Biochemical Pharmacology 80 (2010) 2032–2041

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Inflammation dysregulates Notch signaling in endothelial cells: Implication of Notch2 and Notch4 to endothelial dysfunction

Thibaut Quillard [a,b,c,1], Julie Devallière [a,b,c], Stéphanie Coupel [a,b,c], Béatrice Charreau [a,b,c,*]

[a] INSERM, U643, Nantes F44000, France
[b] CHU Nantes, Institut de Transplantation et de Recherche en Transplantation, ITERT, Nantes F44000, France
[c] Université de Nantes, Faculté de Médecine, Nantes F44000, France

ARTICLE INFO

Article history:
Received 27 April 2010
Accepted 8 July 2010

Keywords:
Endothelium
Inflammation
Notch
Cell signaling
Apoptosis
TNF

ABSTRACT

Although the involvement of the Notch pathway in several areas of vascular biology is now clearly established, its role in vascular inflammation at the endothelial level remains to be elucidated. In this study, we demonstrated that pro-inflammatory cytokines drive a specific regulation of the Notch pathway in vascular endothelial cells (ECs). In arterial ECs, TNFα strongly modulates the pattern of Notch expression by decreasing Notch4 expression while increasing Notch2 expression. Changes in Notch expression were associated with a reduction in hes1 and hey2 expression and in CBF1 reporter gene activity, suggesting that TNFα regulates both Notch expression and activity. Notch2 and Notch4 regulations occurred independently and were found to be mostly mediated by the NFκB signaling pathways and PI3-kinase signaling pathways, respectively. Functionally, TNF-mediated Notch regulation promotes caspase-dependent EC apoptosis. Finally, our findings confirmed that dysregulated Notch signaling also occurs upon inflammation *in vivo* and correlates with caspase activation and apoptosis. In conclusion, inflammatory cytokines elicit a switch in Notch expression characterized by Notch2 predominance over Notch4 leading to a reduced Notch activity and promoting apoptosis. Thus, here we provide evidence for a role of soluble mediators of inflammation (i.e. cytokines) in the regulation of Notch signaling and for the implication of a dysregulated Notch pathway to endothelial and vascular dysfunction.

1. Introduction

Notch signaling is an evolutionarily conserved pathway that allows cell communication through molecular cell/cell interactions [1]. Notch encodes a single pass transmembrane protein with epidermal growth factor (EGF) repeats in the extracellular domain and ankyrin repeats in the intracellular domain that can binds to two different ligands, Delta and Serrate/Jagged. Vertebrates express multiple Notch receptors (Notch1 to 4) and ligands including Delta-like (Dll) 1, 3 and 4, and Jagged 1 and 2. The Notch receptors undergo three successive cleavages before allowing transcription of downstream targets. The first proteolytic event occurs in the trans-Golgi network by a furin-like convertase and leads to the cell surface presentation of a functional heterodimeric form of the receptors. The second cleavage, mediated by a disintegrin and metalloprotease (ADAM) family member, occurred after interaction with a ligand expressed on neighboring cells. Finally, the γ-secretase complex allows the cytoplasmic release of the intracellular domain of the receptor. This fragment is then translocated into the nucleus where it binds to the mammalian transcription factor CBF1/RBP-Jκ docked in a transcriptional repressor complex. This interaction ultimately leads, through displacing the silencing complex and by the recruitment of coactivator factors, to the expression of primary target genes such as the hes and herp/hey genes [2]. Many studies have reported that the Notch pathway plays a fundamental role in drosophila and mammal development [1]. More recently, it was shown that Notch also plays major roles in the adult in several contexts involving cell plasticity, such as proliferation, oncogenesis [3], immune recognition [4], and angiogenesis [5].

Endothelial cells (ECs) control vascular tone, leukocyte adhesion, coagulation and thrombosis by a fine-tuned regulation of many cell surface and soluble molecules [6]. EC activation is considered to be an early event which subsequently leads to EC dysfunction and ultimately to vascular injury, key events

Abbreviations: ECs, endothelial cells; HAECs, human arterial ECs; HUVECs, human umbilical vein ECs; ICD, intracellular domain; SMCs, smooth muscle cells.

* Corresponding author at: INSERM U643, ITERT, CHU Hôtel-Dieu, 30 Bd Jean Monnet, F-44093 Nantes, France. Tel.: +33 240 087 416; fax: +33 240 087 411.
E-mail addresses: Beatrice.Charreau@univ-nantes.fr, brlcharreau@gmail.com (B. Charreau).
[1] Present address: Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.07.010

associated with acute and chronic inflammation, including sepsis, atherosclerosis and acute vascular and chronic allograft rejection [7,8]. EC changes involve membrane damage, increased permeability, swelling, apoptosis and necrosis. The EC loss of function could be as a result of changes in hemodynamic forces (shear and/or hoop stress), direct drug-induced cytotoxicity, mechanical device implant-induced injury and/or immune-mediated mechanisms [9,10]. Inflammatory signaling cascades alter EC integrity by enhancing expression of cellular adhesion molecules, activation of cytotoxic T cells and/or induction of antibodies directed against EC surface [7]. Local release of inflammatory cytokines, including TNFα and IL-1β, and chemokines activate ECs to upregulate soluble adhesion molecules, activate neutrophils and generate reactive oxygen species that amplify the initial inflammation leading to dysregulated apoptosis, secondary necrosis and overt vascular injury lesions. Considering the role of the endothelium in the initiation and propagation of vascular wall injury, there is a need for the discovery of molecular targets to serve as inhibitors of EC activation, dysfunction and vascular injury [6].

Both embryonic and adult ECs express Notch receptors and Notch ligands [2]. Notch signaling has been extensively implicated in endothelial cell-fate determination along vasculogenesis and angiogenesis [11]. Several studies examining the effects of activated Notch signaling on EC phenotype and function have identified potential mechanisms including endothelial-to-mesenchymal (EMT) transformation [12], EC proliferation [13] and control of apoptosis [14]. Recent findings further suggest a potential role for deregulated Notch signaling in tumor angiogenesis and metastasis [15]. It has also been reported that Notch may be necessary for the establishment and/or maintenance of quiescent EC phenotype [16]. However, implication of Notch signaling in activated EC phenotype and function upon inflammation has not been documented.

In previous studies, we investigated signaling pathways regulated by TNFα in vascular ECs [17–20]. Of particular interest, we have shown that the desintegrin and metalloproteinase known as Kuzbanian or ADAM-10 is strongly upregulated at mRNA and protein level in ECs activated with TNFα [17]. ADAM-10 is involved in the processing of Notch receptors and ligands [21], suggesting a potential crosstalk between TNF signaling and Notch pathway that may contribute to changes in EC phenotype and functions. We also reported on the contribution of Notch signaling in transplant arteriosclerosis and endothelial injury [22,23]. In this study, we investigated the regulation of Notch receptors and effector molecules in human vascular ECs upon stimulation with TNFα and other pro-inflammatory mediators *in vitro* and *in vivo*. Moreover, the overall Notch activity and the respective involvement of TNFα-mediated signaling pathways, including NFκB, PI-3 kinase and JNK MAPK, in Notch regulation was also examined.

2. Materials and methods

2.1. Cell culture and reagents

Primary cultures of human ECs issued from segments of renal artery (HAEC) or from human umbilical veins (HUVEC) were isolated and cultured as we previously reported [19]. ECs were grown in endothelial basal growth medium (ECBM, Promocell, Heidelberg, Germany) supplemented with 10% fetal calf serum (FCS), 0.4% EC growth supplement/heparin, 0.1 ng/mL human epidermal growth factor, 1 ng/mL human basic fibroblast growth factor, 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, and 50 ng/mL amphotericin B (Promocell). Before activation, confluent EC monolayers were growth factor and serum depleted by culture for 24 h in basal ECBM supplemented with only 2% FCS. For activation, confluent EC monolayers were cultured with 100 U/mL recombinant human gamma interferon (IFNγ) (Imukin, Boehringer Ingelheim, Germany) or human tumor necrosis factor-alpha (TNFα) (provided by Professor P. Neuman, BASF, BASF Ludwigshafen, Germany). Human Recombinant IL1β (R&D Systems, Abingdon, UK) was used at 5 ng/mL. For inhibition experiments, SP600125 (10 μM), N-acetyl-cysteine (NAC, 10 mM), pyrrolidine dithiocarbamate (PDTC; 100 μM) and wortmannin (100 nM) (all purchased from Sigma–Aldrich, Saint Quentin Fallavier, France) were added to cells 1 h before TNFα treatment.

2.2. Recombinant adenovirus, SiRNAs, plasmids and transfection

The recombinant adenovirus for Notch2NICD and GFP (AdN2ICD) was generated as we previously described [23] and produced in the 293 cells by the vector core laboratory of the University Hospital of Nantes (INSERM UMR649 Gene Therapy Laboratory, Nantes, France). The recombinant adenovirus AdTrack-GFP was used as control (AdGFP). Adenoviral infection was carried out in ECGM supplemented with 1% FCS for 3 h at 37 °C, 5% CO_2 under agitation. Transduction efficiency was analyzed 24 h after infection through GFP detection by direct microscopy imaging and Flow Cytometry using a FACScalibur® (BD Biosciences, Franklin Lakes, NJ, USA).

For gene silencing, cells were transfected according to manufacturer's recommendations with RNAiMax lipofectamine® (Invitrogen, Cergy Pontoise, France) and siRNA targeting Notch4 (#107458, 95% knockdown, #107459, 74% knockdown), or a scrambled negative control (#AM4611) (Ambion, Austin, TX, USA) at a final concentration of 10 nM. Specific expression knockdown by siRNA was attested by qRT-PCR and functional assays were assessed 48 h post-transfection.

2.3. Semi-quantitative RT-PCR, quantitative real-time PCR and Southern blotting

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase (Ambion) before reverse transcription (RT). Subsequent to RT, cDNA were amplified by PCR and analyzed in agarose gels stained with ethidium bromide. For semi-quantitative RT-PCR, PCR conditions were optimized for each primer set and performed for 18–35 cycles of amplification to allow semi-quantitative analysis (Table 1). For Southern blotting, PCR products were purified according to the nucleospin extract II protocol (Macherey-Nagel, Hoerdt, France). Purified amplicons were then cloned into the TOPO TA cloning® vector (Invitrogen) and sequenced before their use as cDNA probes. Southern blotting and hybridizations were performed as we previously described [17]. Quantitative PCRs were performed using the ABI PRISM 7700 sequence detection application program (PE Applied Biosystems, Foster City, CA, USA). For quantification, duplicates were normalized by the concomitant quantification of hypoxanthine-guanine phosphoribosyl transferase (HPRT). Normalization was made with the control samples in the human cells and with an additional reference sample for the rat study. Relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method, as previously described [24]. Custom primers were obtained from MWG (High Point, NC, USA) and used for semi-quantitative PCR and qPCR (Table 1). Transcript levels were quantified by qRT-PCR with the following primers and probe from Applied Biosytems (Foster City, CA, USA): Notch1 (Hs00413187_m1), Notch2 (Hs00225747_m1; Rn00577522_m1), Notch4 (Hs00270200_m1; Rn01525737_g1), hey1 (Hs00232618_m1), VCAM-1 (Hs00365486_m1; Rn00563627_m1) and HPRT (Rn01527838_g1).

2.4. Immunoblotting

Cells were lysed on ice in 20 mmol/L Tris–HCl (pH 7.4), 137 mmol/L NaCl, 0.05% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride supplemented with protease inhibitors (PIC,

Table 1
Oligonucleotide primer pairs and PCR conditions for semi-quantitative analysis.

Target	Forward (sequence 5′–3′)	Reverse (sequence 5′–3′)	Number of PCR cycles
Notch2	GCAGGAGGTGGATGTGTTAG	CCAGGATCAGGGGTGTAGAG	21
Notch4	TGTTTGATGGCTACGACTGT	TCCTTACCCAGAGTCCTACC	27
hes1	AGAGGCGGCTAAGGTGTTTG	GAGAGGTGGGTTGGGGAGTT	25
hes2	TCATCCTGCCGCTGCTGGG	TACCCTGGAGCTGCTGAAG	30
hes3	TCCTCCTCCCCGAAAGTCTC	CACGACCAGAACGGACGACT	35
hes4	CTCAGCTCAAAACCCTCATC	GCGGTACTTGCCCAGAACGG	30
hes5	TGGGGTTGTTCTGTGTTTGC	CAGACCACCAGGCACACTCA	35
hes6	CCCTGAGGCTGAACTGAGTC	CTACCCCACCACATCTGAAC	30
hes7	TAGGGGTGGGGTAGAGACTC	AGACAGAAGGGAAGGGAAAG	35
hey1	CAGGCAACAGGGGGTAAAGG	GTGGAGCGGATGATGGTGGT	27
hey2	GTCGCCTCTCCACAACTTCA	CTGGACGTGGCTGATACTGA	27
hey3	TGGGACAGGATTCTTTGATG	GGTAAGCAGGAGAGGAGACA	35
VCAM-1	AATGTTGCCCCCAGAGATAC	TCTCCTGTCCTCGCTTTTTT	27
β-actin	TCTGGCACCACACCTTCTAC	CAGCTTCTCCTTAATGTCAC	18

Sigma–Aldrich). Lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (6–10%) and subjected to Western immunoblot analysis using specific antibodies against Notch2 (C651.6DbHN, Developmental Studies Hybridoma Bank, IA, USA), Notch4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), total and cleaved caspase 3 and 7 (Cell Signaling Technology, St Quentin-en-Yveline, France) and tubulin (Oncogene, MERCK EuroLab, Val de Fontenay, France), and secondary horseradish peroxidase-labeled anti-rabbit, anti-mouse, or anti-goat antibodies (Cell Signaling Technology). Antibody-bound proteins were detected using an enhanced chemiluminescence kit (ECL kit, Amersham, Les Ulis, France).

2.5. Apoptosis assays

Measurement of caspase activity – Caspase activity was analyzed by western blot using antibodies against cleaved Caspase-3 (Asp175), cleaved Caspase-7 (Asp198), and antibodies against total caspase-3 and -7 (all from CST).

The cell-permeable fluorogenic substrate PhiPhiLux-G2D2 (OncoImmunin, Gaithersburg, MD) containing the cleavage site DEVD was used to monitor caspase-3-like activity in intact cells. ECs were incubated with the substrate solution for 1 h at 37 °C in the dark, according to the manufacturer's instructions. Caspase 3 activation/apoptosis was examined during 18 h by time lapse imaging using a microscope DMI6000B (Leica Microsystems SAS. Rueil Malmaison) equipped with an objective lens X40 (HCX FL Plan), and a CCD camera (Coolsnap HQ2, Photometrics Roper Scientifics SAS Evry). Caspase 3 positive cells/field were counted every 2 h between 48 h and 66 h post-infection with AdN2ICD and controls adenovirus. Results were expressed as the percentage of caspase-positive ECs.

2.6. Animal model of vascular inflammation

The care and use of animals in this study complied with institutional guidelines. Male Sprague Dawley rats (300–400 g body weight) purchased from Charles River (Saint-Aubin, les Elbeuf, France) were injected intravenously, under anesthesia, with 10 μg/kg of recombinant rat TNFα (PreproTech, Neuilly-Sur-Seine, France) or PBS as vehicle. Animals were euthanized for organ collection 1, 4 or 6 h after treatment. Rat RNA and proteins were isolated using TriZol® (Invitrogen) and RIPA (0.5% sodium deoxycholic acid, 0.1% SDS, 1% NP40, PBS, protease inhibitors) buffers, respectively, and were then treated as reported above.

2.7. Statistics

Data are represented as means ± SE for replicates experiments ($n = 3$ independent experiments). Statistical analysis was performed on Graphpad Prism® Software (Graphpad Software, San Diego, CA) with the parametric or Kruskal Wallis non-parametric analysis of variance test as appropriate. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Constitutive and cytokine-regulated expression of the Notch2 and Notch4 receptors in vascular cells

To further characterize the vascular changes in expression and activity of the Notch pathway molecules upon inflammatory processes, we sought to analysis the pattern of mRNA levels for Notch receptors in resting and cytokine-activated vascular cells. To this aim, primary cultures of ECs from two different vascular beds (HAEC from arteries and HUVEC from veins) were treated for 0–24 h with recombinant tumor necrosis factor-alpha (TNFα) and transcript levels were analyzed by semi-quantitative RT-PCR. As shown in Fig. 1A and B, transcripts for the receptor Notch4 were detected in untreated vascular ECs whereas only minimal levels of Notch2 mRNA were found, indicating that quiescent ECs differentially express Notch receptors. TNF elicits an upregulation of Notch2 and a downregulation of Notch4 in both HAEC and HUVEC.

Quantitative PCRs confirmed that, in ECs, TNFα selectively modulates the mRNA steady-state levels for the Notch receptors. TNFα decreased transcript levels for Notch4 with a significant effect starting 2 h after treatment and maximal inhibition of 78 ± 2% as compared with basal levels ($^*p < 0.05$) (Fig. 1C). In contrast, an enhanced mRNA level for Notch2 was found in response to TNFα, corresponding to a maximal 3.3 ± 0.3-fold increase at 24 h ($^*p < 0.05$) as compared to the basal mRNA level. Western blotting analysis (Fig. 1D) indicates that regulation in of Notch2, and 4 protein level paralleled changes in steady state mRNA levels for these molecules (up to 2.69 ± 1.26 and 0.28 ± 0.02-fold the baseline for Notch2 and Notch4, respectively; $^*p < 0.05$), suggesting that TNFα triggers an effective and selective Notch regulation at both mRNA and protein levels in ECs.

Next, we tested whether Notch expression could be regulated by cytokines other than TNFα. To address this question, qRT-PCR was used to compare mRNA levels for Notch2 and Notch4 in ECs treated with the cytokines TNFα, interleukin-1β (IL1β) and interferonγ (IFNγ) (Fig. 2). A comparable regulation in both time course and magnitude was observed for Notch2 in ECs activated with TNFα, IL1β or IFNγ (up to a 2.3 ± 0.3-fold increase for Notch2 as compared with untreated cells). Notch4 exhibited the same pattern of regulation upon TNFα and IL1β stimulation, with a maximal decrease in mRNA of 65 ± 5% and 69 ± 1% for TNFα and IL1β, respectively. Regulation of vascular cellular adhesion molecule-1 (VCAM-1) is shown as a control of EC activation. Similarly to

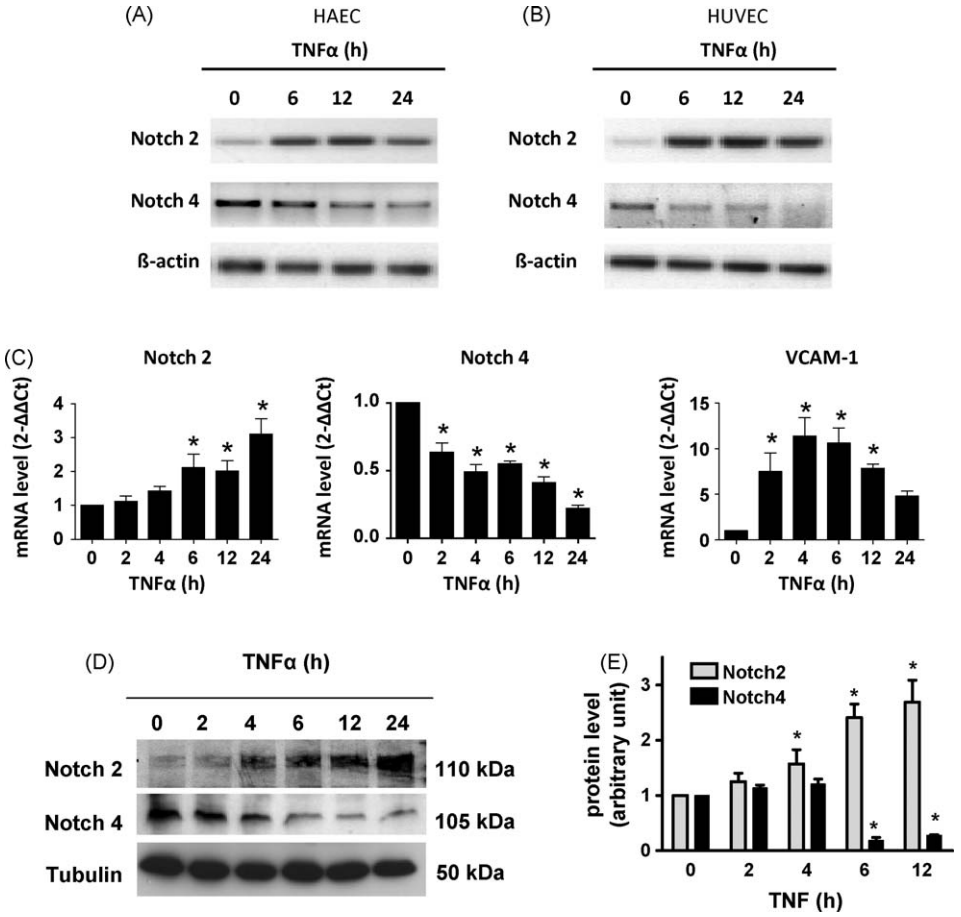

Fig. 1. Expression of Notch receptors in resting and TNFα-activated endothelial cells. Confluent cells monolayers were incubated with 100 U/mL TNFα for the indicated periods. Cells were lysed in parallel experiments to allow RNA and protein analysis. Semi-quantitative RT-PCR analysis of mRNA levels for Notch2 and 4 in vascular cells: HAEC (A), HUVEC (B). PCR products were separated on 1.2% agarose gels, blotted onto nylon membranes and hybridized with specific radiolabeled PCR probes. β-Actin mRNAs were amplified as normalization controls. Representative autoradiographs of three independent experiments are shown. (C) Real-time quantitative PCR for Notch2 Notch4, VCAM-1 in HAECs. Results shown are the mean ± SEM from three independent experiments performed in duplicates and are expressed as relative expression, calculated according to the $2^{-\Delta\Delta Ct}$ method (*$p < 0.05$ versus control). (D) A representative analysis of Notch protein expression in HAEC by Western blotting. Blots were reprobed with an anti-tubulin antibody to ensure equal loading. (E) Quantification of western blot analysis for Notch2 and Notch4 from at least 4 independent experiments.

VCAM-1, IFNγ had no significant effect on the reduction of Notch4 transcripts.

3.2. TNFα-mediated regulation of Notch effectors and impact on basal Notch activity in cultured ECs

Expression of Hairy/Enhancer of split (hes) and Hairy-related (hey/hrt/herp) transcription factors has been shown to be regulated by activation of Notch receptors [25]. Consequently, activity of the Notch pathway should be reflected by the expression of the hes and hey transcripts. Cells were incubated with recombinant TNFα for 0–24 h and mRNA levels for hes-1, 2, 3, 4, 5, 6, and 7 and hey1, 2 and 3 were analyzed by RT-PCR. As shown in Fig. 3A, ECs basally express significant levels of hey2, hes1 and hes2 mRNAs. We found that transcript levels of hes2, 4 and 6 were lower than hes1/hey1-2 (detection achieved at >30 cycles of PCR amplification, see also Table 1). In addition, no mRNA for hes3, 5 or 7 or hey3 was detected by RT-PCR (at 35 PCR cycles), suggesting that these effectors molecules are weakly expressed in ECs and play minor roles in Notch signal in ECs (data not shown) as compared to hes1/hey1-2. Among these molecular targets of Notch activity, only hey1 was found upregulated in TNFα-activated ECs. In contrast, after TNFα treatment, hey2 and hes1 showed a significant decrease in their expression.

In parallel, the expression of effector molecules in response to TNFα, IL1β and IFNγ was investigated. As shown in Fig. 3, a drastic downregulation of both hes-1 and hey-2 was found in response to TNFα (79 ± 1% and 78 ± 1% inhibition at 24 h, respectively). Similarly, IL1β induced a comparable regulation pattern with a maximal inhibition achieved at 6 h for hes1 and at 12 h for hey2 (52 ± 3% and 71 ± 4% of decrease, respectively, as compared to untreated cells). In addition, the enhancement of hey1 (2.2 ± 0.3-fold increase versus control) by TNFα was further observed with IL1β (6.5 ± 1.6-fold increase as compared to basal expression level). However, no significant regulation of these effector genes was obtained after IFNγ treatment, suggesting selective Notch receptors/effectors regulations and therefore functions in response to inflammatory stimuli.

3.3. TNF-mediated regulation of Notch2 and Notch4: involvement of NFκB, PI-3 kinase and MAP kinase signaling pathways

The selective effects of inflammatory cytokines by TNFα, IL1β and IFNγ on Notch molecules also suggest that specific signaling pathways are implicated in this process. In ECs, TNFα activates several signaling pathways including the phosphatidylinositol 3-kinase (PI3-K), nuclear factor-κ B (NFκB) and mitogen-activated protein kinase (MAPK) pathways [26]. The respective involvement of these pathways in Notch regulation mediated by TNFα in ECs was examined. For this purpose, HAECs were pretreated with or without signaling pathway inhibitors (N-acetyl cysteine (NAC), PDTC, wortmannin and SP600125) for 1 h and then activated with TNFα for 24 h, a time point leading to maximal regulation as

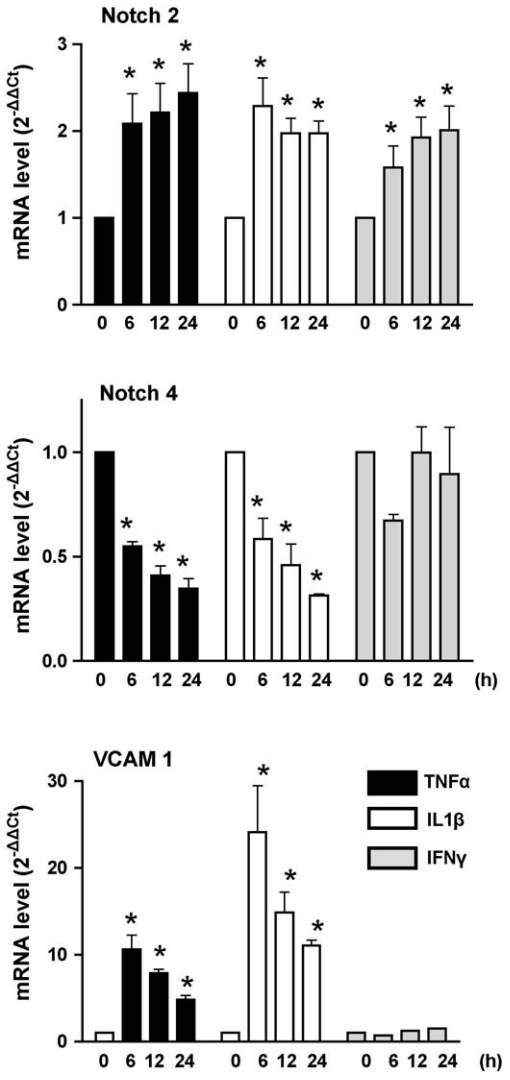

Fig. 2. Comparative effects of various cytokines on Notch signaling. HAECs were treated for 0, 6, 12 and 24 h with TNFα (100 U/mL), IL1β (5 ng/mL) or IFNγ (100 U/mL). Transcriptional regulation was analyzed by Real-time quantitative RT-PCR for Notch2, Notch4 and VCAM-1. Results shown are the mean ± SEM from three independent experiments performed in duplicates and are expressed as relative expression, calculated according to the $2^{-\Delta\Delta Ct}$ method, after normalization to HPRT levels (*$p < 0.05$ versus control).

shown above. Transcript levels for Notch2 and 4 were determined by qRT-PCR.

We found that blocking PI3-K using wortmannin does not affect Notch4 downregulation triggered by TNFα (Fig. 4). In contrast, Notch2 transcriptional upregulation was totally inhibited. Inhibition of the PI3K as well as inhibition of JNK also significantly prevents transcriptional regulation of hey1. An efficient prevention of TNFα-dependent Notch4 and hes1 downregulation was obtained after NFκB inhibition using PDTC or NAC. The blockade of c-Jun N-terminal kinase (JNK) MAPK with SP600125 has no effect on Notch2, Notch4 and hes1 suggesting that this pathway is not involved. Overall, our data demonstrated for the first time that opposite regulations of Notch2 and Notch4 in activated EC require selective signaling pathways suggesting that Notch receptors exert non-redundant, complementary, functions upon inflammation. Moreover, our data showing that hes/hey effector molecules are also selectively regulated by the NFκB and PI3-kinase pathways substantiated the hypothesis that Notch receptors control specific functions through the regulation of selected effectors (i.e. Notch2/hey1, Notch4/hes1).

Next, we used silencing experiments to mimic the changes in Notch4 expression mediated by TNFα in vascular ECs. To this aim, silencing of Notch4 was achieved using two different siRNAs targeting Notch4 (SiN4#1 and SiN4#2) or a scramble non-targeting SiRNA (scramble) as we previously described [22]. Cells were then analyzed by qPCR for Notch2 and Notch4 transcript levels. Notch1 mRNA was used as a control for potential off-target effects. We found that silencing Notch4 has no significant effect on Notch1 and Notch2 expression indicating that downregulation of Notch4 does not trigger the regulation of Notch2. Conversely, we also observed that silencing or overexpressing Notch2 has no regulatory effect of Notch4 expression in ECs (data not shown).

Overall, our findings suggest that TNFα-mediated changes of Notch2 and 4 transcription may occur independently and are dependent, at least partially, on the PI3K and NFκB pathways and support a major role for NFκB in the control of Notch4 and hes1 [27].

3.4. Endothelial changes in Notch2 and Notch4 expression promote EC apoptosis

Next, to functionally assess the impact of Notch regulation, apoptosis assays were performed after modulation of Notch2 and/or Notch4 in cultured ECs. We used gene transfer to mimic the changes in both Notch2 and Notch4 mediated by TNFα in vascular ECs. To this aim, silencing of Notch4 was achieved as above using siRNAs while Notch2 was modulated and activated using an adenoviral vector encoding Notch2-ICD and GFP (AdN2ICD) as we previously described [23]. Controls include a non-targeting siRNA (scramble) and a recombinant adenovirus for the reporter gene GFP (AdGFP). Transduced and knock-down cells were then analyzed by Western blots for caspase activation. We found that silencing Notch4 and overexpression of Notch2 (NICD) similarly induce the cleavage of caspase-3 (Asp175) and caspase-7 (Asp198) indicating that both events are pro-apoptotic in vascular ECs (Fig. 5A). To confirm these results, caspase-3 activity was monitored in live ECs by videomicroscopy using a cell-permeable substrate (PhiPhiLux®) to detect real-time activation of caspase (Fig. 5B–E). The PhiPhiLux probe becomes fluorescent (red) when cleaved by active caspase-3. For these experiments, ECs were silenced for Notch4, transduced using AdN2ICD or both. Higher basal caspase-3 activity in treated cells compared to control reflects the pro-apoptotic effect of Notch modulation (Fig. 5B and C). Consistent with immunoblotting, we show a time-dependent increase in caspase-3 activity in ECs with a sustained Notch2 NICD expression or with a knocked-down for Notch4. Moreover, we found that combination of both further elicits caspase-3 activity suggesting that despite partly independent regulation of Notch2 and 4, apoptosis is a common effector mechanism. These data were further confirmed by annexin V labeling and facs analysis (data not shown).

3.5. Modulation of Notch2 and Notch4 in vascular inflammation in vivo

In order to establish a preliminary evidence *in vivo* for the biological relevance of our *in vitro* findings related to Notch regulation in activated ECs, we investigated Notch expression in rats treated with recombinant TNFα. EC activation was assessed by measuring VCAM-1 expression, a representative marker of EC activation *in vitro* and *in vivo* [7]. To induce vascular inflammation and EC activation, rats were treated intravenously with recombinant TNFα or vehicle as control. At 0–6 h postinjection, kidney, heart and lung were collected for analysis. First, basal expression of Notch2 and 4 transcripts in the different tissues from untreated rats was compared by qRT-PCR. As shown in Fig. 6A, the transcript level of Notch2 and 4 molecules varied greatly according to tissues,

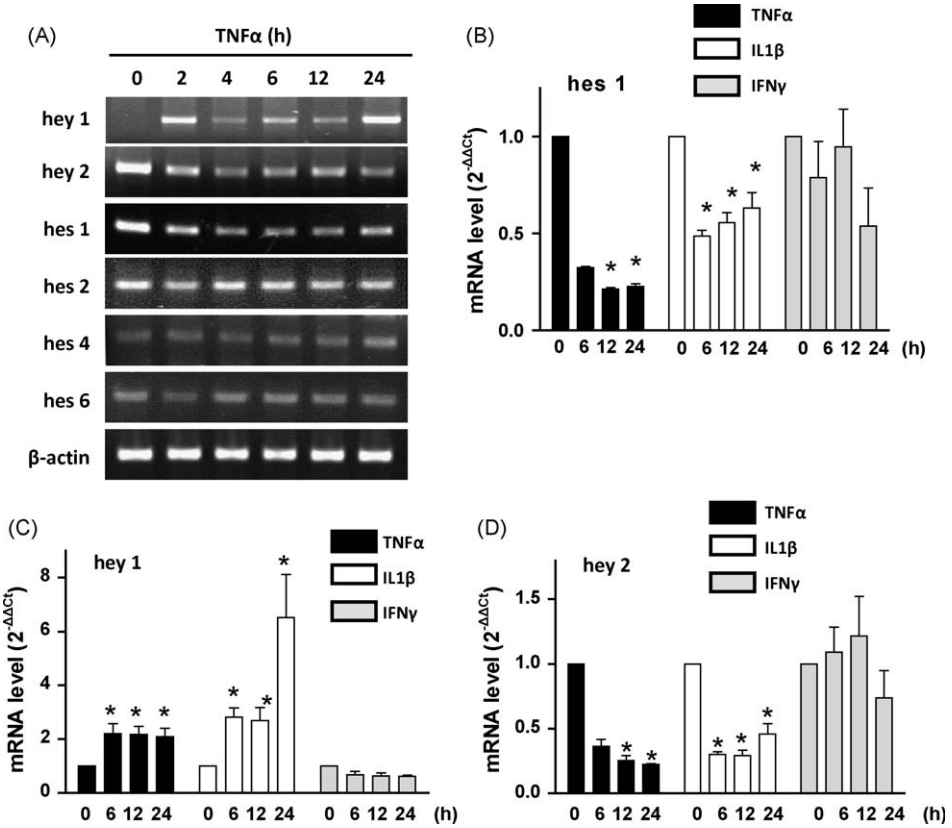

Fig. 3. Regulatory effects of TNF on Notch effectors and Notch activity. HAECs were treated for 0, 2, 6, 12 and 24 h with TNFα (100 U/mL), IL1β (5 ng/mL) or IFNγ (100 U/mL). (A) Expression pattern of Notch effectors hes and hey was determined by semi-quantitative RT-PCR. PCR products were separated on 1.2% agarose gels and stained with ethidium bromide. β-actin mRNA was amplified as a control. Results are representative of three experiment performed. Transcriptional regulation was analyzed by real-time quantitative PCR for hes1 (B), hey1 (C) and hey2 (D). Results shown are the mean ± SEM from three independent experiments performed in duplicates and are expressed as relative expression, calculated according to the $2^{-\Delta\Delta Ct}$ method, after normalization to HPRT levels (*$p < 0.05$ versus control).

with the highest expression levels consistently observed in the lung. The lower levels of Notch2 and 4 transcripts were found in the heart. Ratios of expression levels in the lung compared with the heart were 16.2 ± 3.0-fold (*$p < 0.05$) for Notch2, and, 6.4 ± 1.1-fold (*$p < 0.05$) for Notch4.

Expression of Notch receptors was further examined in lung from rats treated with TNFα for 1, 4 and 6 h (Fig. 6B). Notch2 mRNAs were significantly and transiently increased (1.8 ± 0.1-fold increase as compared to untreated rats; $p < 0.05$ at 1 h). In contrast, Notch4 was downregulated upon TNFα treatment, with a maximal 2.2 ± 0.1-fold decrease in mRNA level, ($p < 0.05$ versus control). Western blotting for Notch2 and Notch4 further confirmed the respective up- and downregulation at the protein level in tissues (Fig. 6C). We also performed immunochemistry analysis on lung sections and we confirmed the decrease at endothelial level of Notch4 in TNF-treated animals (data not shown). Unlike Notch4, Notch2 is ubiquitously expressed in cells and tissues and we were not able to appreciate a clear quantitative increase in Notch2 expression in the endothelium (data not shown). Immunoblotting experiments also associated Notch regulation with pro-apoptotic events reflected by the activation of caspase-3 and caspase-7. The 19-kDa form of cleaved caspase-3 (Asp175) and the 20-kDa form of cleaved caspase-7 5(Asp198) were detected in lung from TNF-treated but not in controls (Fig. 6D).

4. Discussion

Although the impact of the Notch pathway in several areas of vascular biology is now clearly established, its role in vascular inflammation at the endothelial level remains to be elucidated. A large number of studies demonstrated, mostly through modulation of Notch pathway activity, that Notch is involved in EC differentiation, apoptosis and proliferation [12,16,28]. In addition, recent studies also investigated the effect of various effectors, such as soluble mediators of cell growth (VEGF or FGF-2) [28,29], differentiation (TGFβ) [30] or activation (LPS) [31] on the Notch pathway in various cell types. In the present study, we asked whether inflammatory mediators could also modulate the Notch signaling and the pattern of Notch molecules expressed in vascular endothelial cells. To this aim, the effect of cytokines on Notch receptors expression and on Notch activity in human ECs was examined.

Here, we demonstrated that TNFα, the prototype of pro-inflammatory cytokines, drives a specific regulation of the Notch pathway in vascular ECs. In arterial ECs, TNFα strongly modulates the pattern of Notch molecules expression by decreasing Notch4 expression while increasing Notch2 expression. Changes in Notch levels were further observed at the protein level, and were associated with a reduction in hes-1 and hey-2 expression and CBF1 reporter gene activity as previously reported [23], suggesting that inflammation regulates both Notch expression and activity. Interestingly, regulation of Notch4 expression seems to be cytokine-specific since no regulatory effect was found in response to IFNγ, similar to VCAM-1 that is not affected by IFNγ. This TNFα-driven transcriptional regulation was found to be mostly mediated by the NFκB and the PI3-kinase signaling pathways. *In vivo* analysis confirmed that in the lung, TNFα regulates Notch2 and Notch4 at both transcriptional and protein levels.

Four distinct Notch receptors, Notch1, 2, 3, and 4, and five different Notch ligands, Jagged-1 and 2, and Delta-1, 3, and 4, have been identified and characterized in mammals. ECs express

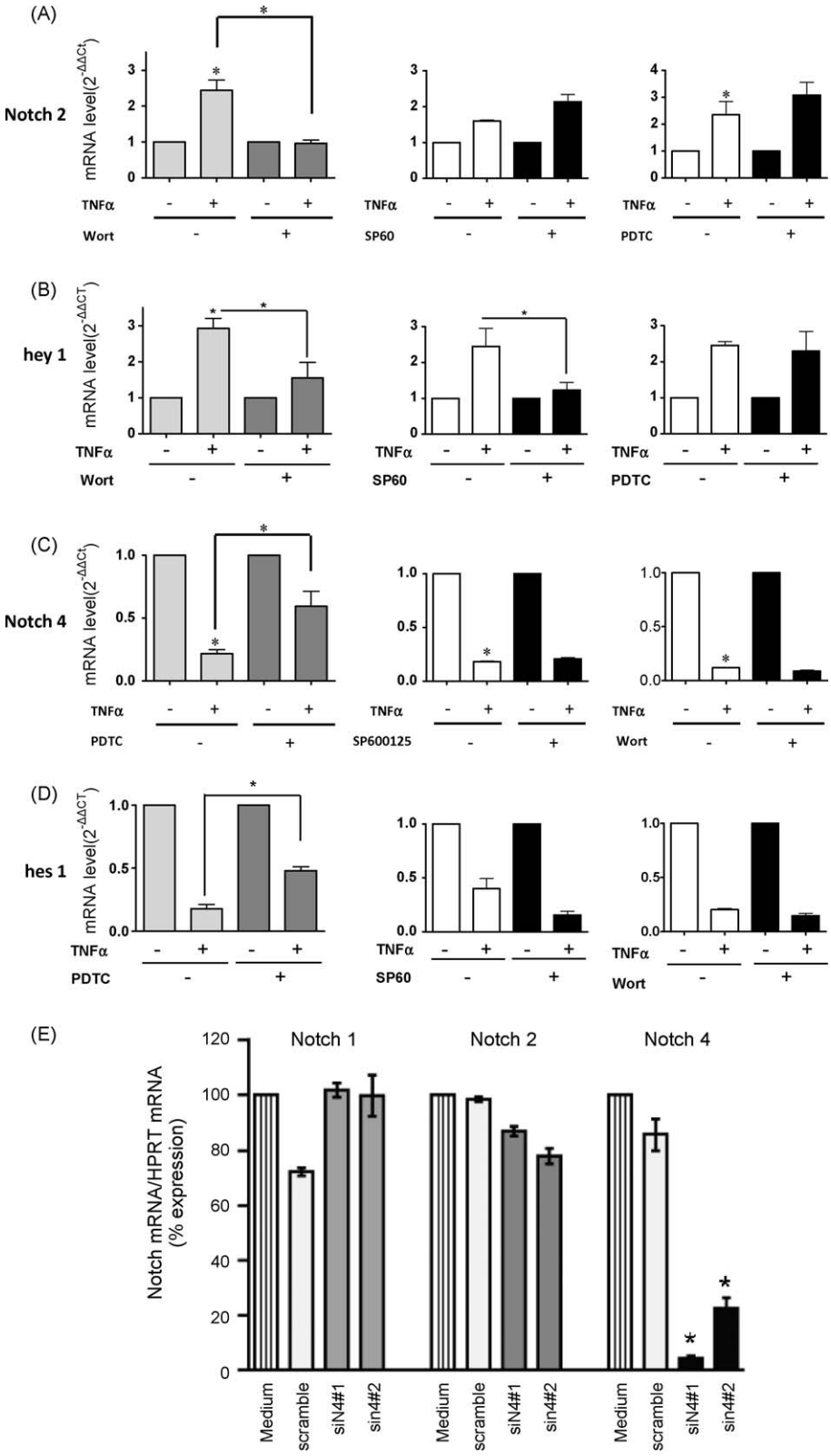

Fig. 4. Signaling pathways involved in TNFα-dependent Notch regulation. HAECs were preincubated with PDTC (100 μM), SP600125 (10 μM) or wortmannin (100 nM) for 1 h before 24 h treatment with TNFα (100 U/mL). Cells were lysed to allow RNA analysis by quantitative RT-PCR for Notch2 (A), hey1 (B), Notch4 (C) and hes1 (D). Results shown are the mean ± SEM from three independent experiments and are expressed as relative expression, calculated according to the $2^{-\Delta\Delta Ct}$ method, after normalization to β-actin levels. $^{*}p < 0.05$ versus TNFα-untreated cells (ctrl) and between non-pre-treated and pre-treated TNFα-activated cells with inhibitors. (E) ECs were transfected with siRNAs targeting Notch4 (SiN4#1 and SiN4#2) or a non-targeting scramble siRNA. Notch1, Notch2 and Notch4 mRNA steady states were analyzed by qRT-PCR 48 h post-transfection. Results shown are means ± SEM from 3 independent experiments and are express as a percentage of control expression (medium).

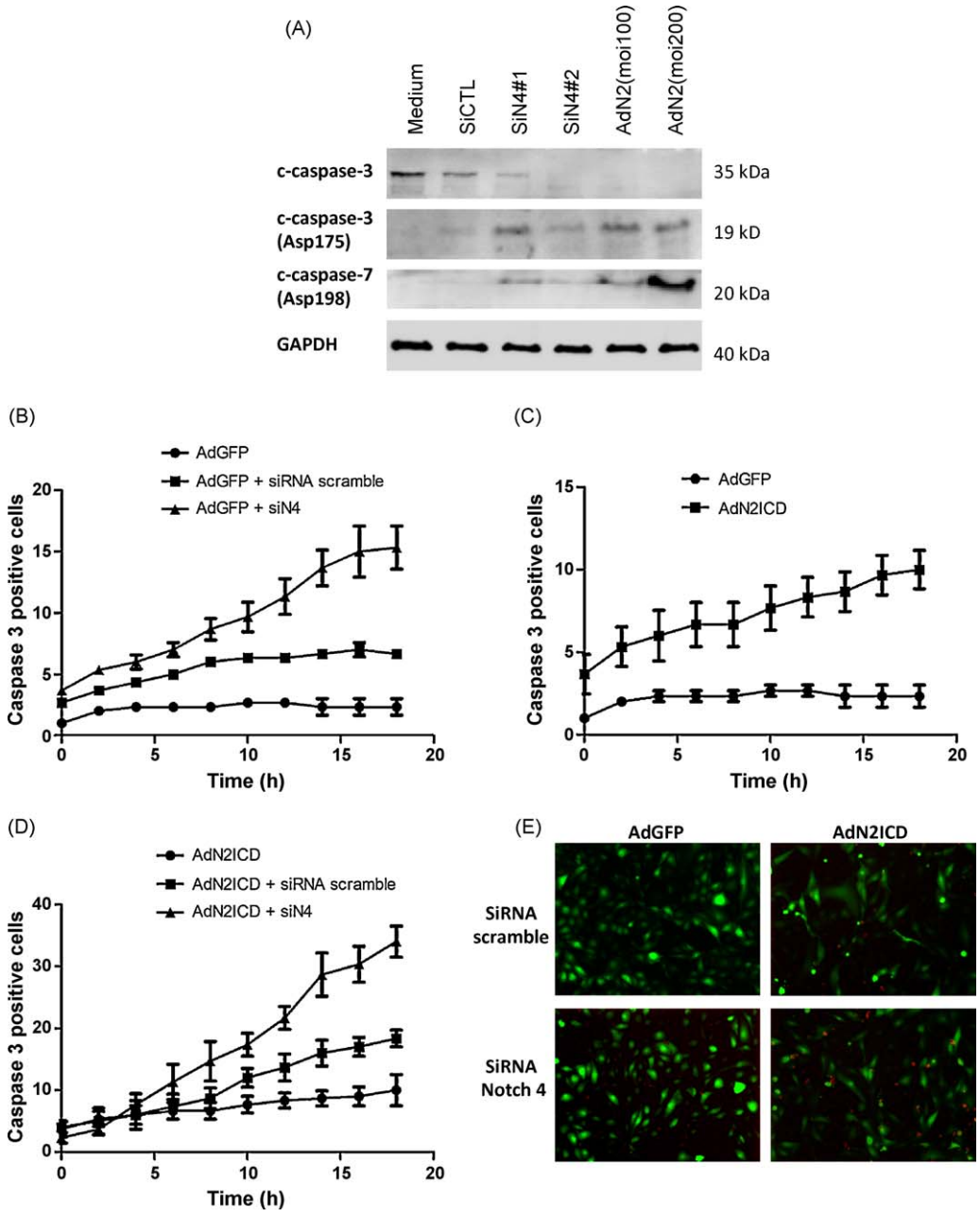

Fig. 5. TNF-mediated Notch regulation and EC apoptosis. (A) A representative Western blot analysis showing caspase-3 and caspase-7 cleavage in ECs silenced for Notch4 or overexpressing Notch2NICD. Immunoblotting was performed using specific anti-cleaved or total form of caspases antibodies. Blots were reprobed with anti-GAPDH antibodies (B, C, D, E) Caspase-3 activity in EC transduced with AdN2ICD or AdGFP and/or transfected with either non-targeting (scramble) or Notch4 siRNAs. Caspase-3-like activity was visualized in individual, live ECs by time lapse fluorescence videomicroscopy. Cultures were incubated with cell-permeable PhiPhiLux-G_2D_2 substrate at 37 °C, 5% CO_2. The quenched fluorescence PhiPhiLuxG2D2 substrate is cleaved intracellularly by caspase-3-like proteases, greatly enhancing red fluorescence. Non-apoptotic ECs expressing Notch2NICD-GFP or GPF alone appeared in green while apoptotic ECs are round red fluorescent cells. ECs were examined under a 20× objective and the total number of apoptotic cells determined by counting. (B, C, D) Results are expressed as the percentage of caspase-3 positive EC (*$p < 0.05$). (E) Representative pictures of fields analyzed.

endothelium-specific Notch members, including Notch4 and Dll-4. However, whether normal, quiescent, human ECs express basal levels of other Notch receptors and ligands is not clearly established. Here we show that Notch2 is also expressed in cultured ECs and is upregulated in response to TNFα. However, the concomitant downregulation of Notch4 expression and Notch activity may suggest that Notch4 is the major Notch receptor in arterial ECs or that Notch2 partly exerts its functions by a non-canonical mechanism.

In contrast to ECs and consistent with previous data [32], we also found that vascular SMCs express Notch2 and Notch3 but not Notch4 at mRNA level. Consistent with our results on ECs, vSMCs responded to TNFα with a significant upregulation of Notch2 (about a 4.2-fold increase as compared to untreated cells) and a strong downregulation of Notch3 expression (data not shown).

Associated with the constitutive expression of Notch receptors, we found a basal expression of a selective pattern of effector molecules of the *Hairy/Enhancer of split* (Hes) and Hairy-related transcription factors (Hey, also known as Hrt, Hesr, Hey, CHF, grl, and Herp) family. Previous studies showed basal transcript levels for hes1, hey1 (herp2, hrt1, hesr1) and hey2 (herp1, hrt2, hesr2) in ECs [25,33]. Consistent with these results, we reported significant expression of hes-1 and 2 and hey-1 and 2 associated with a basal CBF1/luciferase activity (data not shown), confirming that endoge-

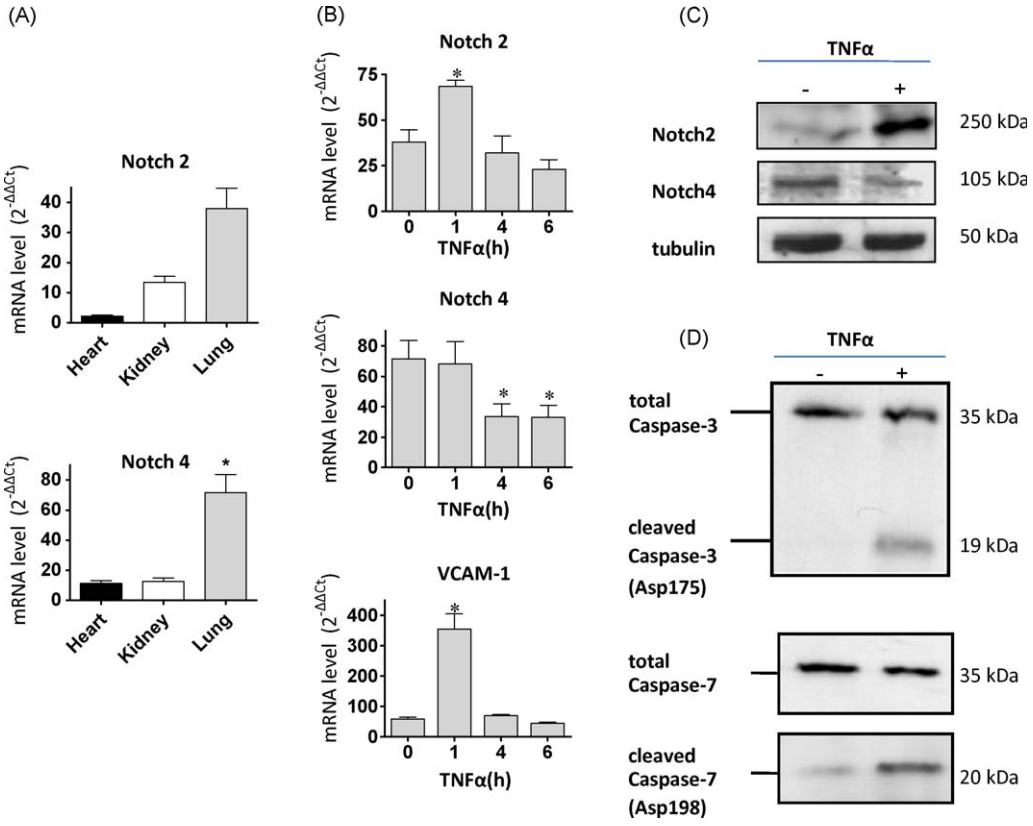

Fig. 6. TNFα-dependent Notch regulation upon inflammation *in vivo*. Rats ($n = 3$) were treated intravenously with rat TNFα (10 μg/kg) for 0, 1, 4 or 6 h. Lungs, heart and kidneys were collected and frozen for mRNA and protein analysis. Real-time quantitative PCR was used for mRNA analysis. Results shown are the mean ± SEM of three independent experiments and are expressed as relative expression, calculated according to the $2^{-\Delta\Delta Ct}$ method, after normalization with β-actin levels ($^*p < 0.05$ versus control). (A) Basal levels of Notch2 and Notch4 transcripts in heart, kidney and lung from untreated rats; $^*p < 0.05$ versus transcript level in heart. (B) Time-course analysis of transcript levels in lung in response to TNFα ($^*p < 0.05$ versus untreated rats). (C, D) Western blotting for Notch2, Notch4, cleaved (Asp175) and total caspase-3, cleaved (Asp198) and total caspase-7 and tubulin in lung in response to TNFα (4 h). A representative experiment out of 3 independent experiments is shown.

nous Notch activity occurs in quiescent ECs and is probably implicated in the maintenance of endothelium quiescence [34]. A microarray comparison of large series of human EC lines confirmed arterial-specific expression for hey2 [35]. Further, those authors showed that ectopic expression of hey2 in HUVECs specifically induces expression of a series of genes that are characteristic of arterial endothelia, implicating hey2 as a key regulator of the arterial phenotype. Consistent with our results, Espinosa et al. provided evidence that TNFα triggers an important decrease in the level of hes1 mRNA, while a lower effect was found on hey1 [36]. Our findings further indicate that, consistent with an overall decreased expression for the major effector molecules hes1 and hey1, TNFα reduces basal CBF1 reporter activity in activated ECs. Considering that CBF1 activity reflects canonical Notch pathway activity, we may extrapolate that TNFα decreases Notch activity in ECs.

The functional consequences of Notch modulation mediated by TNFα in the endothelium appear to promote EC apoptosis. Notch4 has been implicated in the control of proliferation, apoptosis and migration of SMCs and ECs [14,16,37]. Notch2 has mostly been involved in monocyte and T lymphocyte maturation and differentiation [38–40]. Its role in EC biology is still unclear. We recently demonstrated that Notch2 signaling sensitizes EC to apoptosis [23]. TNF elicits a broad array of cellular effects via two receptors TNFR1 and TNFR2. TNFR1 mediates inflammation and cell death while TNFR2 serves to enhance TNFR1-induced apoptosis or to promote cell activation, migration, growth or proliferation in a cell-specific manner [41]. Here, our results suggest that concomitant changes in Notch2 and Notch4 expression elicited by TNFα may have an additive pro-apoptotic effect that triggers endothelial injury and vascular damage.

To conclude, inflammatory cytokines trigger a selective expression pattern of Notch receptors in the endothelium associated with a reduced canonical Notch activity. Consistent with previous models where growth factors modulate the expression of Notch receptors and ligands at both qualitative and quantitative levels, our findings suggest that inflammation may provide additional control of Notch signaling.

Contributors

T.Q. contribute most of the experimental work and participated in the design or the study, data analysis and drafting the manuscript. J.D. contributed significantly to the *in vitro* experiments and apoptosis assays. S.C. performed initial *in vivo* experiments and analysis. B.C. performed conception and design of the study, contributed substantially to interpretation of the data and drafting of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Flora Coulon and Nathalie Gérard for excellent technical assistance and Philippe Hulin and the "confocal microscopy and cellular imaging platform" of IFR26 for time lapse study. This work was supported by "Xenome", a European Commission-funded Integrated Project, Life Sciences, Genomics and Biotechnology for Health LSHB-CT-2006-037377, and by grants from La Société Francophone de Transplantation, La Société de Néphrologie and La Fondation Progreffe. T.Q. was supported by a grant from la Fondation pour la Recherche Médicale.

References

[1] Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284(5415):770–6.
[2] Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. Arterioscler Thromb Vasc Biol 2003;23(4):543–53.
[3] Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 2003;3(10):756–67.
[4] Maillard I, Adler SH, Pear WS. Notch and the immune system. Immunity 2003;19(6):781–91.
[5] Limbourg FP, Takeshita K, Radtke F, Bronson RT, Chin MT, Liao JK. Essential role of endothelial Notch1 in angiogenesis. Circulation 2005;111(14):1826–32.
[6] Pober JS. Endothelial activation: intracellular signaling pathways. Arthritis Res 2002;4(Suppl. 3):S109–16.
[7] Mantovani A, Bussolino F, Introna M. Cytokine regulation of endothelial cell function: from molecular level to the bedside. Immunol Today 1997;18(5): 231–40.
[8] Pober JS. Immunobiology of human vascular endothelium. Immunol Res 1999;19(2–3):225–32.
[9] Pober JS, Cotran RS. The role of endothelial cells in inflammation. Transplantation 1990;50:537–44.
[10] Briscoe DM, Alexander SI, Lichtman AH. Interactions between T lymphocytes and endothelial cells in allograft rejection. Curr Opin Immunol 1998;10(5): 525–31.
[11] Leong KG, Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. Blood 2006;107(6):2223–33.
[12] Noseda M, McLean G, Niessen K, Chang L, Pollet I, Montpetit R, et al. Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ Res 2004;94(7):910–7.
[13] Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. Blood 2006;107(3):931–9.
[14] MacKenzie F, Duriez P, Wong F, Noseda M, Karsan A. Notch4 inhibits endothelial apoptosis via RBP-J{kappa}-dependent and -independent pathways. J Biol Chem 2004;279(12):11657–63.
[15] Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 2006;444(7122):1083–7.
[16] Noseda M, Chang L, McLean G, Grim JE, Clurman BE, Smith LL, et al. Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. Mol Cell Biol 2004;24(20):8813–22.
[17] Boulday G, Coupel S, Coulon F, Soulillou JP, Charreau B. Antigraft antibody-mediated expression of metalloproteinases on endothelial cells. Differential expression of TIMP-1 and ADAM-10 depends on antibody specificity and isotype. Circ Res 2001;88(4):430–7.
[18] Boulday G, Coulon F, Fraser CC, Soulillou JP, Charreau B. Transcriptional up-regulation of the signaling regulatory protein LNK in activated endothelial cells. Transplantation 2002;74(9):1352–4.
[19] Coupel S, Leboeuf F, Boulday G, Soulillou JP, Charreau B. RhoA activation mediates phosphatidylinositol 3-kinase-dependent proliferation of human vascular endothelial cells: an alloimmune mechanism of chronic allograft nephropathy. J Am Soc Nephrol 2004;15(9):2429–39.
[20] Fitau J, Boulday G, Coulon F, Quillard T, Charreau B. The adaptor molecule Lnk negatively regulates tumor necrosis factor-alpha-dependent VCAM-1 expression in endothelial cells through inhibition of the ERK1 and -2 pathways. J Biol Chem 2006;281(29):20148–59.
[21] Six E, Ndiaye D, Laabi Y, Brou C, Gupta-Rossi N, Israel A, et al. The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. Proc Natl Acad Sci USA 2003;100(13):7638–43.
[22] Quillard T, Coupel S, Coulon F, Fitau J, Chatelais M, Cuturi MC, et al. Notch4 activity elicits endothelial cell activation and apoptosis: implication for transplant arteriosclerosis. Arterioscler Thromb Vasc Biol 2008;28(12):2258–65.
[23] Quillard T, Devalliere J, Chatelais M, Coulon F, Seveno C, Romagnoli M, et al. Notch2 signaling sensitizes endothelial cells to apoptosis by negatively regulating the key protective molecule survivin. PLoS One 2009;4(12):e8244.
[24] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001;25(4):402–8.
[25] Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 2003;194(3):237–55.
[26] Madge LA, Pober JS. TNF signaling in vascular endothelial cells. Exp Mol Pathol 2001;70(3):317–25.
[27] Aguilera C, Hoya-Arias R, Haegeman G, Espinosa L, Bigas A. Recruitment of IkappaBalpha to the hes1 promoter is associated with transcriptional repression. Proc Natl Acad Sci USA 2004;101(47):16537–42.
[28] Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, et al. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol 2003;23(1):14–25.
[29] Matsumoto T, Turesson I, Book M, Gerwins P, Claesson-Welsh L. p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. J Cell Biol 2002;156(1):149–60.
[30] Hiratochi M, Nagase H, Kuramochi Y, Koh CS, Ohkawara T, Nakayama K. The Delta intracellular domain mediates TGF-beta/Activin signaling through binding to Smads and has an important bi-directional function in the Notch-Delta signaling pathway. Nucleic Acids Res 2007;35(3):912–22.
[31] Monsalve E, Perez MA, Rubio A, Ruiz-Hidalgo MJ, Baladron V, Garcia-Ramirez JJ, et al. Notch-1 up-regulation and signaling following macrophage activation modulates gene expression patterns known to affect antigen-presenting capacity and cytotoxic activity. J Immunol 2006;176(9):5362–73.
[32] Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, et al. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 2000;14(11):1343–52.
[33] Henderson AM, Wang SJ, Taylor AC, Aitkenhead M, Hughes CC. The basic helix–loop–helix transcription factor HESR1 regulates endothelial cell tube formation. J Biol Chem 2001;276(9):6169–76.
[34] Liu ZJ, Xiao M, Balint K, Soma A, Pinnix CC, Capobianco AJ, et al. Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. FASEB J 2006;20(7): 1009–11.
[35] Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, et al. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci USA 2003;100(19):10623–8.
[36] Espinosa L, Ingles-Esteve J, Robert-Moreno A, Bigas A. IkappaBalpha and p65 regulate the cytoplasmic shuttling of nuclear corepressors: cross-talk between Notch and NFkappaB pathways. Mol Biol Cell 2003;14(2):491–502.
[37] Sweeney C, Morrow D, Birney YA, Coyle S, Hennessy C, Scheller A, et al. Notch 1 and 3 receptor signaling modulates vascular smooth muscle cell growth, apoptosis, and migration via a CBF-1/RBP-Jk dependent pathway. FASEB J 2004;18(12):1421–3.
[38] Ohishi K, Varnum-Finney B, Flowers D, Anasetti C, Myerson D, Bernstein ID. Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. Blood 2000;95(9):2847–54.
[39] Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity 2003;18(5):675–85.
[40] Witt CM, Hurez V, Swindle CS, Hamada Y, Klug CA. Activated Notch2 potentiates CD8 lineage maturation and promotes the selective development of B1 B cells. Mol Cell Biol 2003;23(23):8637–50.
[41] Al-Lamki RS, Wang J, Vandenabeele P, Bradley JA, Thiru S, Luo D, et al. TNFR1- and TNFR2-mediated signaling pathways in human kidney are cell type-specific and differentially contribute to renal injury. FASEB J 2005;19(12): 1637–45.