Notch4, a member of the Notch family of transmembrane receptors, is expressed primarily on endothelial cells. Activation of Notch in various cell systems has been shown to regulate cell fate decisions, partly by modulating the propensity of cells to live or die. Various studies have demonstrated a role for Notch1 in modulating apoptosis, either in a positive or negative manner. In this study, we determined that constitutively active Notch4 (Notch4 intracellular domain) inhibited endothelial apoptosis triggered by lipopolysaccharide. Notch signals are transmitted by derepression and coactivation of the transcriptional repressor, RBP-Jκ, as well as by less well defined mechanisms that are independent of RBP-Jκ. A Notch mutant lacking the N-terminal RAM domain showed only partial antiapoptotic activity relative to Notch4 intracellular domain but stimulated equivalent RBP-Jκ-dependent transcriptional activity. Similarly, constitutively active RBP-Jκ activated a full transcriptional response but only demonstrated partial antiapoptotic activity. Additional studies suggest that Notch4 provides endothelial protection in two ways: inhibition of the JNK-dependent proapoptotic pathway in the absence of NotchIC and activation of an antiapoptotic pathway through an RBP-Jκ-independent up-regulation of Bcl-2. Our findings demonstrate that Notch4 activation inhibits apoptosis through multiple pathways and provides one mechanism to explain the remarkable capacity of endothelial cells to withstand apoptosis.

The Notch proteins comprise a family of transmembrane receptors that have been highly conserved through evolution as mediators of cell fate and are comprised of four members in mammals (Notch1 to -4) (1). Following intracellular processing of the full-length protein by a furin-like convertase, Notch is expressed at the cell surface as a heterodimeric receptor (2, 3). Engagement by ligand results in a two-step cleavage of the Notch heterodimer. These cleavage events release the intracellular domain of Notch (NotchIC) from its membrane tether, whereupon NotchIC translocates to the nucleus and interacts with the DNA-binding factor, RBP-Jκ (CBF1). RBP-Jκ is a DNA binding protein that has dual function: it represses transcription in the absence of NotchIC and activates transcription in its presence (2). In the nucleus, NotchIC and RBP-Jκ associate with other factors to form a multimeric complex that results in transcriptional activation of various basic helix-loop-helix factors of the HES (Hairy and enhancer of split) and HRT (Hairy-related transcription factor, also called HEY, HESR, Gridlock, and HERP) families, through the release of a corepressor complex from RBP-Jκ, and recruitment of a co-activator complex (2). There is cell type-dependent activation of the HRTs, but all three HRTs have been shown to be expressed in vascular cells (4–7). RBP-Jκ-independent Notch activity has also been described, but Notch signaling through this mechanism is less well elucidated.

Because Notch function requires ligand-dependent cleavage of the intracellular domain, enforced expression of NotchIC results in a constitutively active, signaling form of the receptor, which results in altered cell fate decisions in several models (2, 3). The intracellular domain can be divided into two major subdomains; C-terminal to the transmembrane domain, there is a RAM domain, which is followed by an ankyrin domain composed of six Cdc10/ankyrin repeats. The region C-terminal to the ankyrin repeats acts as a putative transactivation domain in Notch1 but not Notch4 (4).

Notch1, -2, and -4 have been reported to be expressed in endothelial cells in vivo, and similar results have been reported in cultured endothelial cells (9). Several studies point to a role for Notch and its ligands in influencing vascular development (10). Notch signaling is required for arterial-venous differentiation in zebrafish (11). Mutant mice that are null for Notch1 show defects in the vasculature, and the severity of these vascular defects is enhanced in mice that are null for both Notch4 and Notch1 (12). A homozygous Notch2 hypomorphic allele disrupts development of vasculature of the glomerulus, heart, and eye (13). Interestingly, constitutive activation of Notch4 also causes defects in vascular remodeling (14, 15).

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Notch4 Activation Inhibits Endothelial Apoptosis

The regulation of vascular cell death and survival is critical during vascular development and homeostasis as well as in diverse pathological processes including inflammation (16–18). Despite continual exposure to various inflammatory cytokines and exogenous toxins, endothelial cells have a remarkable capacity to resist apoptosis, and this may be a mechanism to preserve vascular integrity in pathological situations (19, 20). However, we have shown that in certain circumstances inflammatory mediators, such as tumor necrosis factor and bacterial lipopolysaccharide (LPS), are able to induce endothelial apoptosis. In particular, we have shown that LPS induces endothelial apoptosis by activating the mitogen-activated protein kinase family member, c-Jun NH2-terminal kinase (JNK) (20). Although Notch1 activation appears to promote endothelial viability when cells are starved of serum, little is known about the mechanism (9). Since Notch and its ligands are expressed at high levels in vascular endothelium, we postulated that Notch activation may play a protective role in maintaining endothelial survival in inflammatory situations (21–23).

In this paper, we examined the functional activity of Notch4, a Notch member that is expressed selectively in the endothelium. We demonstrate that activated Notch4 is able to inhibit endothelial apoptosis in response to the inflammatory mediator, LPS, in at least two different ways. First, Notch activation is able to inhibit LPS-mediated JNK activation, through an RBP-Jκ-dependent pathway. Notch also provides antiapoptotic activity by up-regulating Bcl-2 via an RBP-Jκ-independent mechanism. This dual antiapoptotic mechanism makes the activation of Notch a particularly potent inhibitor of the intrinsic apoptotic pathway.

EXPERIMENTAL PROCEDURES

Cell Culture

Transformed human microvascular endothelial cells (HMEC-1, hereafter referred to as HMEC) were provided by the Centers for Disease Control and Prevention (Atlanta, GA) and cultured as previously described (14, 24). Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described (25). Cells were maintained at 37 °C in 5% CO2.

Plasmid Constructs and Gene Transfer

The Notch4 intracellular region (Notch4IC) construct, described previously (14), contains a C-terminal hemagglutinin epitope tag (HA) and includes amino acids 1476–2003 of the 2003 residue full-length Notch4. The Notch4IC deletion mutants were constructed by PCR, using Notch4IC as a template, and inserted into the LNCX retroviral vector. The Notch4IC mutants (see Fig. 3A) include constructs (i) lacking the RAM and N-terminally fused with an SV40-derived NLS (NLS-RAM; encodes amino acids 1518–1614), (ii) containing the RAM and N-terminally fused with an SV40-derived NLS (NLS-ΔRAM; the NLS tag codes for the amino acid sequence DPKKKKKV), and (iii) lacking all six ankyrin repeats (ΔAnk encodes amino acids 1476–1578 and 1801–2003). Notch4IC was also cloned into the MSCV-IRES-YFP (MYF) retroviral vector, as was RBP-VIP16, a constitutively active RBP-Jκ. RBP-VIP16 was constructed by PCR amplification of the 3′ region of the murine RBP-VIP16 cDNA (gift of E. Manet) containing the coding region for the VIP16 transactivation domain (26). The product was digested with AFI1 and ligated to the corresponding AFI1 site of the cDNA for FLAG-RBP-Jκ derived from the RBP-2N isoform of human RBP-Jκ (gift of R. Schmid) (27). The 4×FLAG-RBP-Jκ luciferase plasmid (gift of S. D. Hayward) includes four copies of an RBP-Jκ binding element cloned into the pGL2pro (Promega) firefly luciferase plasmid (28). HMEC and HUVEC were transduced with the various constructs as described previously (19). Polyclonal HMEC lines were isolated by selection into 300 μg/ml of G418 (Invitrogen) for the LNCX constructs and 200 μg/ml of ALLN (concentrations as indicated with ALLN (25 μM)) or cycloheximide (50 μg/ml). After 16 h, viable cell numbers were estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (19). Viability was expressed as a proportion of cells incubated without LPS.

Loss of ΔNκ—To measure mitochondrial transmembrane potential (ΔΨm), 5 × 104 cells were incubated with tetramethyl rhodamine ethyl ester (TMRM) (TMRM Probes, Inc., Eugene, OR) and analyzed for fluorescence on a flow cytometer. The mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (Sigma) was used as a positive control for the detection of loss of ΔΨm.

Caspase Activity Assay—DEVD-p-nitroaniline (pNA) (caspase 3/7) cleavage activity was quantitated with a colorimetric assay kit according to the manufacturer’s instructions (R & D Systems). Briefly, 200 μg of whole cell lysates from HMEC cells exposed to LPS (100 ng/ml) was combined with DEVD-p-nitroaniline (200 μM) in a 96-well plate and incubated at 37 °C. The release of the chromophore by active caspases was quantitated at 405 nm and normalized to untreated cell lysates.

Immunofluorescence

Transduced HMEC lines were cultured overnight on chamber slides, fixed with 4% paraformaldehyde for 15 min, and then permeabilized with cold methanol for 3 min. Nonspecific binding was blocked by incubation with 5% goat serum. Cells were stained with the mouse anti-Notch4 monoclonal antibody (1:100 dilution) for 1 h and then for 50 min with an AlexaFluor 488-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole for 5 min, and coverslips were mounted with 50% glycerol. Slides were viewed using a Zeiss Axiosplan II Imaging inverted microscope (Carl Zeiss Canada), and images were captured with a 1350EX cooled CCD digital camera (QImaging).

Transient Transfection and Luciferase Assays

Transient transfection of luciferase reporter plasmids was carried out by electroporation as described (29). Transduced HMEC lines were grown until ~80% confluence and then trypsinized and resuspended in HMEC medium. Cells (1.5 × 104 transfected) were pelleted at 1000 rpm for 5 min, washed with PBS, pelleted as previous, and then resuspended in 0.4 ml of electroporation buffer (20 mM HEPES, 137 mM sodium chloride, 5 mM potassium chloride, 0.7 mM sodium phosphate, 6 mM m-glucone, pH 7.0) containing luciferase reporter plasmid DNA. The cell/DNA mixture was transferred to a 4-mm gap electroporation cuvette (Bio-Rad), left for 10 min at room temperature, and then electroporated at a fixed capacitance of 900 microfarads and 200 V using a Bio-Rad Gene Pulser II instrument. For each transfection, 2.5 μg of 4×FLAG-RB-p-binding promoter luciferase and 1 μg of RL-CMV was used. The RL-CMV reporter contains the Renilla luciferase cDNA expressed under control of the CMV immediate early enhancer/promoter and serves as a normalization control for transfection efficiency. After electroporation, the cells were left for 10 min at room temperature before plating in prewarmed HMEC medium. The medium was changed 24 h later, and cells were harvested for assay 48 h after transfection. Lysis and dual luciferase reporter assays were performed according to the manufacturer’s recommendations (Promega) with lucinescence measured on a Tropix tube luminescent (BIO/SCAN Scientific). Luminescence values of mock transfections were subtracted from sample luminescence readings to give the net firefly and net Renilla luciferase units. The net firefly units divided by the net Renilla units determined the relative luciferase units.

RNA Isolation and RT-PCR

Total RNA was isolated from confluent cell monolayers using TRIzol reagent (Invitrogen). First strand cDNA was synthesized using 50-μM reactions containing 2.5 μg of RNA and 200 units of SuperScript II reverse transcriptase (Invitrogen). Following RNase H (2 units/reaction) (Invitrogen) treatment, PCRs were performed, and amplicons were resolved on TAE-agarose gels. No PCR products were detected in the negative control reactions performed without reverse transcriptase. Conditions used for amplification were as follows: HRT2, sense primer (5′-tgacatagagctggaaggag-3′) and antisense primer (5′-gaggaagggtagttg-3′) amplified at 57 °C annealing temperature for 28 cycles; glyceraldehyde-3-phosphate dehydrogenase, sense primer (5′- cctggcagtctctgcctcag-3′) and antisense primer (5′-ggccctggtgagcttcagg-3′) amplified at 55 °C annealing temperature for 22 cycles.

RESULTS

Activated Notch4 Inhibits Endothelial Cell Apoptosis—Members of the Notch family have previously been shown to have
either anti- or proapoptotic effects depending on the Notch member, cell type, or apoptotic stimulus (30, 31). Because Notch4 is structurally distinct from the other Notch members and exhibits endothelium-selective expression, we tested whether Notch4 was able to regulate endothelial apoptosis elicited by inflammatory mediators. We have previously shown that LPS can induce endothelial apoptosis; thus, HMEC expressing an activated form of Notch4 were exposed to LPS to induce cell death (20). As seen in Fig. 1A, Notch4IC inhibited HMEC death in response to LPS, as measured by MTT assays.

LPS utilizes a mitochondria-dependent death pathway to induce apoptosis (20). We thus confirmed that endothelial cells were able to maintain mitochondrial integrity by confirming the ability of Notch4IC-expressing cells to retain their transmembrane potential. The cationic fluorophore, TMRE, partitions preferentially to the mitochondria. Loss of mitochondrial transmembrane potential (ΔΨm) is indicated by a decrease in fluorescence as measured by flow cytometry. The net proportion of cells with low ΔΨm is shown. C, HMEC lines were treated with LPS (100 ng/ml) for the times indicated prior to incubation with the lipophilic dye, TMRE. Loss of mitochondrial transmembrane potential over time is plotted as the proportion of cells with low ΔΨm (B) and expressed as a proportion of cells not treated with LPS. Results represent mean ± S.E. of three experiments.

Fig. 2. Activated Notch4 inhibits apoptosis of primary endothelial cells. Notch4IC- or vector-transduced HUVECs were exposed to LPS for 8 h followed by incubation with annexin V-PE. The proportion of YFP-positive, annexin V-positive cells was determined as a proportion of the total YFP population. Results represent the mean ± S.E. of three experiments.

The Ankyrin Repeats Are Required for the Notch4 Antiapoptotic Function—The Notch intracellular domain contains two major subdomains that have been implicated in binding to the downstream effector, RBP-Jk. These are the RAM domain at the NH2 terminus and the ankyrin repeat region. To determine which of these domains are important in the antiapoptotic activity of Notch, deletion mutants were generated lacking each of these subdomains (Fig. 3A, ΔRAM and ΔAnk). Expression of these constructs was confirmed by immunoblotting (Fig. 3B) and immunofluorescent staining (Fig. 3C). Whereas the intact Notch4 intracellular domain and the ΔAnk mutant localized mainly to the nucleus, the ΔRAM mutant was expressed mainly in the cytoplasm, although a small amount of nuclear expression was also seen. The localization pattern of the ΔRAM mutant was confirmed by subcellular fractionation studies (data not shown). Although there is some nuclear expression of this construct, the mainly cytoplasmic localization of the ΔRAM mutant is consistent with the presence of an important nuclear localization signal in the region NH2-terminal to the ankyrin repeats as has been previously suggested (32). In order to ensure nuclear localization of the ΔRAM mutant, an SV40 nuclear localization signal (NLS) was fused to the NH2 terminus of the ΔRAM construct (NLS-ΔRAM), and this construct showed mainly nuclear expression (Fig. 3C).

The various Notch4 mutants were tested for their ability to protect HMEC against LPS-initiated apoptosis. As seen in Fig. 3D, deletion of the ankyrin repeats abrogated the cytoprotective effect of Notch4. In contrast, the ΔRAM mutant only partially lost cytoprotective activity. Because the ΔRAM mutant exhibited a defect in nuclear entry, we tested whether the ΔRAM mutant targeted to the nucleus would provide similar antiapoptotic activity to Notch4IC. Interestingly, the NLS-ΔRAM mutant was not able to protect endothelial cells to the same extent as Notch4IC, but rather this mutant showed a similar degree of protection as the nontargeted ΔRAM mutant (Fig. 3E). Thus, increased nuclear expression is not sufficient for the ΔRAM mutant to provide full cytoprotective activity. These findings indicate that the ankyrin repeats are essential for antiapoptotic function, and the partial protection conferred by the ΔRAM mutant suggests that the RAM motif may signal one of multiple cytoprotective pathways induced by Notch4. Alternatively, The RAM domain may be required for “full” derepression and activation of its downstream effector RBP-Jk.

Only the Ankyrin Repeats Are Required for Notch4-activated RBP-Jk-dependent Signaling—Little is known about the down-
Notch4 Activation Inhibits Endothelial Apoptosis

Fig. 3. The Notch4 ankyrin repeats are required for Notch4 antiapoptotic function. A, structure diagrams of the HA-tagged Notch4IC and deletion constructs. The amino acid (a.a.) residues of the 2003-amino acid human Notch4 protein included in each mutant are indicated in parentheses. B, expression of the Notch4IC constructs in HMEC as detected by immunoblotting of total cellular lysates with a monoclonal anti-HA antibody. Immunoblotting for α-tubulin demonstrates equivalent loading of total protein. C, subcellular localization of the Notch4IC constructs were detected by immunofluorescent staining transfused HMEC lines with a monoclonal anti-HA primary antibody and an Alexa Fluor 488-conjugated secondary antibody. Nuclei are counterstained with 4',6-diamidino-2-phenylindole. Original magnification was × 400. D, Notch4IC and mutant HMEC lines were exposed to LPS at various concentrations for 16 h, and viability was quantitated by an MTT assay and expressed as a proportion of cells not treated with LPS. E, viability of ΔRAM and NLS-ΔRAM HMEC lines was compared with Notch4IC and vector-transduced HMEC by exposing cells to LPS at various concentrations for 16 h, and viability was quantitated by an MTT assay. Results represent the mean ± S.E. of three experiments.

endothelial cells and appears to play an important role in endothelial function (4). Thus, to confirm the ability of the ΔRAM mutant Notch4IC construct to activate an endogenous RBP-Jκ-dependent promoter, RT-PCR for HRT2 from RNA of the mutant Notch4 cell lines was performed. These results confirmed that the ΔAnk mutant was not able to activate the HRT2 promoter, whereas both the ΔRAM and the NLS-ΔRAM mutants increased the expression of HRT2 mRNA to a similar extent as wild-type Notch4IC (Fig. 4B). Thus, our findings suggest that only the ankyrin repeats are necessary for Notch4 to signal through RBP-Jκ, whereas the RAM domain is dispensable for this activity. Further, the finding that the RAM and the NLS-ΔRAM mutants showed similar protective activity is in keeping with the idea that minimal nuclear localization is sufficient for functional RBP-Jκ activation by Notch.

Notch4 Inhibits Apoptosis through RBP-Jκ-dependent and -independent Pathways—The NLS-ΔRAM mutant activates RBP-Jκ-dependent promoters to a similar extent as the wild-type Notch4IC construct, yet this mutant only provides partial protection against apoptosis. Given these findings, we posited that Notch4 is able to protect endothelial cells against apoptosis through RBP-Jκ-dependent and -independent pathways. To test whether blocking RBP-Jκ activation would only partially inhibit Notch-driven antiapoptotic activity, we co-transduced a dominant negative RBP-Jκ construct into Notch4IC endothelial cells. Although this mutant only partially blocked the Notch4 antiapoptotic function, we were also not able to completely block RBP-Jκ-promoter activity using this approach (data not shown). Thus, this approach was not robust enough to determine whether complete inhibition of RBP-Jκ would reveal a Notch-dependent, RBP-Jκ-independent cytoprotective activity. We therefore attempted an alternative approach using a constitutively active RBP-Jκ mutant. To generate a constitutively active RBP-Jκ mutant, the
VP16 activation domain was fused to the COOH terminus of RBP-Jκ, and this chimeric construct (RBP-VP16) was transduced into HMEC. RBP-VP16 was able to activate the RBP-Jκ-dependent promoter to the same extent as Notch4IC (Fig. 5A) but only partially protected HMEC against LPS-induced apoptosis (Fig. 5B). Taken together with the function of the ΔRAM mutant, our findings demonstrate that activated Notch4 is able to inhibit endothelial apoptosis induced by LPS and that RBP-Jκ-dependent and -independent signals are both required for full cytoprotective activity.

**Notch4 Inhibits JNK Activation and Up-regulates Bcl-2 Expression via RBP-Jκ-dependent and -independent Signals, Respectively**—Notch has been shown to modulate JNK activity, and we have previously shown that inhibition or delay of JNK activation protects endothelial cells against LPS-induced apoptosis (20, 35). We therefore tested whether activated Notch4 was able to inhibit JNK activation in endothelial cells, as one potential mechanism of its antiapoptotic activity. Fig. 6A demonstrates that Notch4 significantly attenuates JNK activation induced by LPS and that deletion of the RAM domain does not abrogate this function. In contrast, deletion of the ankyrin repeats does not inhibit LPS-stimulated JNK activation. Since the ΔRAM mutant is able to activate RBP-Jκ-dependent signaling, the above finding suggested that the constitutively active RBP-VP16 mutant would also potentially inhibit JNK activation. Fig. 6B demonstrates that RBP-VP16 does indeed block JNK activation by LPS, thus providing one mechanism for the Notch4-induced, RBP-Jκ-dependent antiapoptotic activity.

Others have demonstrated that Notch1 induces the antiapoptotic protein Bcl-2 in some but not all T cell lines (36). We have seen that various Bcl-2 family members are able to protect endothelial cells against apoptosis induced by LPS or serum starvation (25, 37). We thus tested whether Notch4 and the deletion mutants were able to induce Bcl-2 expression. Interestingly, only wild-type Notch4IC was able to up-regulate Bcl-2 expression, whereas neither mutant had any effect on Bcl-2 levels (Fig. 7A). Bcl-XL and Bax levels were unchanged by Notch4 activation (Fig. 7A). Given that deletion of the RAM domain did not affect Notch4-induced RBP-Jκ activation, we theorized that Notch4 probably induces Bcl-2 expression via an RBP-Jκ-independent pathway. As seen in Fig. 7B, constitutive activation of RBP-Jκ was not sufficient to induce Bcl-2 in endothelial cells, thus confirming that Bcl-2 up-regulation by activated Notch4 occurs through an RBP-Jκ-independent pathway.

**DISCUSSION**

Our findings show that activated Notch4 is able to inhibit endothelial apoptosis through multiple mechanisms and suggest that Notch activation plays a role in maintaining vascular stability. Indeed, studies by Taylor et al. (38) raise the notion that Notch activation may be required for the establishment of a mature, quiescent endothelial phenotype by down-regulating vascular endothelial growth factor receptor-2. Further, the prominent vascular defects observed in Notch- and Notch ligand-deficient mice suggest that inappropriate apoptosis may play a role in the observed phenotypes. Interestingly, the lack of Notch1 and/or Notch4 does not prevent endothelial differentiation in mice (10). The primary vascular plexus is laid down, but remodeling of this initial endothelial network does not take place (10). Thus, it is possible that Notch activation is required to maintain endothelial viability only in reorganizing or mature vasculature.

Notch plays a critical role in the determination of cell fate, and in many cases this is related to the regulation of apoptosis. Notch1 activation has been implicated both in promoting and in inhibiting cell death. In T lymphocytes and tumor cells, Notch1 shows antiapoptotic activity (30, 39). Another study has suggested that activated Notch1 synergizes with papilloma virus oncoproteins and inhibits apoptosis through the activation of phosphatidylinositol-3-kinase (40). In contrast, in chicken B
lymphocytes and human monocytes, Notch1 activation has been reported to promote apoptosis (31, 41).

In T cells, different studies suggest multiple modes of apoptosis inhibition by Notch1. On the one hand, Jehn et al. (39) isolated Notch1 in a yeast two-hybrid screen using Nur77 as bait. Notch1IC inhibited Nur77-dependent transcription and prevented T cell receptor-mediated but not glucocorticoid-dependent apoptosis in D011.10 cells (39). In contrast, Deftos et al. (36) have demonstrated that in the AKR1010 and 2B4.11 T cell lines, Notch1IC protected against glucocorticoid-triggered apoptosis. Interestingly, these investigators demonstrated up-regulation of Bcl-2 in AKR1010, but not 2B4.11, cells, although Notch1IC protected both cell lines against death (36). The above findings demonstrate the cell type specificity of Notch signaling activity and highlight the multiple potential pathways of cytoprotection.

We used a Notch4IC mutant lacking the RAM domain to attempt to separate distinct pathways of Notch-mediated protection of endothelial cells. The RAM mutant showed partial cytoprotective activity despite the ability to activate RBP-Jκ-dependent transcriptional activity to the same level as the intact Notch4IC. Nevertheless, the RAM domain alone is not sufficient to provide cytoprotective activity, since deletion of the ankyrin repeats abrogates all of the Notch-mediated antiapoptotic effect. Thus, the functionality provided by the RAM domain must require the cooperation of the ankyrin repeats. Interestingly, a constitutively active RBP-Jκ mutant also displayed robust transcriptional activity but only partial antiapoptotic function. Taken together, the above findings would suggest that Notch4IC signals antiapoptotic activity that is both dependent and independent of RBP-Jκ. Indeed, neither the RAM mutant nor the constitutively active RBP-Jκ constructs were able to induce Bcl-2 expression, whereas both mutants inhibited JNK activation. One recognized Notch-mediated, RBP-Jκ-independent signal is transmitted via Deltex (42, 43). Interestingly, in the study by Deftos et al. (36), AKR1010 cells that up-regulated Bcl-2 in response to Notch1IC also induced Deltex expression. Although it was not reported whether the T cell line that lacked Bcl-2 induction (2B4.11) also lacked Deltex up-regulation, it is conceivable that the RBP-Jκ-independent induction of Bcl-2 is Deltex-dependent. In contrast, our data indicate that inhibition of JNK activation by Notch4 activation in endothelial cells is RBP-Jκ-dependent.

Our data implicate Notch in inhibition of the intrinsic, mitochondria-directed apoptotic pathway. We have previously seen that enforced expression of Bcl-2 family members is sufficient to inhibit endothelial cell death in response to LPS as well as other apoptotic triggers (25, 37). Similarly, inhibition of JNK activation using a dominant negative JNK mutant inhibited endothelial apoptosis in response to LPS stimulation or serum starvation (29, 35). Both mechanisms of cytoprotection identified in this study are mitochondrionally mediated. Bcl-2 is known to localize to the mitochondrial membrane and inhibit loss of mitochondrial transmembrane potential and release of cytochrome c (44). JNK promotes apoptosis by activating proapoptotic Bcl-2 members of the BH3 family and potentiating Bax-induced cell death (45–47). Thus, by inhibiting JNK and up-regulating Bcl-2, Notch signals a two-pronged antiapoptotic pathway that limits the intrinsic mitochondria-dependent death pathway. Although we did not specifically test the effect of Notch1IC in these studies, a previous study showed that Notch1IC delays endothelial death induced by serum starvation (9). Given that serum starvation-induced endothelial death is also dependent on JNK activation and is inhibited by Bcl-2, our findings suggest that the cytoprotective mechanisms described herein would explain the protection seen in the previous study (25, 35). Our preliminary studies with tumor necrosis factor-induced endothelial apoptosis demonstrate that there is only a slight protective effect of Notch4IC.2 The greater effect of Notch cytoprotective activity on LPS-induced apoptosis is consistent with the intrinsic pathway being critical for LPS but not tumor necrosis factor-induced endothelial cell death, which is mainly mediated through the extrinsic death receptor pathway (20, 37, 48).

Our study thus demonstrates that activated Notch4 is able to inhibit multiple apoptotic pathways converging on the mitochondria. The Notch4 activity is separable into a pathway that is signaled by RBP-Jκ and one that is independent of RBP-Jκ. Endothelial cells are remarkably resistant to various apoptotic triggers in vivo, despite continual exposure to various toxins and proapoptotic cytokines (49). The findings reported in this paper implicate Notch activation in providing one mechanism to explain this capacity for survival.

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\[\text{FIG. 7. Activated Notch4 induces Bcl-2 by an RBP-Jκ-independent mechanism. Cell lysates from HMEC cell lines expressing Notch4IC mutants (A) or RBP-VP16 (B) were immunoblotted to detect expression of Bcl-2, Bcl-X\_\text{L}, and Bax. Membranes were stripped and reprobed with anti-α-tubulin to confirm equal loading and transfer. The relative patterns of protein expression are representative of at least three separate experiments.}\]
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