Determinants of Notch-3 Receptor Expression and Signaling in Vascular Smooth Muscle Cells
Implications in Cell-Cycle Regulation
Alexandre H. Campos, Wenli Wang, Matthew J. Pollman, Gary H. Gibbons

Abstract—The Notch family of receptors and ligands plays an important role in cell fate determination, vasculogenesis, and organogenesis. Mutations of the Notch-3 receptor result in an arteriopathy that predisposes to early-onset stroke. However, the functional role of the Notch signaling pathway in adult vascular smooth muscle cells (VSMCs) is poorly characterized. This study documents that the Notch-3 receptor, the ligand Jagged-1, and the downstream transcription factor, HESR-1, are expressed in the normal adult rat carotid artery, and that this expression is modulated after vascular injury. In cultured VSMCs, both angiotensin II and platelet-derived growth factor (PDGF) markedly downregulated Notch-3 and Jagged-1 through ERK-dependent signaling mechanisms and prevented the glycosylation of Jagged-1. The downregulation of Jagged-1 and Notch-3 was associated with a decrease in CBF-1–mediated gene transcription activation and a fall in the mRNA levels of the downstream target transcription factor HESR-1. To test the hypothesis that the Notch pathway was coupled to growth regulation, we generated VSMC lines overexpressing the constitutively active form of Notch-3 (A7r5-N3IC). These cells exhibited a biphasic growth behavior in which the growth rate was retarded during the subconfluent phase and failed to decelerate at postconfluence. The lack of cell-cycle arrest in postconfluent A7r5-N3IC was associated with an attenuated upregulation of the cell-cycle inhibitor p27kip relative to control cells. This study documents the regulation of the Jagged-1 and Notch-3 genes in VSMCs by growth factor stimulation as well as a role for Notch-3 as a determinant of VSMC growth. (Circ Res. 2002;91:999-1006.)

Key Words: angiotensin II ■ platelet-derived growth factor ■ vascular remodeling ■ neointima ■ glycosylation
esis, our findings indicate that Jagged-1, Notch-3, and HESR-1 are expressed in the adult artery and that growth factors such as angiotensin II (Ang II) and platelet-derived growth factor (PDGF) promote a downregulation of Jagged-1, Notch-3, and HESR-1 expression via an ERK-dependent signaling pathway. Furthermore, this study documents that the Notch-3 signaling pathway is a contextual determinant of VSMC growth behavior. These findings suggest that the pathogenesis of vascular disease may involve alterations in cell proliferation that are governed by the activity of the Notch-3 receptor-signaling pathway.

Materials and Methods

Cell Culture
Quiescent primary cultures from adult rat aortic smooth muscle cells (RASMCs) were analyzed at a postconfluent state between passages 4 and 16. Clonal rat embryonic VSMCs (A7r5) were studied between passages 3 and 20. Cell lines overexpressing the Notch-3 IC (N3IC; pCMX-PL2-N3IC was a kind gift from Dr U. Lendahl, Karolinska Institute, Stockholm, Sweden) were generated from A7 r5 cells by retroviral vectors (pLNCX2, Clontech) and selected in the presence of geneticin (500 μg/mL; G418).

Rat Carotid Artery Balloon Injury
Male Sprague-Dawley rats (12 animals, 300 to 400 g; Charles River Laboratories, Wilmington, Mass) were submitted to balloon injury according to previously described methods, in accordance with a protocol approved by the Standing Committee on Animals, Morehouse School of Medicine. Rats were anesthetized with an intraperitoneal injection of xylazine (5 mg/kg of body weight) and ketamine hydrochloride (90 mg/kg of body weight). Vessels were harvested 28 days later for mRNA and/or protein analysis. Injured vessels were perfused (100 mm Hg) with 0.9% saline and then 10% formalin, 5 minutes each. Vessels were fixed in 10% formalin overnight and then paraffin-embbeded. Sections (5 μm) were prepared, and conventional IH using Jagged-1 antibody (1 μg/mL) was performed ( Vectastain ABC kit, Vector Labs). Antigens were unmasked with 0.1 mol/L acetate buffer (pH 6). Normal nonimmune goat IgG (Santa Cruz; 1 μg/mL) was used as a negative control.

Analysis of Jagged-1 Glycosylation
Tunicamycin (5 μg/mL; Biomol Research Laboratory,) or vehicle (DMSO, 0.1%) was added to control or Ang II–treated RASMCs for 18 hours, and protein samples were extracted. Glycosylation of Jagged-1 was investigated with an enzymatic protein deglycosylation kit (Bio-Rad), according to the manufacturer’s instructions. In brief, protein samples were treated with NANase II and O-glycosidase DS [removes all Ser/Thr-linked Gal[b1,3GalNAc(a1)], and all sialic acid substituted Gal[b1,3]GalNAc(a1)]. PNGase F (removes all Asn-linked oligosaccharides), or with the three enzymes combined. Jagged-1 expression was then analyzed by Western blot.

Experimental Procedures
Expression levels of Notch-1, Notch-2, Notch-3, Jagged-1, HESR-1, and CBP-1 were analyzed in RASMCs treated with Ang II (300 nmol/L) or PDGF (20 ng/mL) by QRTPCR and confirmed by Western blot with available reagents. The mediator role of ERK, p38, and phosphatidylinositol 3-kinase (PI3K) pathways was evaluated in Ang II–treated RASMCs with the following inhibitors, respectively: U0126 (10 μmol/L, Biomol Research Laboratory, Inc), SB 202190 (25 μmol/L, Biomol Research Laboratory), or LY 294002 (50 μmol/L, Sigma-Aldrich Co). The efficacy of pathway blockade at these doses has been confirmed in our laboratory as previously described by others. Twenty–two Finally, the expression levels of Notch family members in rat carotid arteries were investigated at different time points after balloon injury.

 Luciferase Assays
Three tandem repeats of wild-type (3xWTcBF1be; 5'-CTGGTGTAAACACGCCGTTGGAAAAAATT-3') or mutated (3xMTcBF1be; 5'-CTGGTGTAAACACGCCGTTGGAAAAAA-ATT-3') CBP-1 binding elements were inserted into the PGL3 promotor luciferase reporter plasmid (Promega). A7r5 cells were cotransfected (Effectene transfection reagent; Qiagen) with a combination of reporter plasmids, pCMX-PL2-N3IC, or empty vector, and pEGFPc1 (Clontech). In some experiments, to study CBP-1 pathway repression, cells were transiently transfected with the reporter plasmids using the electroporation system Nucleofector (Amaxa), according to the manufacturer’s instructions. Luciferase activity was measured 48 hours after transfection by using a commercially available kit (Promega) and normalized by GFP fluorescence levels. In a separate series of experiments, stable cell lines were transfected with the reporter plasmids and pEGFPc1, and luciferase activity was analyzed.

Analysis of Cell Proliferation
Changes in cellularity were determined by electronic cell counting (Coulter counter). In addition, bromodeoxyuridine (BrdU) (10 μmol/L) was added to cell cultures for 24 hours, and BrdU incorporation was analyzed with a commercially available kit (BrdU flow kit, BD PharMingen), according to the manufacturer’s instructions. Anti-BrdU fluorescence data were normalized by total cell counts.

Statistical Analysis
Comparisons were analyzed via Student’s t test (for two groups; P>0.05) or ANOVA followed by Student-Newman-Keuls test (for three or more groups; P<0.05). Results were presented as mean±SEM. At least 3 different samples were analyzed in each experimental group.

Results
Expression of Jagged-1, Notch-1, -2, -3, and HESR-1 in VSMCs
Our initial studies using QRTPCR and/or Western blot analysis are consistent with a recent report that documents
basal expression of Jagged-1, Notch-3, and HESR-1 in normal adult rat carotid arteries. We observed biphasic expression of this pathway with an initial downregulation of Jagged-1, Notch-3, and HESR-1 mRNA levels within 3 days of balloon injury and later an upregulation of Jagged-1 mRNA within neointimal cells at 28 days after injury compared with uninjured vessels (Figure 1A). Furthermore, Figure 1B confirms that Jagged-1 protein levels were significantly increased in extracts selectively obtained from neointimal tissue compared with medial tissue from contralateral control vessels. This finding was corroborated by immunohistochemical analysis (Figure 1C).

By QRTPCR, we documented the mRNA expression of three receptors (Notch-1, -2, and -3) and at least one ligand (Jagged-1) of the Notch pathway family in quiescent RASMCs maintained in defined serum-free medium. Western blot analysis confirmed the constitutive expression of Jagged-1, Notch-2, and -3, but not Notch-1 protein expression in RASMCs under basal conditions.

Ang II and PDGF Downregulate Jagged-1 and Notch-3 in Cultured VSMCs

Ang II induced a time-dependent downregulation of Jagged-1 mRNA levels in RASMCs within 3 hours of administration (Figure 2A), with a maximal 8-fold inhibitory effect observed after 6 hours (P<0.001). As shown in Figure 2A, Jagged-1 expression levels returned to baseline after 12 hours of Ang II incubation and remained stable for up to 24 hours.

Ang II also induced a time-dependent, 5-fold decrease in Notch-3 mRNA levels (P<0.001). In contrast to the relatively short-lived effect on Jagged-1 expression, Ang II effect on Notch-3 was more sustained over the full 24-hour time course (Figure 2A). The specificity of that response was indicated by the fact that Notch-2 mRNA levels were not significantly affected by Ang II within the first 12 hours of incubation. The profile of changes in Jagged-1/Notch-3 mRNA expression in response to PDGF was similar to that observed with Ang II administration (Figure 2B). Western blot analysis confirmed that Ang II significantly decreased Jagged-1 expression and almost abolished Notch-3 protein levels in RASMCs within 24 hours (Figure 2C).

Regulation of Jagged-1 and Notch-3 Expression by ERK

The QRTPCR analysis demonstrated that the Ang II–induced downregulation of Jagged-1 could be abolished by ERK blockade. Similarly, the inhibition of p38 activation also
attenuated the decrease of Jagged-1 mRNA expression induced by Ang II. In contrast, PI3K inhibition had no effect on the Ang II response (Figure 3A). The capacity of Ang II to induce a decrease in Notch-3 mRNA levels also appeared to be attenuated in the context of either ERK or p38 blockade (Figure 3B). However, the exquisite sensitivity of the QRT-PCR method revealed effects on the basal expression of Notch-3 in response to signal blockade that limits the interpretation of the mediator pathway involved in the Ang II–induced inhibition. To further define the role of each signaling pathway, we examined the overall effect on Jagged-1 and Notch-3 protein expression at 24 hours. As shown in Figure 3C, only the MEK inhibitor (U0126) prevented the Ang II–induced downregulation of both Notch-3 and Jagged-1 protein levels in RASMCs.

**Jagged-1 Glycosylation in VSMCs: Effect of Ang II**

The inhibition of Jagged-1 protein expression by Ang II was associated with the detection of two molecular weight (MW) forms on the Western blots, one ~30 kDa larger than the other (Figure 4A). Similar findings were observed after treatment of RASMCs for 16 hours with PDGF (Figure 4A). The specificity of the immunoreactive bands was verified by confirming that saturation of the primary antibody with the peptide epitope of Jagged-1 abolished both bands (data not shown). We postulated that the higher MW form of Jagged-1 reflects the effects of posttranslational modifications of the nascent form, and that Ang II might also influence Jagged-1 expression by inhibiting this process. We then assessed the effect of blocking a critical step of glycosylation at the Golgi apparatus with the inhibitor tunicamycin on Jagged-1 protein expression. As shown in Figure 4A, tunicamycin treatment resulted in greater expression of the same nascent (lower MW) form of Jagged-1 that is present after Ang II and PDGF stimulation. To further verify this finding, we performed glycosidase treatment of protein extracts from unstimulated RASMCs. We demonstrated that deglycosylation induced by PNGase F (but not by the combination of O-glycosidase and NANase II) results in a shift of Jagged-1 back to its lower MW form (Figure 4B). This finding suggests that Jagged-1 undergoes N-linked glycosylation.
conditions, CBF-1 tonically represses gene expression. This result indicated that under basal activity (Figure 5A). Transient transfection of the Notch-1 IC had a stimulatory effect on CBF-1–dependent gene transcription that was similar to the activation response to Notch-3 IC (Figure 5A). Moreover, we confirmed previous reports that the previously described dominant-negative CBF-1 construct prevents the activation induced by Notch-3IC (data not shown). These findings suggested that the balance between CBF-1–dependent gene activation versus repression in VSMCs is influenced by the level of Notch receptor activation.

Based on this model, we hypothesized that the steady-state level of CBF-1–dependent promoter activity reflected a balance between the intrinsic basal repression versus a tonic, constitutive activation of Notch signaling in VSMCs. According to this hypothesis, the growth factor–induced downregulation of Notch-3 expression should reduce the tonic activation mediated by endogenous Notch IC and result in a further repression of CBF-1–dependent gene expression. As shown in Figure 5B, we observed a 2-fold decrease in luciferase activity in the promoter-reporter construct with the wild-type sequence relative to the construct with the mutant CBF-1 binding element. Of interest, we observed that this basal repression of CBF-1–dependent gene transcription was further enhanced to a 3.4-fold decrease after the treatment with PDGF. Taken together, these findings suggest that the growth factor–induced downregulation of constitutive Jagged-1 and Notch-3 expression is associated with a decline in the tonic activation of CBF-1–dependent transcription in VSMCs.

As an additional test of this working hypothesis, we analyzed the endogenous expression levels of well-defined downstream target genes of CBF-1. Although members of the HES family are well-characterized CBF-1–dependent target genes in other cell systems, we could not demonstrate significant mRNA expression of either HES-5 or HES-1 in RASMCs (data not shown). However, we observed constitutive expression of HESR-1 mRNA in cultured RASMCs under basal conditions. We next examined whether the inhibitory effect of Ang II on Notch-3 expression is transmitted into a similar decrease in HESR-1 expression in RASMCs. Although no significant effect of Ang II stimulation on HESR-1 levels was noted after 6 hours of incubation, we observed a significant 3-fold decline of HESR-1 mRNA levels at 12 hours (Figure 2D). It is notable that this inhibition of HESR-1 mRNA level lags behind the peak downregulation of Jagged-1 and Notch-3 expression by 6 hours. These results suggest that there is constitutive activation of the Notch signaling pathway that maintains tonic constitutive level of HESR-1 expression in VSMCs. Overall, these findings are consistent with the postulate that growth factors are capable of inhibiting the expression and activity of downstream elements of the Notch-3 signaling pathway in VSMCs.

Effect of Constitutive Notch-3-Receptor Activation on VSMCs

To begin to define the functional role of Notch in VSMC biology, we established stably transfected VSMC lines that overexpress the constitutively active intracellular domain of the Notch receptor (Notch-3 IC). To begin to define the functional role of Notch in VSMC biology, we established stably transfected VSMC lines that overexpress constitutively active intracellular domain of the Notch receptor (Notch-3 IC).
the Notch-3 receptor (N3IC). Studies confirmed that N3IC mRNA and protein levels were markedly elevated in A7r5-N3IC cells, compared with neomycin-selected control cells transfected with empty vectors (data not shown). To verify that stable transfection of the N3IC construct resulted in constitutive activation of the Notch signaling pathway, VSMCs were transiently transfected with CBF-1 reporter constructs. As shown in Figure 5C, CBF-1 repression of gene transcription was reversed in A7r5-N3IC, such that luciferase activity levels were significantly higher (4-fold; \( P < 0.001 \)) than in control cells.

The analysis of the A7r5-N3IC phenotype revealed a growth rate significantly slower than that of stably transfected control cells (A7r5-Neo) at the preconfluent stage (Figure 6, upper curves). On reaching the postconfluent state, stably transfected control VSMCs exhibited the typical deceleration in growth, with an eventual plateau in cell number. However A7r5-N3IC continued to grow and accumulate despite reaching postconfluence (Figure 6, upper curves). We verified that this growth pattern was independent of initial plating conditions. When plated at a 10-fold lower density, the time required to achieve confluence was increased as anticipated, but the biphasic pattern of growth exhibited by A7r5-N3IC cells remained intact compared with controls (Figure 6, lower curves).

To confirm that the abnormal increase in cellularity induced by constitutive Notch-3 activation reflected perturbations in cell-cycle regulation, BrdU incorporation was measured after 5 days of postconfluence. Figure 7 demonstrates that DNA synthesis was significantly increased (8.8-fold; \( P < 0.005 \)) in A7r5-N3IC cells compared with A7r5-Neo controls, even after normalization by cell counts. We hypothesized that the Notch-3 signaling pathway may be coupled to the Notch cell fate program. As depicted in Figure 8, both QRTPCR analysis and Western blotting detected significantly lower mRNA and protein levels of the cell-cycle inhibitor p27kip in A7r5-N3IC cells at 5 days postconfluence compared with controls. Thus, constitutive activation of the Notch-3 receptor pathway in postconfluent VSMCs induced an inappropriately persistent state of cell-cycle progression that was associated with the failure to upregulate the cell-cycle inhibitor p27kip.

**Discussion**

Although the role of Notch receptor and ligands in vasculogenesis and organogenesis is well established, the potential influence on vascular function and structure in the adult animal remained to be defined. The present study documents that major components of the Notch signaling pathway, including Jagged-1 and Notch-3, and its downstream effector gene, HESR-1, are constitutively expressed in adult VSMCs both in vitro and in vivo. Lindner et al have recently reported that all four Notch receptors are expressed in the adult vasculature and are upregulated in the context of vascular injury. Similarly, Jagged-1 and Jagged-2 are expressed in regenerating endothelium as well as VSMCs after vascular injury. In an initial study, we observed a biphasic response in which Jagged-1, Notch-3, and HESR1 were acutely downregulated in medial VSMCs within the first 2 days after vascular injury and became upregulated 7 to 14 days after injury compared with uninjured vessels. Taken together, these data indicate that many of the critical elements of the Notch receptor signal transduction pathway are present within the adult vasculature and the expression levels of these mediators are modulated in response to vascular injury.

Our findings indicate that Ang II acutely induces a downregulation of Jagged-1, Notch-3, and HESR-1 in cultured VSMCs. The observation that PDGF induces a similar inhibitory effect suggests that it may be a common feature of factors activated in the context of vessel injury and repair. Furthermore, we showed that an ERK-dependent pathway mediates the downregulation of Jagged-1 and Notch-3 expression by Ang II. This finding suggests a possible nexus by which the activation of a growth regulatory signaling pathway is coupled to the Notch cell fate program.

Recent studies indicate that glycosylation of residues within the EGF repeat domains of Notch plays a critical role in modulating ligand-receptor activation by Jagged versus.
Delta. Fringe is a critical determinant of this process within the Golgi apparatus by its intrinsic fucose-specific acetylglucosaminyltransferase activity. Although Jagged/Delta exhibits a similar EGF repeat domain structure amenable to glycosylation, we are unaware of previous reports that this posttranslational modification is also a potential locus of regulation of the Notch ligands. Our study provides evidence that Jagged-1 undergoes substantial glycosylation in VSMCs through a mechanism (N-glycosylation) distinct from the effect of Fringe on Notch receptors and that this process can be modulated by factors such as Ang II and PDGF. Further studies are needed to clarify the functional consequences of inhibiting Jagged glycosylation, as this may represent a novel additional mechanism for modifying selective ligand-receptor interactions by Notch family members.

To further test the hypothesis that growth factor stimulation inhibits the distal, postreceptor elements of the Notch signaling pathway in VSMCs, we assessed the effect on downstream transcriptional events. It is noteworthy that we observed a basal level of transcriptional repression using the promoter-reporter assays with wild-type versus mutant CBF-1 binding site constructs. Moreover, we observed that the growth factor–induced downregulation of Jagged-1 and Notch-3 was also associated with a further repression of CBF-1–mediated transcription. These findings suggest that there is a constitutive, basal activation of the Notch signaling pathway in these cultured VSMCs and that the downregulation of this pathway potentiates the tonic repression by CBF-1. Given that we failed to detect an upregulation of CBF-1 expression induced by growth factor stimulation, we speculate that enhancement of the tonic repression primarily reflects a fall in the levels of endogenous N3IC in response to decreased levels of Jagged-1 and/or Notch-3. The enhancement of CBF-1–mediated repression in response to growth factor stimulation is consistent with our observation that endogenous mRNA levels of HESR-1 also fall acutely in response to growth factor stimulation.

By utilizing stably transfected VSMC lines (A7r5-N3IC), we demonstrated a bifunctional effect of constitutive Notch-3 receptor activation on the regulation of VSMC growth. It is apparent that under conditions in which there is little cell-cell contact, this pathway promotes a decrease in the rate of cell accumulation. This may be consistent with our finding that factors promoting VSMC proliferation induce a downregulation of this pathway. Conversely, we observed that in the postconfluent context, the Notch-3 signaling pathway appeared to perturb the usual process of growth deceleration. Given that the downregulation of p27 expression often involves posttranslational mechanisms, it is interesting that N3IC appeared to induce a suppression of both mRNA and protein levels. Additional studies are needed to explore whether CBF-1 or HESR-1 is sufficient to induce the observed downregulation of p27 mRNA expression. In preliminary studies, we observed that overexpression of HESR-1 is sufficient to induce the same growth behavior noted in the N3IC cells. Taken together, these studies provide the first demonstration that the Notch-3 receptor is an important context-dependent determinant of VSMC cell-cycle progression. It is likely that the response to Notch-3 receptor activation is modulated by cues within the cellular milieu. It is noteworthy that putative loss-of-function mutations in the Notch pathway are associated with an arteriopathy in humans that predisposes to early-onset stroke. It is intriguing to consider that the link between Ang II and Notch-3 may provide a potential mechanistic basis for the clinical observation that blockade of Ang II generation is effective in preventing stroke. The downregulatory response induced by Ang II on Notch-3, but not Notch-2, mRNA expression levels in VSMCs is also noteworthy, because mutations in Notch-2 seem to be involved in Alagille syndrome, in which the vasculature is not a major disease target, whereas Notch-3 modifications affect primarily blood vessels in CADASIL. It is anticipated that future investigation of the regulation of the Notch signaling pathway in VSMCs will provide new insights into the molecular mechanisms of vascular complications.

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