-induced apoptosis in Notch1 overexpressing HUVECs. (A) HUVECs were transfected with a plasmid encoding the Notch1 intracellular domain (Notch1ICD) and, 24 hours later, were treated with TNFα (10 ng/ml) for 24 hours. The empty vector (pcDNA3) was used as a control. Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val1744) antibody. β-actin antibody was used to ensure equal loading. (B) HUVECs transfected for 24 hours were treated for 24 hours with TNFα (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 ± SD of three independent experiments. *P < 0.05.

3.4 17β-estradiol protection of HUVECs against TNFα-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 (164) and TNFα (165). Therefore, in order 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 TNFα for 1, 2 and 24 hours, with or without E2. Short-term treatments were included because phosphorylation is an early event in the pro-survival activity of Akt (166,167). Western blot analysis showed that 1 and 2 hours of treatment with TNFα activated Akt, whereas, 24 hours later, Akt phosphorylation was reduced compared to untreated cells. TNFα also had a biphasic effect on N1ICD, which increased during early treatment and decreased, compared to control, after 24 hours (Fig. 12A and B). The addition of DAPT abolished Notch1 activation and strongly decreased Akt phosphorylation induced by TNFα (Fig. 12C and D). In the presence of E2, at each time point tested, TNFα-treated cells showed a more pronounced phosphorylation of Akt and higher levels of N1ICD compared to cells treated with TNFα only (Fig. 12A and B). Also in the presence of E2, DAPT treatment abolished Notch1 activation and strongly reduced the effects of E2 on Akt phosphorylation, both in control and TNFα- treated cells (Fig. 12E and F). In conclusion these experiments show that, in HUVECs, early treatment with TNFα induces phosphorylation of Akt, which requires N1ICD and is enhanced by E2. After 24 hours, both pAkt and N1ICD levels decline more pronouncedly in TNFα- compared to E2/TNFα-treated cells. These data suggest that Notch1-dependent Akt phosphorylation contributes to the pro-survival action of E2 in the presence of TNFα.


A
E2
CTRL
TNFα 1h
TNFα 2h
TNFα 24h
CTRL
TNFα 1h
TNFα 2h
TNFα 24h
Notch1ICD
β-actin
pAkt
Total Akt
100 kDa
43 kDa
60 kDa
60 kDa
B
E2
E2
N1ICD / β-actin
pAkt / total Akt
CTRL
TNFα 1h
TNFα 2h
TNFα 24h
C
DAPT
Notch1ICD
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N1ICD / β-actin
pAkt / total Akt

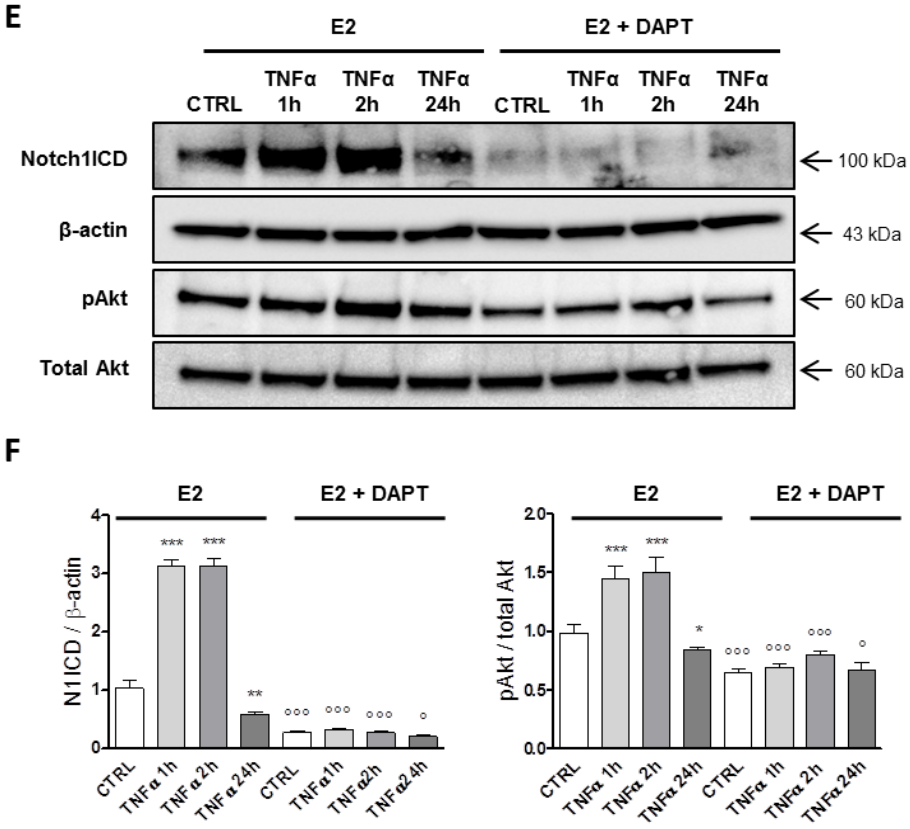


FIGURE 12. Role of 17β-estradiol (E2) and Notch1 on TNFα regulation of Akt phosphorylation in HUVECs. (A, B) Western blotting and densitometry of HUVECs treated with TNFα (10 ng/ml) for 1, 2, or 24 hours in the presence of E2 (1 nM). Cells treated with DMSO were used as control (CTRL). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val1744), pAkt (Ser^{473}) and total Akt antibodies. β-actin antibody was used to ensure equal loading. (B) Graphs show protein levels after 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 ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (comparison between TNFα treatment and corrispective control); °P < 0.05, °°P < 0.01, °°°P < 0.001 (pairwise comparison between plus or minus E2). (C, D) Western blotting and densitometry of HUVECs treated with TNFα (10 ng/ml) for 1, 2, or 24 hours in the presence of DAPT (5 μM). Cells treated with DMSO were used as control (CTRL). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val1744), pAkt (Ser^{473}) and total Akt antibodies. β-actin antibody was used to ensure equal loading. Graphs show protein levels after 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 ± SD of three independent experiments. *P < 0.05, ***P < 0.001 (comparison between TNFα treatment and corrispective control); °P < 0.05, °°°P < 0.001 (pairwise comparison between plus or minus DAPT). (E, F) Western blotting and densitometry of HUVECs treated with TNFα (10 ng/ml) for 1, 2, or 24 hours in the presence of E2 (1 nM) or E2 (1nM)/DAPT (5μM). Cells treated with DMSO were used as control. Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val1744), pAkt (Ser^{473}) and total Akt antibodies. β-actin was used to ensure equal loading.

Graphs show protein levels after 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 ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (comparison between TNFα treatment and corrispective control); °P < 0.05, °°°P < 0.001 (pairwise comparison between E2 and E2 plus DAPT treatment).

To confirm the role of Akt in the E2-Notch1 mediated protection against TNFα-induced apoptosis, we assessed apoptosis in the presence of wortmannin, a specific phosphatidylinositol 3-kinase (PI3-K) 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 TNFα-induced apoptosis (Fig. 13A and B).

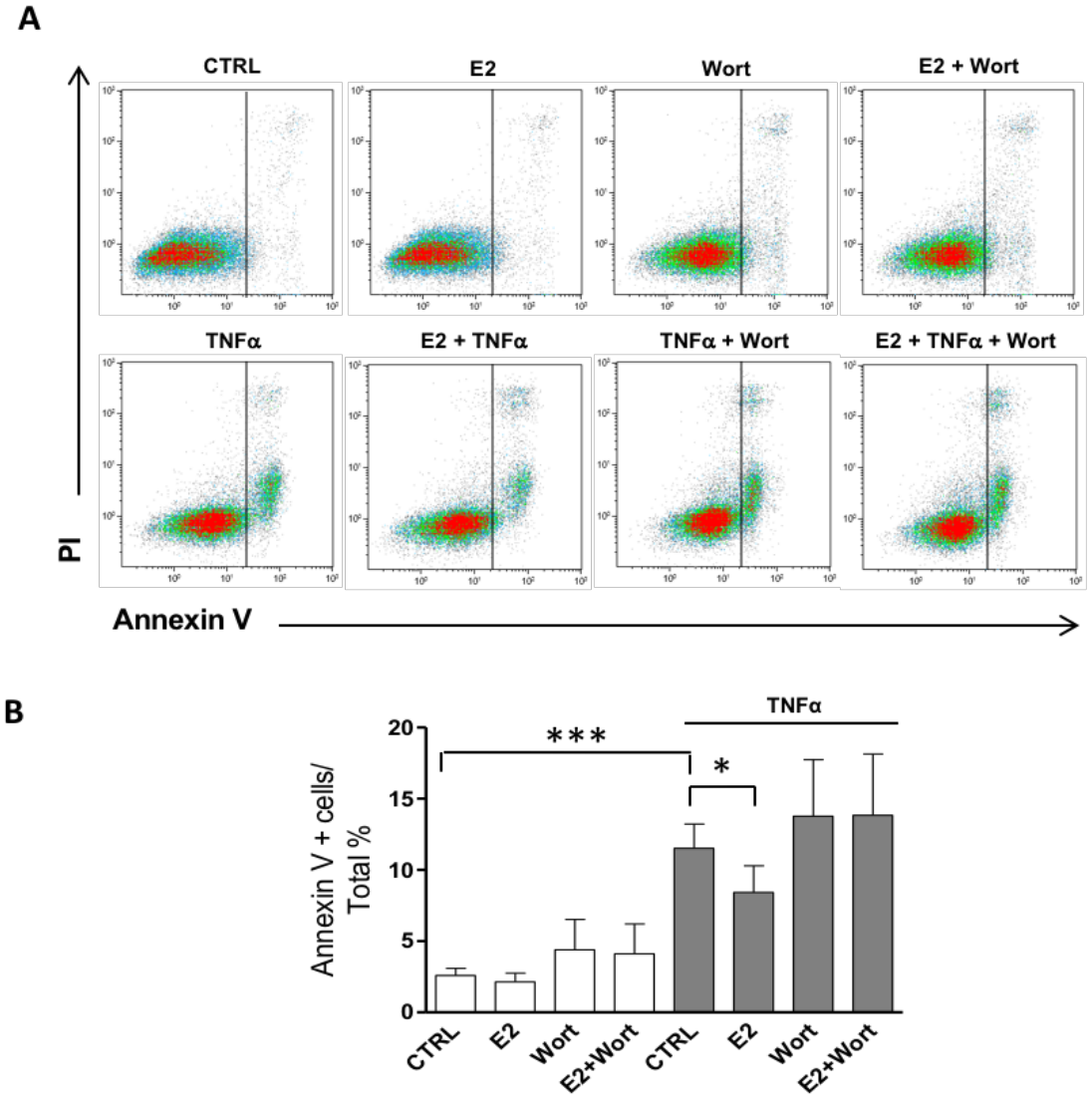


FIGURE 13. Effect of wortmannin on TNFα-induced apoptosis in HUVECs. (A) HUVECs treated for 24 hours with DMSO (CTRL), 17β-estradiol (E2, 1 nM) and TNFα (10 ng/ml) in the presence or absence of wortmannin (100 nM), were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. (B) Percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) is shown. Data are expressed as mean ± SD of three independent experiments. *P < 0.05, ***P < 0.001.

The wortmannin-mediated reduction of Akt phosphorylation was confirmed by western blot analysis (Fig. 14).

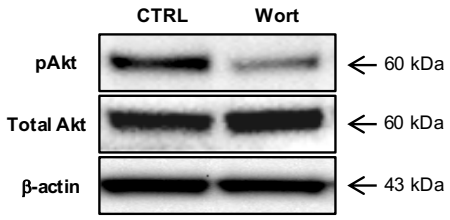


FIGURE 14. Effect of wortmannin on Akt phosphorylation. HUVECs were treated for 24 hours with wortmannin (100 nM). Lysates were electrophoresed and immunoblotted with pAkt (Ser^{473}) and total Akt antibodies. β-actin antibody was used to ensure equal loading.

3.5 Estrogen receptor-β is required for protection against TNFα-induced apoptosis

To test the involvement of ERs in the E2-mediated protective action against TNFα-induced apoptosis, HUVECs were treated for 24 hours with E2, TNFα and ICI 182.780 (SERD, selective ERs down-regulator), alone or in combination and we have performed apoptosis assay. Flow cytometric analysis showed that in the presence of ICI 182.780, E2 was not longer able to protect against TNFα-induced apoptosis (Fig. 15A and B).

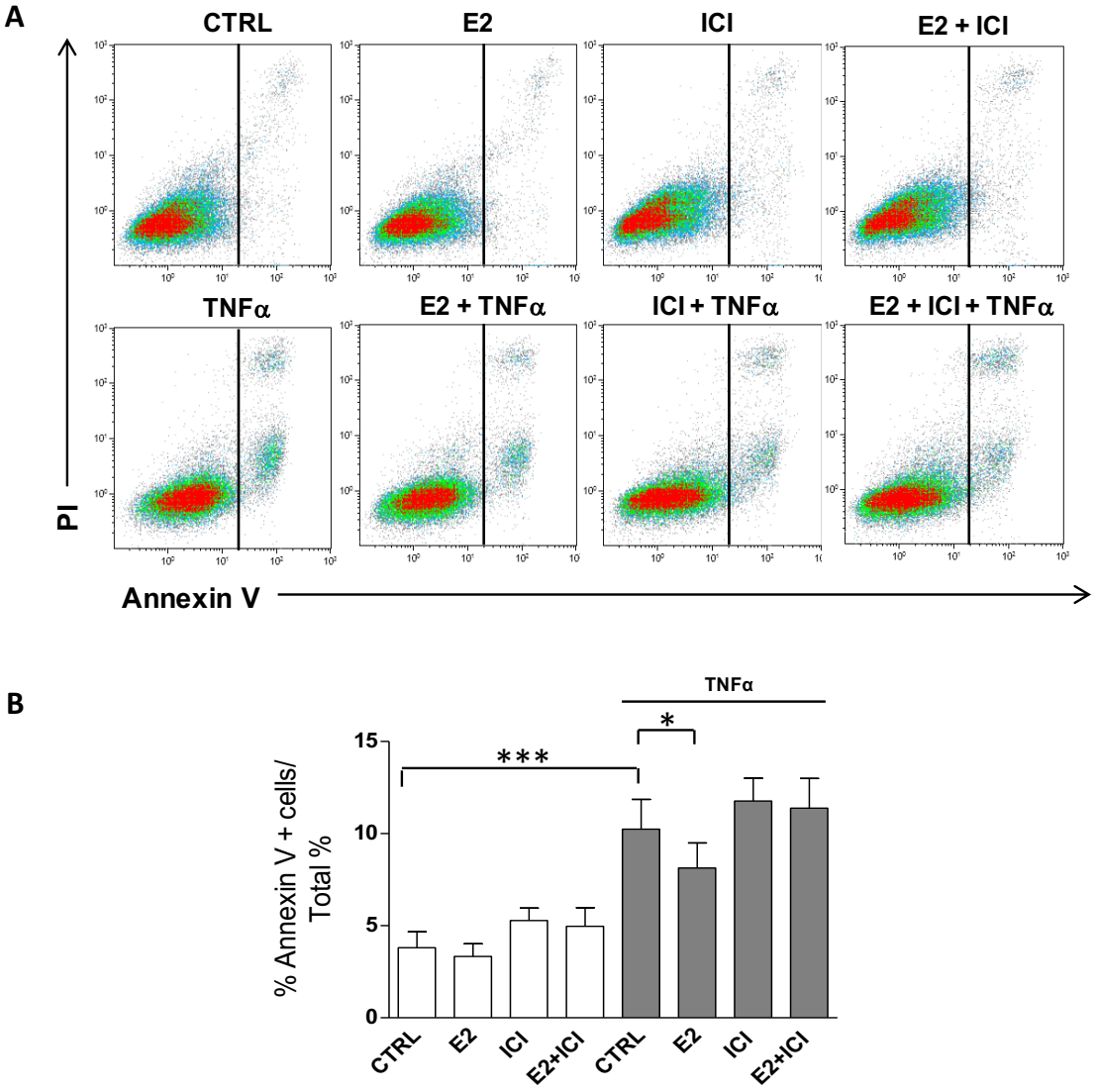
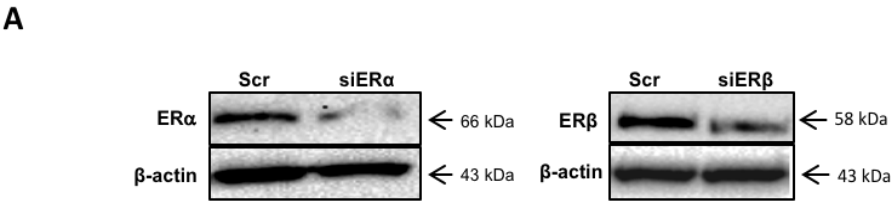


FIGURE 15. Effect of 17β-estradiol (E2) and ICI 182.780 (ICI) treatments on TNFα-induced HUVECs apoptosis. (A) HUVECs treated for 24 hours with DMSO (CTRL), E2 (1 nM) or ICI 182.780 (1 μM) and TNFα (10 ng/ml), alone and in combination, were stained with Annexin V and PI and cytometric analysis was performed. 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 ± SD of three independent experiments. *P < 0.05, ***P < 0.001.

To establish which estrogen receptors (ERs), ERα and/or ERβ, is involved in the anti-apoptotic action of E2, HUVEC were transfected with siRNA targeting ERα or ERβ. ERα and ERβ mRNA knockdown was confirmed by western blot (Fig. 16A) and apoptotic, Annexin V-positive, cells were detected by flow cytometry. As shown in Fig. 16B and C, TNFα increased the percentage of apoptotic cells, compared to untreated cells, independently of the type of transfected siRNA, whereas co-treatment with E2 protected cells transfected with scrambled siRNA and ERα siRNA, but not with ERβ siRNA (Fig. 16B and C). These findings indicate that ERβ, but not ERα, is involved in E2-mediated counteraction of TNFα-induced apoptosis. Of interest, knockdown of ERβ, but not of ERα, led to increased apoptosis compared to scrambled siRNA-transfected cells, even in the absence of TNFα (Fig. 16B and C), which suggests that ERβ is a pro-survival factor in HUVECs.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ERα
66 kDa
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Scr
siERβ
ERβ
58 kDa
β-actin
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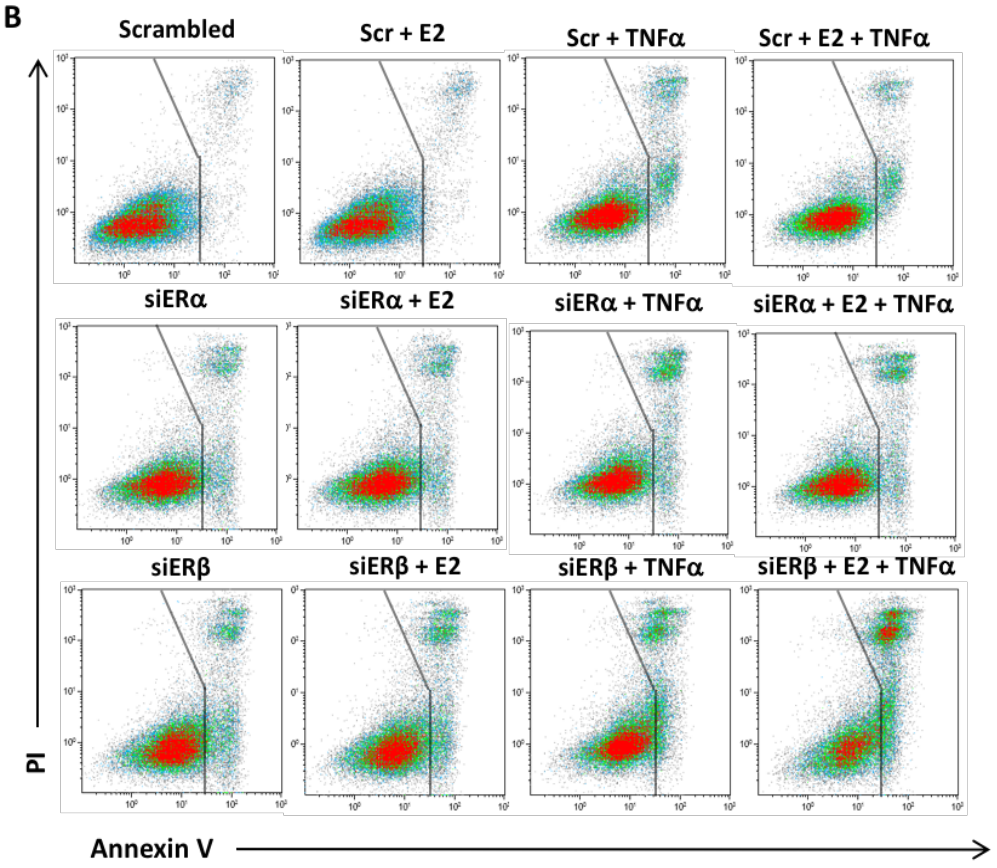
B
Scrambled
Scr + E2
Scr + TNFα
Scr + E2 + TNFα
siERα
siERα + E2
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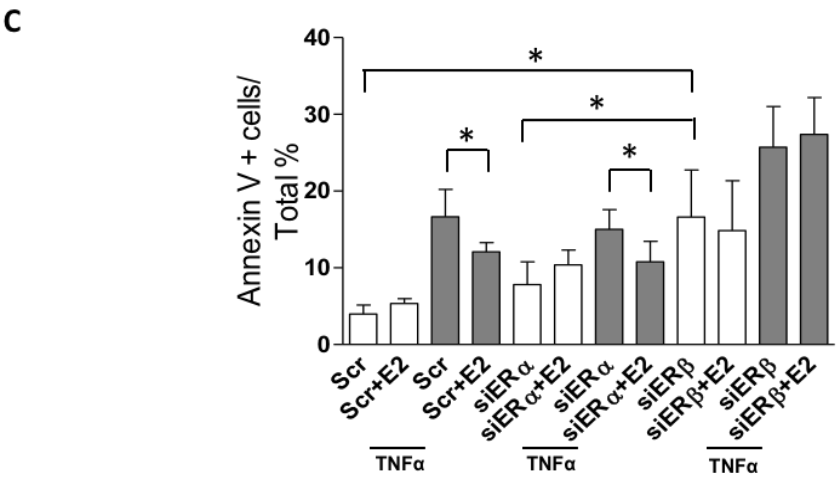
siERβ + E2 + TNFα
PI
Annexin V


C
Annexin V + cells/
Total %
40
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10
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Scr
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siERα
siERα+E2
siERα
siERα+E2
siERβ
siERβ+E2
siERβ
siERβ+E2
TNFα
TNFα
TNFα

FIGURE 16. Role of estrogen receptor β on Notch1 activation and protection against TNFα-mediated apoptosis in HUVECs. (A) HUVECs were transfected with siRNA against ERα or ERβ for 48 hours. Lysates were electrophoresed and immunoblotted with ERα and ERβ antibodies, respectively. β-actin antibody was used to ensure equal loading. (B) HUVECs were transfected with siRNA against ERα and ERβ and, 24 hours later, they were treated for 24 hours with E2 (1 nM) and TNFα (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. (C) The histogram depicts the percentage of apoptotic cells as ratio of Annexin V-positive cells/total cells. Data are expressed as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

Furthermore, while in the cells transfected with scrambled siRNA, E2 treatment increased the levels of N1ICD, both in the presence or absence of TNFα, in ERβ siRNA-transfected cells, E2 capacity to increase N1ICD levels was abrogated (Fig. 17A and B). These results show that ERβ is required for E2-mediated counteraction of TNFα-induced N1ICD downregulation.

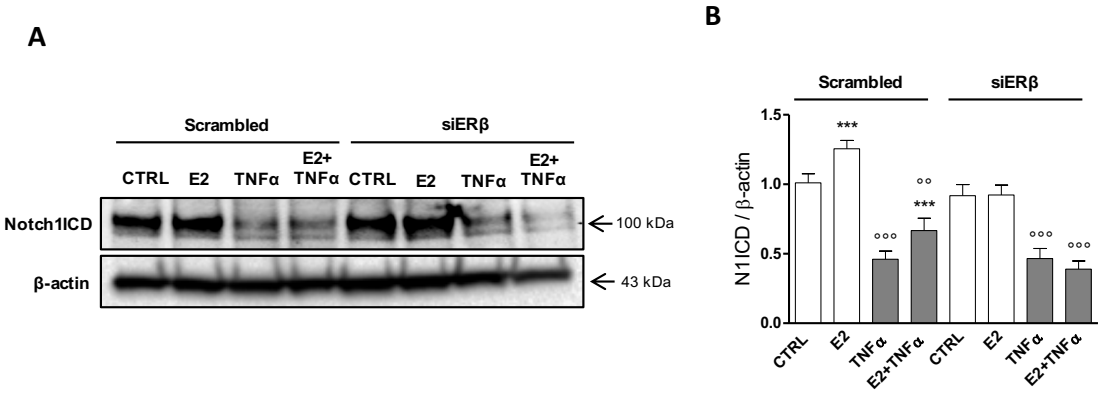


FIGURE 17. Role of estrogen receptor β on Notch1 activation against TNFα-mediated effects in HUVECs. (A) Western blot analysis for cleaved Notch1 (Val1744) in HUVECs after the transfection with siRNA against ERβ and TNFα (10 ng/ml) and 17β-estradiol (E2, 1 nM) treatments for 24 hours. β-actin antibody was used to ensure equal loading. (B) Densitometric analyses of western blot assays. Graphs show protein levels after indicated treatment normalized to untreated control levels, after signal comparison to β-actin expression, included as a loading control levels. Results are expressed as mean ± SD of three independent experiments. ***P < 0.001 (comparison between corresponding control and E2 treatment); °°P < 0.01, °°°P < 0.0 01 (pairwise comparison between plus or minus TNFα treatment).

To determine whether TNFα affects HUVECs survival by regulating the expression levels of this ER isoform, we assessed ERα and ERβ protein levels after treatment with TNFα, alone and in combination with E2. As shown in Fig. 18A and B, TNFα decreased ERβ while increasing ERα protein levels, compared to control, and E2 did not counteract this effect. Nevertheless, since E2 downregulated ERα but not ERβ protein, the ERα/ERβ ratio increased in TNFα-treated cells but not in co-treatment E2. These data suggest that an increased ERα/ERβ ratio could be linked to the reduced survival observed when HUVECs are exposed to TNFα.

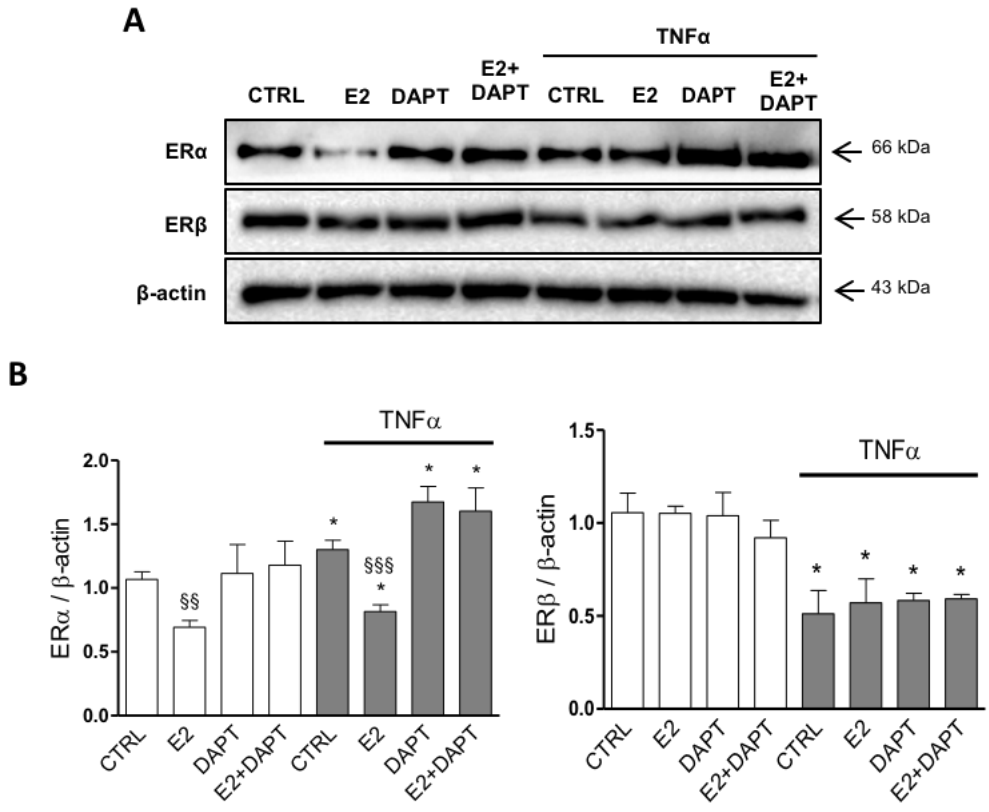


FIGURE 18. ERs protein level after TNFα-treatment in HUVECs. (A) HUVECs were treated for 24 hours with E2 (1 nM), TNFα (10 ng/ml) and DAPT (5 μM), alone and in combination. Lysates were electrophoresed and immunoblotted with ERβ or ERα antibodies. β-actin antibody was used to ensure equal loading. (B) Densitometric analyses of western blot assays. Graphs show protein levels after indicated treatment normalized to untreated control levels, signal comparison to β-actin expression, included as a loading control levels. Results are expressed as mean ± SD of three independent experiments. ***P < 0.001 (comparison between corresponding control and treatments); °P < 0.05, °°P < 0.01, °°°P < 0.001 (pairwise comparison between plus or minus TNFα treatment).

The role of ERβ in regulating apoptosis and N1ICD levels in the presence of TNFα was confirmed by experiments utilizing specific agonists of both ER isoforms. Specifically, treatment with a specific agonist of ERβ (DPN), but not with an agonist of ERα (PPT), increased N1ICD levels (Fig. 19A and B).

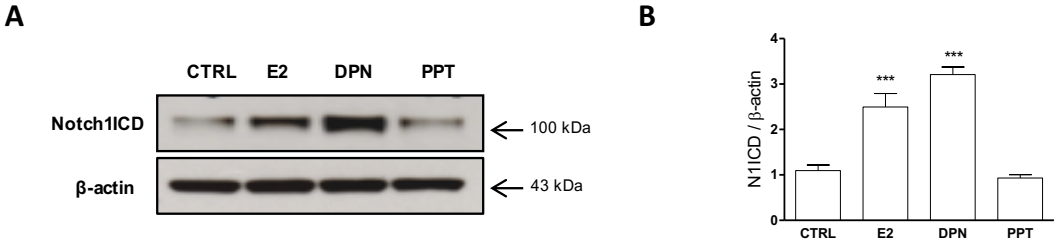
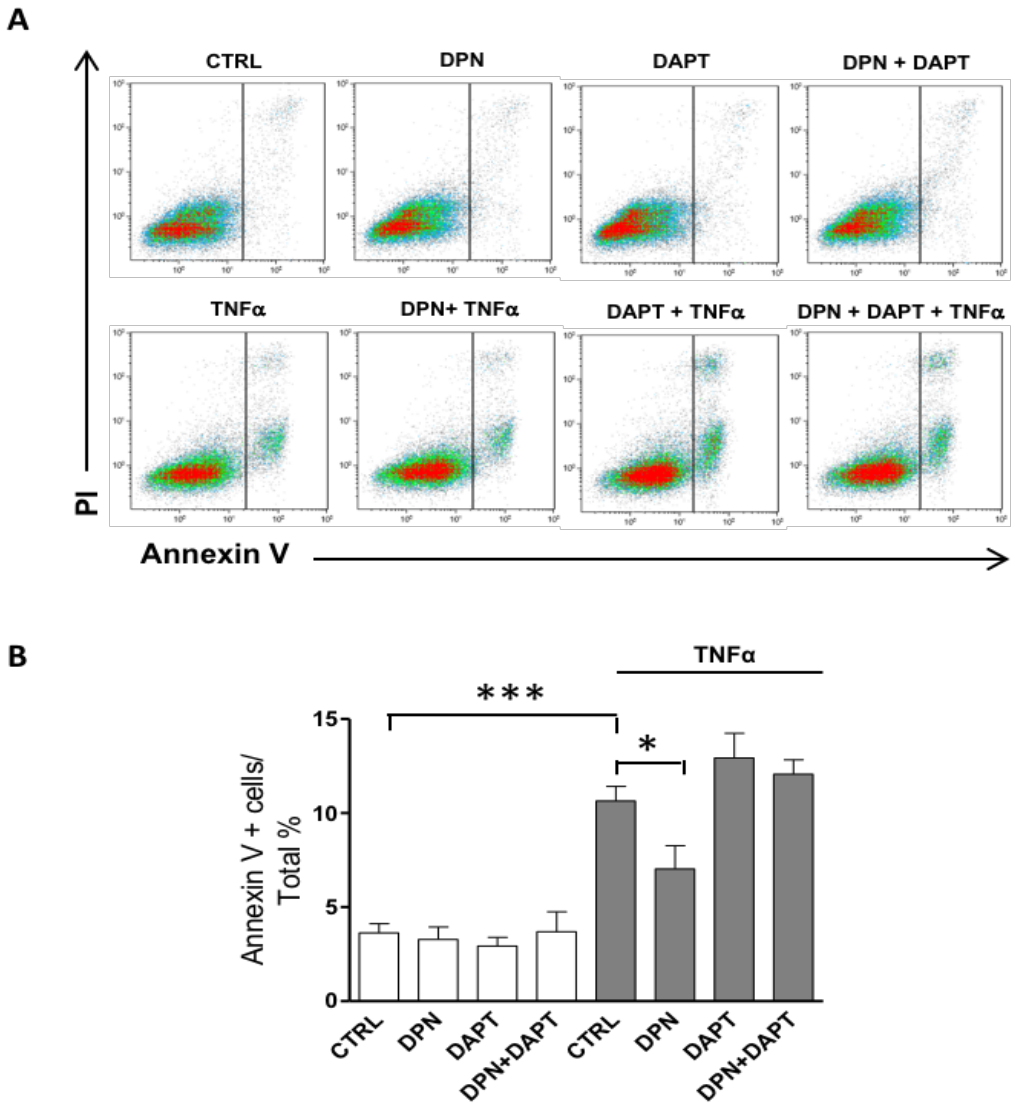


FIGURE 19. Effect of DPN and PPT on active Notch1 in HUVECs. (A) HUVECs were treated for 24 hours with 17β-estradiol (E2, 1 nM), DPN (ERβ agonist, 10 nM) and PPT (ERα agonist, 10 nM). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val1744) antibody. β-actin antibody was used to ensure equal loading. (B) Densitometric analysis**.** Graphs show protein levels after indicated treatments normalized to untreated control levels, after signal comparison to β-actin expression. Results are expressed as mean ± SD of three independent experiments. ***P < 0.001 (comparison between control and agonist treatment).

Additionally, DPN (ERβ-agonist) but not PPT (ERα-agonist), reduced the ratio of apoptotic cells, compared to TNFα-treated cells, but not in the presence of DAPT (Fig. 20 A-D).



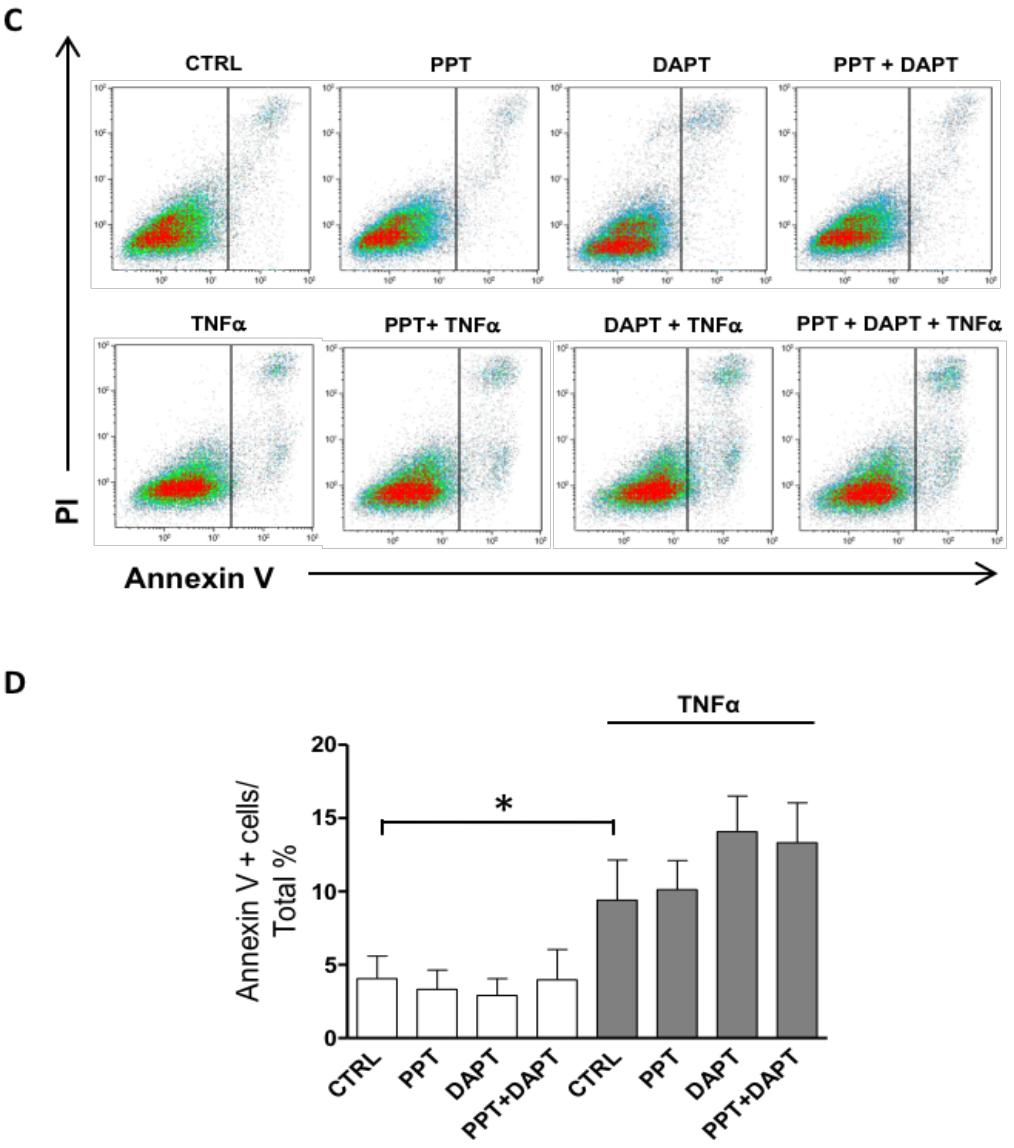


FIGURE 20. Effect of DPN and PPT on TNFα-induced apoptosis in HUVECs. (A, B) HUVECs treated for 24 hours with DMSO (CTRL), DPN (10 nM), TNFα (10 ng/ml), DAPT (5 μM), alone and in combination, were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are shown for each treatment. The histogram depicts the percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells) following treatment is shown. Data are expressed as mean ± SD of three independent experiments. *P < 0.05, ***P < 0.001. (C, D) HUVECs treated for 24 hours with DMSO (CTRL), PPT (10 nM), TNFα (10 ng/ml), DAPT (5 μM), 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 is shown. Data are expressed as mean ± SD of three independent experiments. *P < 0.05.

To further confirm the involvement of ERβ in E2-mediated protection, we used PHTPP, a specific antagonist of ERβ. HUVECs were treated with PHTPP for 20 hours before adding E2 and/or TNFα for 24 hours. As shown in Fig. 21A and B, E2 protected against TNFα-induced apoptosis only in the absence of PHTPP.

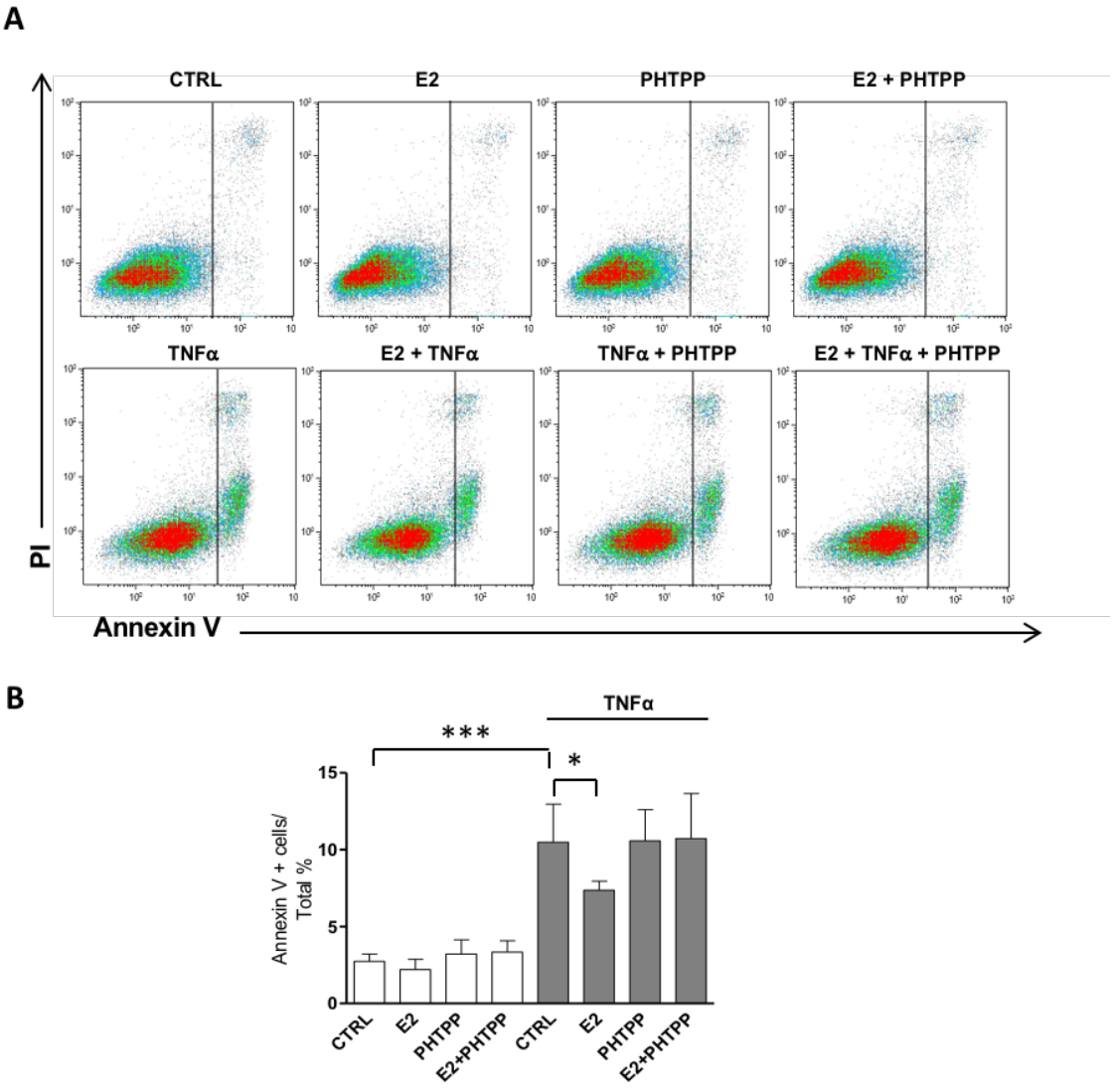


FIGURE 21. Effect of PHTPP on TNFα-induced apoptosis in HUVECs. (A) HUVECs treated for 24 hours with DMSO (CTRL), E2 (1 nM), PHTPP (1 μM) and TNFα (10 ng/ml) alone and in combination, were stained with Annexin V and PI and then cytometric analysis was performed. Representative Annexin V-PI plots are 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 ± SD of three independent experiments. *P < 0.05, ***P < 0.001.

Furthermore, in the presence of PHTPP, E2 was not able to increase the levels of N1ICD and enhance the phosphorylation of Akt (Fig. 22A and B).

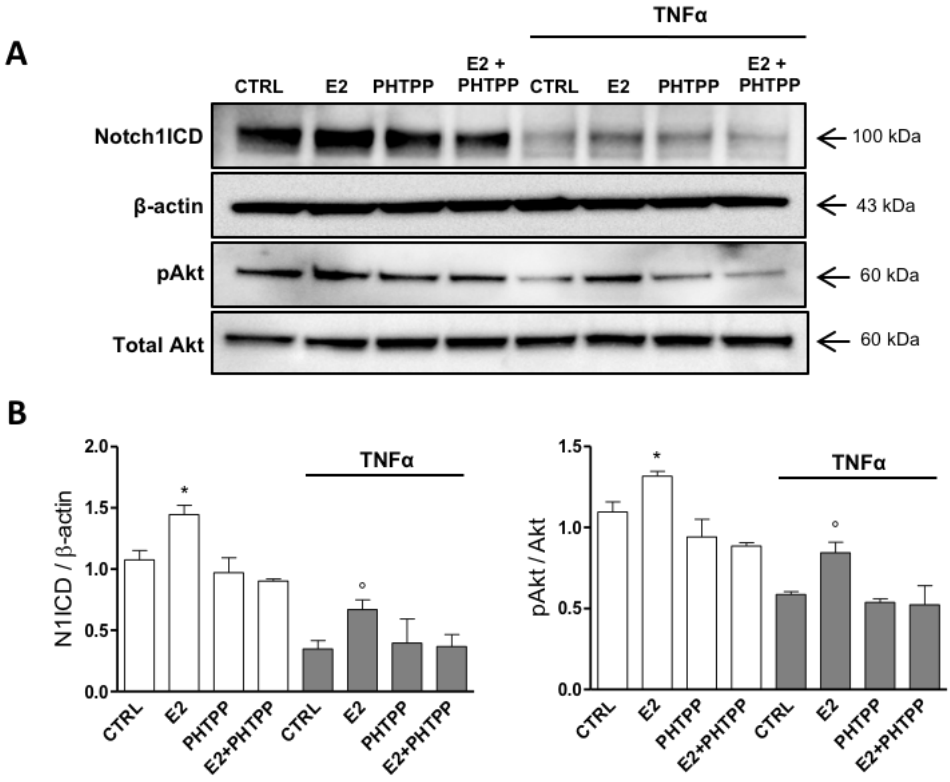


FIGURE 22. Effect of PHTPP on Notch1 activation and Akt phosphorylation in HUVECs. (A) Western blotting analysis of HUVECs treated DMSO (CTRL), 17β-estradiol (E2,1 nM), PHTPP (1 μM) and TNFα (10 ng/ml). Lysates were electrophoresed and immunoblotted with cleaved Notch1 (Val1744), pAkt (Ser^{473}) and total Akt antibodies. β-actin antibody was used to ensure equal loading. (B) Densitometric analysis**.** Graphs show protein levels after 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 ± SD of three independent experiments. *P < 0.05, **P < 0.01 (comparison between treatments and corrispective control); °°P < 0.01, °°°P < 0.001 (pairwise comparison between plus or minus TNFα treatment).

4. DISCUSSION AND CONCLUSIONS

The molecular mechanisms underlying the protective action of estrogens on the endothelium are still not completely understood. In this work, we report that E2 is able to partially counteract the apoptotic action of TNFα in endothelial cells by increasing the levels of intracellular Notch1, through a mechanism dependent on ERβ. The role of Notch signaling in endothelial cells survival (125) and, in particular, the role of Notch1 in protecting endothelial cells from apoptosis has been previously reported in the context of ischemia (168), disturbed shear stress (142) and, in the endothelium of subjects with pulmonary hypertension (169). In this work, we show for the first time, that in HUVECs, TNFα treatment reduces the intracellular levels of Notch1 and, and this is related to the increase of apoptosis. In fact, cells treated with TNFα and DAPT, that under our experimental conditions, inhibits Notch1 but, not Notch2 and Notch4, show significantly increase of the number of apoptotic cells. Conversely, the ectopic expression of the active form of Notch1 protects against TNFα-induced endothelial cell apoptosis. Together these results suggest that Notch1 plays a fundamental role in endothelial cells survival under the effect of TNFα. Furthermore, our study shows that E2-mediated protection against TNFα-induced apoptosis requires the presence of intracellular Notch1, since E2 is unable to decrease the number of apoptotic cells induced by TNFα treatment when Notch activity is chemically or genetically inhibited with DAPT or siRNA against Notch1, respectively. In addition, we show that E2 counteracts the TNFα-mediated inhibition of active form of Notch1, but it has no effects on Notch2 and Notch4.

More studies are needed to clarify the molecular mechanisms by which TNFα decreases the levels of active Notch1 and how E2 partially counteracts this effect. We found that TNFα does not modulate Notch1 mRNA, and this finding is in agreement with Quillard et al. (125), but in contrast with Briot et al. (144), who

reported the inhibition of Notch1 transcription in TNFα-treated human aortic endothelial cells. These discrepancies could be due to differences in the time of TNFα treatment (24 hours versus 4 hours) or in the source of endothelial cells (artery vs vein). Our data suggest instead that TNFα could inhibit the activation rate of Notch1 receptor or decrease the stability of N1ICD, and, both actions could be partially counteracted by E2. We confirm that TNFα treatment inhibits Dll4 and induces Jagged1 at protein and mRNA level (160). Jagged1 is a weaker activator of Notch1 (123), thus the TNFα-mediated reduction of active form of Notch1 could be a consequence of an altered Jagged1/Dll4 ratio. Nevertheless, we have observed that E2 did not affect Jagged1 and Dll4 at protein and mRNA level, both in presence and in absence of TNFα, suggesting that E2-mediated induction of active Notch1 does not depend on ligands.

The pro-survival factor Akt is known to play a crucial role in promoting survival of endothelial cells in the presence of TNFα (164). When HUVECs are treated for different times with TNFα, we observed an increase of Akt phosphorylation (pAkt) in the first few hours (1-2 hours), as also shown in cardiac fibroblasts (170), at a later time, after 24 hours, the apoptotic effect of TNFα prevails, and pAkt level decreases. This dual action of TNFα on pAkt is not surprising considered the ability of this cytokine to elicit both survival and death pathway, depending on the cell context (171). Interestingly, we also found that Notch1 processing is also increased by TNFα in the first few hours and that diminished over the time, following the same pattern as Akt activation. Furthermore, we showed that DAPT abrogates Akt phosphorylation induced by TNFα, indicating that the activation of the Akt pro-survival pathway only occurs in the presence of active form of Notch1. The addition of E2 to cells treated with TNFα for 24 hours partially restored N1ICD and pAkt levels, compared to cells treated with TNFα only. These results suggest that E2 might protect endothelial cells from TNFα-induced apoptosis by increasing the levels of active Notch1, which in turn would promote the phosphorylation of Akt. It has recently show that the crosstalk between Notch and Akt also plays an important role for survival of vascular smooth muscle cells (VSMCs), which are

necessary to maintain vascular stability and function (172). More studies are needed to investigate the mechanism by which Notch1 activates Akt in TNFα-treated HUVECs. We have previously shown that E2 treatment increased N1ICD in HUVECs but no changes were detected in Hes1 mRNA (153) suggesting that *non-canonical* Notch signaling could be involved in the Notch1-mediated phosphorylation of Akt.

The protective effects of estrogens are mainly mediated by two isoforms of ERs (ERα and ERβ) (1), which can exert distinct, and sometimes opposite responses through *genomic*, *non-genomic*, and related pathways, depending on the specific cell type and cellular distribution (173). Here, we showed that, in HUVECs, ERβ, is necessary for E2-mediated protective action against TNFα-induced apoptosis. In the presence of siRNA against ERα, E2 is still able to protect against apoptosis, conversely, in the presence of siRNA against ERβ or of PHTPP (ERβ-antagonist), E2 is no more able to protect against apoptosis. In addition, in cells exposed to TNFα and treated with siRNA against ERβ, we showed a reduction E2-mediated of N1ICD accumulation, on the contrary, in the presence of siRNA against ERα, E2 increase the active form of Notch1. Of note, we found that ERβ mRNA knockdown increased the apoptotic rate even in absence of TNFα, suggesting that this form of receptor contributes to endothelial cell survival according to a ligand-independent mechanism (1). Our results are consistent with the observation that E2 prevents early stage atherosclerosis in ERα deficient mice (174). Of note, TNFα down-regulated ERβ, but not ERα, leading to increased ratio of ERα/ERβ, action inhibited in the presence of E2, which, as also observed by others (175), only down-regulates ERα. Based on these results, TNFα could also affect endothelial cells viability by altering ERα/ERβ ratio.

Our results could have important clinical implications, suggesting 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 atherosclerosis. Endothelial impaired Notch signaling may occur in dyslipidemic

conditions (144) or in heart failure patients (130) and as a unwanted side effect of treatment with natural (176,177) and synthetic anti-cancer drugs (178). Furthermore, our data show, for the first time, that ERβ, but not ERα is involved in the E2-mediated anti-apoptotic effects in the endothelium, suggesting that specifically targeting this isoform may result therapeutic options to interfere with atherosclerosis in menopausal women. The different affinity for the ERs could be the basis for the pharmacological differences between the various SERMs and, it should be considered on future clinical trials. Natural compounds, which bind preferentially ERβ, such as liquiritigenin, S-equol, and genistein have been identified (179), and they could be used to protect the cardiovascular system without increasing the risk of breast cancer, in fact, ERβ has an anti-proliferative action on breast cancer cells (180). In line with this evidence, it has been shown that high levels of ERβ expression are associate with a better response to tamoxifen, a SERM commonly used for breast cancer treatment (181,182). Our data could explain why, unlike tamoxifen, raloxifene has no protective effect against coronary heart disease (78), speculating that tamoxifen is better than raloxifene because performs its estrogen agonist action binding ERβ, and not ERα, in the vascular endothelium. Moreover, our data could confirm the *timing hypothesis* of hormone therapy, showing that estrogen acts at the very beginning of atherosclerotic plaque formation, preventing endothelial dysfunction.

Aromatase inhibitors are clinically used for treatment of breast cancer, and they are associated with the increase of coronary artery disease (CAD) (183). Anti-Notch drugs, such as γ-secretase inhibitors, are been tested in association with aromatase inhibitors for the treatment of breast cancer. Our findings show that in the vascular endothelium, the E2-mediated protective effects are dependent on Notch1, thus the combination of aromatase inhibitors with Notch inhibitors could further increase the risk and severity of CAD in women with breast cancer.

In conclusion, determining the mechanisms of action of estrogens, and their role in the cardiovascular function, could lead to the development of novel

pharmacological therapies for cardiovascular disease not only in post-menopausal women but also in men, in whose endothelium, as was observed in mice (184), biologically active levels of testosterone-derived estradiol could be present.

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