Notch Activation Enhances the Microglia-Mediated Inflammatory Response Associated With Focal Cerebral Ischemia

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Background and Purpose—Activation of Notch worsens ischemic brain damage as antisense knockdown or pharmacological inhibition of the Notch pathway reduces the infarct size and improves the functional outcome in a mouse model of stroke. We sought to determine whether Notch activation contributes to postischemic inflammation by directly modulating the microglial innate response.

Methods—The microglial response and the attendant inflammatory reaction were evaluated in Notch1 antisense transgenic (Tg) and in nontransgenic (non-Tg) mice subjected to middle cerebral artery occlusion with or without treatment with a γ-secretase inhibitor (GSI). To investigate the impact of Notch on microglial effector functions, primary mouse microglia and murine BV-2 microglial cell line were exposed to oxygen glucose deprivation or lipopolysaccharide in the presence or absence of GSI. Immunofluorescence labeling, Western blotting, and reverse-transcription polymerase chain reaction were performed to measure microglial activation and production of inflammatory cytokines. The nuclear translocation of nuclear factor-κB in microglia was assessed by immunohistochemistry. The neurotoxic potential of microglia was determined in cocultures.

Results—Notch1 antisense mice exhibit significantly lower numbers of activated microglia and reduced proinflammatory cytokine expression in the ipsilateral ischemic cortices compared to non-Tg mice. Microglial activation also was attenuated in Notch1 antisense cultures and in non-Tg cultures treated with GSI. GSI significantly reduced nuclear factor-κB activation and expression of proinflammatory mediators and markedly attenuated the neurotoxic activity of microglia in cocultures.

Conclusions—These findings establish a role for Notch signaling in modulating the microglial innate response and suggest that inhibition of Notch might represent a complementary therapeutic approach to prevent reactive gliosis in stroke and neuroinflammation-related degenerative disorders. (Stroke. 2011;42:2589-2594.)

Key Words: apoptosis ■ brain ischemia ■ focal ischemia ■ inflammation ■ neuroprotection ■ neuroregeneration

Mammalian Notch signaling is initiated when ligands of either the Delta or the Jagged family bind to 1 of 4 Notch receptors. This results in a series of proteolytic cleavages that is coupled with the release and translocation of the intracellular Notch receptor domain (NICD) to the nucleus, where it directs the transcription of downstream target genes known to regulate cell fate decisions and tissue homeostasis. Several lines of evidence suggest that Notch also plays an important role in regulating the responsiveness of immune cells to stimulation and infection. Upregulation of Notch signaling during the course of macrophage activation elevates responses to interferon-γ, leading to higher expression of intercellular adhesion molecule-1 and class II major histocompatibility complex proteins. Furthermore, inflammatory mediators can increase the expression of Notch. These observations raise the possibility that Notch activation is induced during inflammation and may serve to regulate the adaptive and innate immune responses.

We previously reported that aberrant Notch signaling induced by cerebral ischemia worsens brain damage and functional outcome. Brain damage and postischemic inflammation were significantly attenuated in Notch antisense (NAS) mice and in normal mice treated with γ-secretase inhibitors (GSI) that block the proteolytic cleavage and activation of Notch. How Notch activation contributes to the mechanisms of ischemic brain injury at the cellular and molecular level is still unknown. Here we showed that Notch activation induced by cerebral ischemia results in a protracted...
nuclear factor-κB (NF-κB)-driven microglia-mediated inflammatory reaction and that pharmacological inhibition of Notch may provide a means to limit the neurotoxic potential of microglia.

Materials and Methods
Mice transgenic for antisense Notch (NAS) on the C57BL/6J background were characterized previously. Nontransgenic (non-Tg) mice were used as controls. Mice were fed regular chow and water ad libitum and were maintained under pathogen-free conditions in an animal facility with regulated temperature, humidity, and 12-hour light cycle.

Middle Cerebral Artery Occlusion and Drug Administration
Focal cerebral ischemia was induced by middle cerebral artery occlusion as described previously. For GSI treatment, 4 mg/kg DBZ (propionamide; Calbiochem) was administered through the left femoral vein 30 minutes before middle cerebral artery occlusion induction. All animal procedures were approved by the University of Central Florida Animal Care and Use Committee. Detailed procedures are described in the Supplemental Methods (available online at http://stroke.ahajournals.org).

In Vivo Model of Brain Inflammation
To model brain inflammation without brain cell injury, 50 μg/kg lipopolysaccharide (LPS; Escherichia coli serotype 055:B5; Sigma) was injected intraperitoneally.

Preparation of Neuronal and Microglia Cultures
Mouse cortical neurons were prepared as previously described and used after 14 days in vitro. Primary microglia (PM) cultures were prepared from postnatal day 1 mice as previously described and were used 1 to 2 days after plating. The murine BV-2 microglial cell line was maintained in Dulbecco modified Eagle medium supplemented with 2% fetal bovine serum.

Experimental Treatments
To simulate ischemic conditions, cultures were subjected to oxygen glucose deprivation (OGD). Detailed procedures are described in the Supplemental Methods. In some experiments, microglial cultures were treated with LPS. DAPT (Calbiochem) was added to microglial cultures 2 hours before OGD or LPS treatments.

Immunofluorescence Labeling
Brain sections or cells seeded on cover slips were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 minutes, blocked in 5% normal serum for 1 hour, and incubated with primary antibodies to CD11b (Serotec), ionized calcium binding adaptor molecule (Iba1; Wako), Val1774-NICD (Upstate), and NF-κB/p65 (Cell Signaling) overnight at 4°C followed by Alexa 488-conjugated Alexa 568-conjugated secondary antibody (Molecular Probes) for 1 hour at room temperature. Nuclei in immunolabeled specimens were visualized with 4′,6-diamidino-2-phenylindol (Molecular Probes). Detailed procedures on sample processing for immunofluorescence labeling are described in the Supplemental Methods.

Immunoblotting
Protein concentration was assessed using the bicinchoninic acid protein assay (Bio-Rad); 50 μg of total proteins was separated by SDS/PAGE and transferred onto nitrocellulose membrane blot (Bio-Rad). The blot was blocked and incubated with primary antibodies to NICD (Val1774-NICD; Upstate) and β-actin (Sigma). Protein bands were detected with a secondary antibody conjugated with horseradish peroxidase (Jackson Immunoresearch) and the chemiluminescence detection system (Pierce).

Measurements of mRNA Levels
Total RNA was extracted from brain tissues or cultured cells using the RNAeasy Qiagen Mini Kit (Qiagen) and converted to cDNA using SuperScriptII RNaseH (−) reverse transcriptase (Invitrogen). The mRNA levels were measured by semiquantitative polymerase chain reaction and real-time polymerase chain reaction. Real-time polymerase chain reaction was performed on the ABI Prism 7700 sequence detector utilizing SYBR Green PCR Master Mix (Bio-Rad) and relative quantification by the comparative CT method. The primer sequences are shown in Supplemental Table I (available online at http://stroke.ahajournals.org).

Measurements of Cytokine Protein Levels
Cytokine levels were analyzed with the Bio-Plex multiplex mouse cytokine assay kit (Bio-Rad) according to manufacturer recommendation.

Measurements of Caspase Activity, Neuronal Mitochondrial Membrane Polarization, and Apoptosis
The activation of caspase-3/caspase-7 was quantified in live cells using Magic Red caspase-3/caspase-7 (AbD Serotec) according to manufacturer recommendation. Mitochondrial membrane polarization was assessed in live cells with the fluorescent probe JC-1 (Molecular Probes). Neuronal cell death was quantified by trypan blue exclusion assay or staining with Hoechst 33342 (Molecular Probes). Detailed procedures are described in the Supplemental Methods.

Statistical Analysis
Statistical analysis was performed with 2-way ANOVA, followed by Bonferroni posttests or 1-way ANOVA followed by Newman-Keuls test using GraphPad Prism version 5.0. Statistical significance was accepted at the 95% confidence level (P<0.05).

Results
Microglial Activation Is Markedly Reduced in GSI-Treated Mice After Ischemia-Reperfusion
We previously reported that NAS mice exhibited substantially reduced CD11 immunoreactivity after ischemia reperfusion when compared to non-Tg mice, suggesting that Notch activation induced by cerebral ischemia may regulate microglial activation. To confirm further that reduced CD11 immunoreactivity in NAS brains results from decreased Notch activity, we investigated the effects of pharmacological inhibition of Notch activation with DBZ, a highly active GSI, administered before middle cerebral artery occlusion induction. CD11b immunoreactivity was significantly reduced in the ipsilateral cortex of mice treated with DBZ (Figure 1A) at a dose that has previously shown to markedly suppress the ischemia reperfusion–induced increase of NICD to level detected in NAS mice. To exclude the possibility that the attenuation of microglia activation in NAS mice might be attributed to secondary effects of the reduction in infarct size, we administered LPS, a direct promoter of microglial activation without causing neuronal death. CD11b immunoreactivity in the cortex also was reduced in NAS when compared to non-Tg mice after injection with LPS (Figure 1B). Collectively, these data suggest that γ-secretase–mediated Notch signaling may play a direct role in the activation of resident inflammatory cells.
The Notch Pathway Is Activated in Microglia After OGD

To determine whether brain ischemia activates Notch signaling directly in microglia, we subjected PM and BV-2 cultures to 2 hours of OGD, an in vitro ischemic condition that resulted in significant activation but little death of microglia (data not shown). The PM cultures contained 98.5% ± 0.7% microglia as determined by staining with the microglial marker Iba1 (data not shown). Among the Notch receptors and their ligands that are expressed in PM and BV-2, Notch1 is substantially upregulated by OGD (Figure 2A). Levels of NICD were increased in PM and BV-2 during OGD (Figure 2B), and the OGD-induced increase of NICD was sensitive to inhibition by the GSI DAPT (Figure 2C). The increase of NICD was also detected in CD11b-labeled cells in the ipsilateral cortex 24 hours after ischemia reperfusion (Figure 2D).

The Notch Pathway Regulates Microglial Activation After OGD and LPS Stimulation

To determine whether Notch regulates microglial activation, PM and BV-2 cultures were challenged with OGD or LPS in the presence or absence of DAPT. DAPT markedly suppressed the OGD-induced and LPS-induced upregulation of Iba1 in microglia (Figure 3A, B). NAS PM also exhibited substantially lower Iba1 expression after OGD (Figure 3C) and LPS stimulation (Figure 3D) when compared to non-Tg PM.

**Figure 2.** Oxygen glucose deprivation (OGD) activates the Notch pathway in microglia. A, Time course of mRNA levels for Notch receptors and their ligands in primary microglia (PM) and BV-2 cultures subjected to OGD followed by reoxygenation. Transcript levels were determined at the indicated time points of reoxygenation by semiquantitative polymerase chain reaction. B, Representative blots showing the time course of Notch intracellular domain (NICD) protein level in PM and BV-2 cultures subjected to OGD, followed by reoxygenation. C, Representative immunoblots showing NICD protein level in PM and BV-2 cultures subjected to OGD, followed by 12 hours reoxygenation in the continued presence of the γ-secretase inhibitor (N-[N-(3,5-difluorophenacetyl)l-alanyl]-S-phenylglycine t-butyl ester; DAPT; 10 μmol/L) or vehicle (DMSO). All blots were reprobed with β-actin to control for equal loading. D, Representative images of NICD and CD11b immunoreactivities in the contralateral (left) and ipsilateral cortices (right) of a non-Tg mouse 24 hours after ischemia reperfusion. Brain sections were stained for CD11b (green) and Notch1 intracellular domain (Val1774-NICD; red). Nuclei were visualized with the nuclear stain 4′,6-diamidino-2-phenylindol.
The Notch Pathway Regulates Postischemic Inflammatory Gene Expression by Modulating NF-κB Activity

One potential outcome of microglial activation is the production of inflammation-related cytokines IL-1β and tumor necrosis factor-α (TNF-α), which are considered to be the major culprits of secondary neuronal death associated with brain ischemia.14,15 Pretreatment of microglial cultures with DAPT before stimulation with LPS markedly attenuated the expression of IL-1β and TNF-α (Figure 4A). To determine whether Notch also modulates the expression of proinflammatory mediators in vivo, levels of IL-1β and TNF-α mRNA were measured in the ischemic ipsilateral hemispheres of NAS and non-Tg mice 24 hours after ischemia reperfusion. The ischemia-induced expression of both cytokines was significantly attenuated in NAS mice (Figure 4B). The corresponding TNF-α protein level was substantially lower in NAS mice (Figure 4C). DBZ effectively suppressed the expression of IL-1β and TNF-α (Figure 4A). To determine whether Notch also modulates the expression of proinflammatory mediators in vivo, levels of IL-1β and TNF-α mRNA were measured in the ischemic ipsilateral hemispheres of NAS and non-Tg mice 24 hours after ischemia reperfusion. The ischemia-induced expression of both cytokines was significantly attenuated in NAS mice (Figure 4B). The corresponding TNF-α protein level was substantially lower in NAS mice (Figure 4C). DBZ effectively suppressed the expression of IL-1β and TNF-α (Figure 4A).
ischemia-induced increase of TNF-α protein level (Figure 4C). Because expression of these proinflammatory cytokines is regulated by NF-κB,16 and because NF-κB plays a crucial role in ischemic brain injury,17,18 we next examined whether Notch activation may modulate NF-κB activity in LPS-stimulated microglia. Nuclear accumulation of the NF-κB/p65 subunit was substantially attenuated in NAS microglia after stimulation (Figure 4D), suggesting that Notch modulation of NF-κB activity, as reported previously in T lymphocytes19 and macrophages,6 likely accounts for the differential production of IL-1β and TNF-α in NAS and non-Tg microglia.

**Discussion**

Deleterious microglia activation is crucially involved in the pathophysiology of cerebral ischemia, as demonstrated by the improved outcome through treatments with inhibitors of microglial activation.20,21 Activated microglial cells have been demonstrated in peri-infarct areas in clinical stroke and are thought to orchestrate neuronal damage in the penumbra.22 Hence, elucidation of the mechanisms responsible for the progressive activation of microglia should prove useful for testing potential therapeutic approaches to ameliorate postischemic inflammation.

Recent studies raise the possibility that the Notch pathway regulates adaptive and innate immune responses.4–7 The Notch pathway is activated in multiple sclerosis, an inflammatory demyelinating disease.23 Inhibition of Notch signaling using GSI mitigates demyelination and paralysis in experimental allergic encephalomyelitis, an animal model of multiple sclerosis.23 GSI treatment also suppresses macrophage accumulation in atheromatous plaques and reduces atherosclerotic lesion formation.24

**Figure 5.** Notch regulates the neurotoxic microglial phenotype. Representative images of neurons labeled with JC-1 (A) or stained for caspase-3/caspase-7 (B) after the addition of oxygen glucose deprivation (OGD)-treated nontransgenic (non-Tg) or Notch antisense primary microglia (PM). The histograms show the ratio of the average green/red fluorescence intensity/neuron 24 hours after addition of PM and the number of neurons positively stained for activated caspase-3/caspase-7 at the indicated time points after coculturing. Values are the means±SEM (n=3 independent experiments); **P<0.01; ***P<0.001 compared to non-Tg PM. C, Percentage of apoptotic neurons at the indicated time points after the addition of OGD-treated and lipopolysaccharide-treated PM. Values are means±SEM (n=3 independent experiments); ***P<0.001 compared to non-Tg PM. D, Pretreatment of PM with 10 μmol/L DAPT before 2 hours of OGD ameliorates caspase-3/caspase-7 activation in neurons 24 hours after coculturing (left) and the neurotoxic activity of microglia at the indicated time points after coculturing (right). Values are means±SEM (n=3 independent experiments); *P<0.05 compared to vehicle-treated PM.

**Notch Signaling Regulates Microglia Neurotoxic Activity**

Because Notch signaling promotes microglial production of proinflammatory mediators that contribute to neuronal damage, we tested if inhibition of Notch reduces the neurotoxic potential of PM. To this end, we treated non-Tg and NAS microglia cultured on cover slips with either OGD or LPS, and then transferred the cover slips onto Petri dishes containing cover slips plated with naive cortical neurons derived from non-Tg embryos. Neuronal damage in cocultures was assessed at various time points by measuring mitochondrial membrane potential, effector caspase-3/caspase-7 activation, and cell viability (Figure 5A–C). The magnitude of mitochondrial membrane depolarization (Figure 5A) and activation of caspases-3/caspase-7 (Figure 5B) were markedly attenuated in neurons cocultured with NAS PM when compared to those cocultured with non-Tg PM. The number of apoptotic neurons was also significantly lower in cocultures with NAS PM (Figure 5C). Pretreatment of non-Tg PM with DAPT before OGD caused significant less neuronal damage and apoptosis (Figure 5D), suggesting that Notch regulates the neurotoxic activity of PM.
Our studies are the first to our knowledge to elucidate the Notch-dependent molecular and cellular inflammatory changes associated with cerebral ischemia. Notch is expressed in microglia and is upregulated after cerebral ischemia. Notch activation induced by cerebral ischemia increases the activation of microglia. Although the mechanisms triggering Notch activation in microglia remain to be established, our observations indicate that Notch activation induces a protracted NF-κB–driven postischemic expression of inflammatory genes. NF-κB plays a detrimental role in cerebral ischemia because transgenic mice deficient in the NF-κB/p50 subunit17 or inhibitor of nuclear factor κB kinase18 develop significantly smaller infarcts after middle cerebral artery occlusion than non-Tg mice. Activation of the Notch pathway is required for the full expression of NF-κB activation and production of interferon-γ in lymphocytes, suggesting that the crosstalk between these signaling pathways participates in modulating the responsiveness of immune cells to stimulation.19,20 Pretreatment of microglia with GSI substantially reduces NF-κB/p65 nuclear translocation, coupled with a decrease in microglia proliferation and transcription of IL-1β and TNF-α, 2 inflammation-related cytokines that play a crucial role in mediating neurotoxicity. Consistent with in vitro findings, pharmacological inhibition of the Notch pathway attenuates microglial proliferation and NF-κB–dependent expression of inflammatory genes in the ischemic lesion. Therefore, Notch may play a role in not only expanding the microglia pool but also increasing the inflammatory and neurotoxic responses associated with cerebral ischemia.

Conclusions

In summary, our study unveils a previously unknown role of Notch in the early molecular events leading to cellular inflammatory changes. Aberrant activation of Notch evoked by cerebral ischemia could potentially contribute to a robust inflammatory response leading to neuronal degeneration. Our study also provides insights into the neuroprotective action of GSI in the setting of ischemic brain injury and the basis to explore Notch as a novel prospective target for the treatment of neuroinflammation-related degenerative disorders.

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Disclosure

None.

References

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Supplemental Table S1 Primer pairs for PCR and RT-PCR

The forward (F) and reverse (R) primers are:

**Notch1:** (F) 5’-aacagtgccgaatgtgagtgg-3’ and (R) 5’-aagtgacgcaagagcacctag-3’

**Notch2:** (F) 5’-cagccggtctccgtgtaaaaagaa-3’ and (R) 5’-gcgaagagtggaggtgcagttg-3’

**Notch4:** (F) 5’-gcttgggaaatctgccttac-3’ and (R) 5’-gagcaatggccctaagccat-3’

**Jagged1:** (F) 5’-atctgtccacctggctatggag-3’ and (R) 5’-atcacttcggaggtggtggta-3’

**Jagged2:** (F) 5’-aaggacatactctaccagtgc-3’ and (R) 5’-acgtcctggtacttctgac3’

**GAPDH:** (F) 5’-gtgccagtgagcttcccgtcagc and (R) 5’-accccttcattgacctcaact-3’

**TNF-α:** (F) 5’-catcttctcaaaatcaggttaca-3’ and (R) 5’-tgggagttagacaagttacaaccc-3’

**IL-1β:** (F) 5’-gaggataccactcccaacagacc-3’ and (R) 5’-aagtgcatcatcgttgttcataca-3’

**β-actin:** (F) 5’-catecgtaaagacctctatgcaac-3’ and (R) 5’-atggagcaccgcctcaca-3’

Supplemental Methods

**In vivo model of brain ischemia** Middle cerebral artery occlusion (MCAO) was induced by the intraluminal filament technique as described previously (1). In brief, a 5-0 nylon filament will be inserted through the external carotid artery stump and advanced into the right internal carotid artery until it blocked the origin of the MCA. After 60 min of MCAO, the filament was withdrawn to restore blood flow. Sham-operated mice underwent the same surgical procedure without filament insertion. Rectal temperature was monitored throughout the entire duration of the surgical procedure and maintained at 37.5°C with a feedback-controlled heating pad.

**Oxygen glucose deprivation** OGD was induced by washing and incubating the cultures in a pre-equilibrated glucose-free balanced salt solution in the Billups-Rothenberg anaerobic chamber containing 95 % nitrogen and 5 % CO₂. The chamber was sealed and incubated for 2 h at 37 °C. At the end of the procedure, cultures were removed from the chamber and replaced with serum-containing DMEM prior to returning the neuronal or microglia cultures to normoxia (95 % oxygen and 5 % CO₂). Control cultures were incubated in a similar balanced salt solution containing 5.5 mM glucose for 2 h at normoxia.

**Sample processing for immunofluorescence labeling** Brains were fixed by transcardial perfusion with saline followed by perfusion and immersion in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and immersed into gradient sucrose from 10-30 % solution for cryopreservation. Serial coronal sections (20 μm thickness) were cut with a freezing microtome (Leica 1900) and collected on slides. Cultured cells seeded on coverslips were fixed in 4 % PFA for 20 min.

**Assessment of caspase activity, neuronal mitochondria membrane depolarization and cell death** Microglia grown on coverslips were subjected to 2 h of OGD in the presence or absence of a GSI and two coverslips of each were then placed in 35 mm Petri dishes along with two coverslips seeded with cortical neurons (DIV14) derived from Non-Tg mice. At various time points, coverslips were removed to assess effector caspase-3 / -7 activation using the Magic Red Caspases-3 / -7 Detection Kit (AbD Serotec) per manufacture instruction. The average caspase-3 / -7 fluorescence intensity / neuron was quantified. Additional coverslips seeded with neurons were removed, incubated with JC-1 (50 nM) at 37 °C for 30 min. followed by several washes.
with PBS, and then visualized on a fluorescence microscope. JC-1 is a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green (\(A_{485\text{ nm}}\) excitation/\(A_{530\text{ nm}}\) emission) to red (\(A_{530\text{ nm}}\) excitation/\(A_{590\text{ nm}}\) emission). After subtraction of background values obtained from wells containing JC-1 but devoid of cells, ratios of red:green fluorescence intensity were calculated (2). After assessing mitochondria membrane depolarization, the same neurons on the coverslip were fixed with 4 % PFA for 10 min., stained with Hoechst 33342 (5 \(\mu\)g / ml), and the number of cells with condensed pyknotic nuclei was counted. An average of 9-10 fields (~ 20 neurons /field) per coverslip was counted for each variable.

Supplemental References
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