Combination Therapy With VELCADE and Tissue Plasminogen Activator Is Neuroprotective in Aged Rats After Stroke and Targets MicroRNA-146a and the Toll-Like Receptor Signaling Pathway

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Objective—Activation of the toll-like receptor (TLR) signaling pathway exacerbates ischemic brain damage. The present study tested the hypothesis that combination treatment with VELCADE and tissue plasminogen activator (tPA) modulates the TLR signaling pathway on cerebral vasculature, which leads to neuroprotection in aged rats after stroke.

Methods and Results—Focal cerebral ischemia acutely increased TLR2, TLR4, and interleukin-1 receptor–activated kinases 1 immunoreactivity on fibrin/fibrinogen-positive vessels in aged rats. Monotherapy of tPA further amplified these signals. However, VELCADE in combination with tPA-blocked stroke- and tPA-potentiated vascular TLR signals, leading to robust reduction of infarct volume compared with respective monotherapies. Quantitative reverse transcription polymerase chain reaction analysis of cerebral endothelial cells isolated by laser capture microdissection revealed that the combination treatment increased miR-146a levels, which was inversely associated with the reduction of vascular interleukin-1 receptor–activated kinases 1 immunoreactivity. In vitro, fibrin upregulated interleukin-1 receptor–activated kinases 1 and TLR4 expression and downregulated miR-146a on primary human cerebral endothelial cells. VELCADE elevated miR-146 levels and abolished fibrin-increased interleukin-1 receptor activated kinases 1 proteins.

Conclusion—Stroke acutely activates the TLR signaling pathway on cerebral vasculature. Upregulation of miR-146a and inactivation of ischemia and tPA-potentiated TLR signaling pathway by VELCADE may play an important role in the neuroprotective effect of the combination therapy of VELCADE and tPA for acute stroke. (Arterioscler Thromb Vasc Biol. 2012;32:1856-1864.)

Key Words: endothelium ■ ischemia ■ microRNA ■ thrombolysis ■ toll-like receptors

Toll-like receptors (TLRs) are pattern recognition receptors that are key players of the innate immune responses. In the central nervous system, TLRs are widely distributed in neurons, microglia, and astrocytes and to a lesser extent in cerebral endothelial cells. Although the functional relevance of TLR signaling in these cells remains to be elucidated, emerging evidence shows that the activation of TLR signaling pathway contributes to the pathophysiology of brain ischemia. In experimental stroke, TLR2 and TLR4 are upregulated in neurons as early as 1 hour after stroke onset, whereas the upregulation of TLR2 in microglia appeared in a relatively delayed fashion (24–48 hours) in the mouse brain. In addition, mice with defective TLR2 or TLR4 genes are less susceptible to ischemic brain injury. Most importantly, upregulation of TLR2 and TLR4 has been found in stroke patients and is associated with poor stroke outcome. Thus, these studies establish a direct linkage between TLR signaling and the pathogenesis of stroke. However, the effects of TLR signaling on cerebral endothelium, the major regulatory component of blood–brain barrier, and cerebral inflammation in the ischemic brain have not been studied in vivo, although studies in vitro show that oxidative stress upregulates TLR expression on cerebral endothelial cells, causing downregulation of tight junction proteins.

VELCADE (Millenium Pharmaceuticals, Cambridge, MA), a potent proteasome inhibitor, has been approved for clinical use in patients with multiple myeloma. Evidence from experimental stroke demonstrates that VELCADE provides potent neuroprotection for the ischemic brain. We have demonstrated that VELCADE extends the therapeutic window of thrombolytic therapy for stroke by enhancement of cerebral vascular patency and integrity. Accumulating evidence indicates that VELCADE possesses an immunomodulatory property via suppressing TLR signaling in the treatment of inflammatory-related diseases. Given the observation that cerebral endothelial cells express TLRs, the present study tested the hypothesis that adjuvant treatment of VELCADE and tPA suppresses the endothelial TLR signaling pathway triggered...
by stroke and tPA, leading to neuroprotection in aged rats after stroke.

**Materials and Methods**

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. All outcome measurements were performed by observers blinded to the treatments.

**Embolic Stroke Model**

Male Wistar rats aged 16 to 18 months weighing 450 to 700 g were subjected to embolic middle cerebral artery (MCA) occlusion, as previously described.\(^{18}\)

**Experimental Groups**

Ischemic rats were randomly divided into the following groups: (1) VELCADE at a dose of 0.1 mg/kg was given intravenously 2 hours after stroke onset (n=16); (2) tPA at a dose of 5 mg/kg (generously provided by Genentech, San Francisco, CA) was given intravenously (10% bolus and the remainder as a continuous infusion over a 30-minute interval using a syringe infusion pump) 2 hours after stroke onset (n=17); (3) combination of VELCADE with tPA (n=17); (4) the control rats were treated with the same volume of saline (1 mL) 2 hours after MCA occlusion (n=18).

**Measurement of Lesion Volume**

Animals were euthanized 7 days after MCA occlusion. Infarct volume was measured on hematoxylin and eosin–stained 7 coronal sections using the microcomputer imaging device system (MCID, Imaging Research, St. Catharines, ON, Canada), as described previously.\(^{18}\)

**Functional Outcome**

Neurological functional deficits were graded with the modified neurological severity score on day 1 and 7 after stroke onset, as previously described.\(^{19}\)

**Immunohistochemistry**

Immunohistochemistry was performed on coronal sections obtained from rats sacrificed 1 day after MCA occlusion. To detect TLRs and associated signaling molecules, a goat anti-TLR2 (1:100, Santa Cruz, Santa Cruz, CA), a rabbit TLR4 (1:100, Santa Cruz, CA), a mouse anti–nuclear factor (NF)-κB (P65, 1:150, Chemicon, Temecula, CA), which is specific for the detection of activated NF-κB,\(^ {20}\) and a mouse anti–interleukin-1 receptor–associated kinase-1 (interleukin-1 receptor activated kinases 1 [IRAK1], 1:50, Santa Cruz) antibodies were used. Double immunofluorescence labeling with mAb antifibrinogen/fibrin (1:1000, Accurate Chemical & Scientific, Westbury, NY) and mAb antidiendothelial barrier antigen (EBA, Sternberger Monoclonals Inc, Baltimore, MD; 1:1000) was performed to detect fibrin deposition. Double immunofluorescence stainings were performed to visualize cellular colocalization of TLR2 with neuron-specific marker-neuronal nuclear antigen (1:200, Millipore, Billerica, MA), glial fibrillary acidic protein (GFAP, 1:400, DAKO, Carpinteria, CA), and with a pericyte marker platelet-derived growth factor-β (1:100, R&D, Minneapolis, MN). For quantification, TLR2, TLR4, IRAK1, and EBA immunoreactive area were measured throughout the territory supplied by the right MCA. Data are presented as the area relative to the total EBA immunoreactive vessel area. The numbers of vessels with fibrin/fibrinogen and NF-κB immunoreactivity were counted throughout the territory supplied by the right MCA and are presented as the density of immunoreactive vessels relative to the scan area (mm\(^2\)).

**Laser Capture Microdissection**

Laser capture microdissection was performed according to our published protocol.\(^ {21}\) See Methods in the online-only Data Supplement for details.

**Quantification of mRNA by Real-Time RT-PCR**

All measurements of miRNAs and microRNAs (miRNAs) were performed on an ABI 7000 polymerase chain reaction instrument (Applied Biosystems, Foster City, CA), as previously described.\(^ {21}\) To verify the purity of endothelial cells isolated by laser capture microdissection, mRNA levels of platelet endothelial cell adhesion molecule-1 and GFAP were measured using TaqMan probes specific for rat platelet endothelial cell adhesion molecule-1 and GFAP. After the verification, we examined the mRNA expression of matrix metalloproteinase 9 (MMP-9) and intracellular adhesion molecule-1 (ICAM-1) in cerebral endothelial cells. Relative quantities were calculated using the 2\(^{-\Delta\Delta C T}\) method with glyceraldehyde-3-phosphate dehydrogenase as the endogenous normalization control.

**Quantification of Mature and Primary miRNAs by Real-Time RT-PCR**

For evaluation of miR-146a and primary miR-146a levels, individual reverse transcription and TaqMan (Applied Biosystems) miRNA assays were performed. See Methods in the online-only Data Supplement for details.

**Cell Culture**

Primary human brain microvascular endothelial cells (HBECs) obtained from Applied Cell Biology Research Institute (Kirkland, WA) were cultured in endothelial cell growth medium according to the protocols provided by the manufacturer. To investigate the effect of fibrin and fibrinogen on TLR genes, HBECs were treated with fibrin (0.15 and 1.5 µg/mL; Sigma, St. Louis, MO) or fibrinogen (15, 150, and 300 µg/mL; Sigma). To examine whether VELCADE blocks fibrin-induced TLR signaling, HBECs were treated with fibrin (1.5 µg/mL) and VELCADE (10 ng/mL). At the end of the experiments, the HBECs were collected for Western blot or reverse transcription polymerase chain reaction (RT-PCR) assay.

**Transfection of miRNA Mimics and Inhibitors**

To examine the effect of miR-146a on TLR4 and IRAK1 expression, the cerebral endothelial cells were transfected with miR-146a mimic or inhibitor (Dharmacon, Lafayette, CO). Briefly, 10 microliters (20 µmol/L) of miR-146a mimic, a control miRNA mimic (cel-miR-67), miR-146a inhibitor, or a negative control miRNA inhibitor (Dharmacon) were diluted into OPTIMEM (Life Technologies, Carlsbad, CA) and mixed with 4 µL of Oligofectamine (Life Technologies) according to supplier’s protocol. Before transfection, the growth medium was replaced with 2 mL of OPTIMEM. DNA–Oligofectamine (Carlsbad, CA) complexes were added to the cells and incubated for 6 hours. The transfection medium was then replaced by growth medium, and cells were incubated for an additional 18 hours.

**Western Blot Analysis**

Protein levels of total IRAK1, phosphorylated IRAK1 (p-IRAK1), and TLR4 on cultured cerebral endothelial cells and brain tissue isolated from the ischemic boundary zone were measured by Western blot. The following antibodies were used: IRAK1 (1:1000, Santa Cruz Biotechnology Inc), p-IRAK1 (for brain tissue, 1:100, Santa Cruz Biotechnology Inc), p-IRAK1 (for HBECs, 1:250, Abcam, Cambridge, MA), TLR4 (1:500, Santa Cruz Biotechnology Inc), and β-actin (1:10000, Abcam). Signal bands were visualized by the ECL system (Amersham, Pittsburgh, PA). The relative densities among the blot lanes were analyzed using the MCID system (Imaging Research).
Statistical Analysis
Data are presented as mean±SE. Statistical comparisons were made using ANOVA followed by Bonferroni post hoc multiple comparisons. Comparisons between the 2 groups were evaluated by the Student t test. P<0.05 was considered statistically significant.

Results
VELCADE Attenuates the TLR Signaling Pathway on Cerebral Vasculature
To examine the effect of cerebral ischemia on TLR expression on cerebral vasculature in vivo, double immunofluorescent stainings of EBA with TLR2, TLR4, and IRAK1 were performed. Our data showed that 24 hours after MCA occlusion, EBA immunoreactive cerebral vessels were TLR2 and TLR4 positive throughout the ipsilateral MCA territory, whereas TLR2 and TLR4 immunoreactivity was not detected on EBA-positive vessels in the nonischemic hemisphere 24 hours after stroke (Figure 1A–1D). In addition, TLR2- and TLR4-positive vessels were IRAK1 immunoreactive (Figure 1K–1P), a key downstream kinase in the signaling pathway of TLRs. Furthermore, double immunofluorescent staining revealed that TLR2-positive vasculature was not GFAP and neuronal nuclear antigen positive (Figure I in the online-only Data Supplement). However, some of TLR2-positive vessels were PDGFβR positive, a marker of pericytes, (Figure I in the online-only Data Supplement). These data suggest that stroke acutely activates TLR signaling on cerebral vasculature. Monotherapy with tPA further increased the percentage of TLR2, TLR4, and IRAK1 immunoreactive area compared with saline-treated rats (Figure 1Q–1S). In contrast, treatment with VELCADE significantly reduced the percentage area of TLR2, TLR4, and IRAK1 immunoreactive vessels compared with saline-treated rats (Figure 1Q–1S). In addition, VELCADE in combination with tPA significantly reduced TLR2, TLR4, and IRAK1 immunoreactive vasculature compared with tPA-treated or saline-treated rats (Figure 1Q–1S). The combination therapy also significantly reduced the percentage of IRAK1 immunoreactive area compared with rats treated with monotherapy of VELCADE (Figure 1S).

![Figure 1. Expression of toll-like receptor (TLR) 2, TLR4, and interleukin-1 receptor activated kinases 1 (IRAK1).](image-url)
Western blot analysis of brain tissue isolated from ischemic boundary zone showed reductions of IRAK1 and p-IRAK1 levels by the combination therapy (Figure 1T–1V). These data suggest that exogenous tPA amplifies TLR signals, whereas VELCADE attenuates this signaling pathway triggered by ischemia and tPA.

Fibrinogen activates TLR4.23 Interestingly, we found that TLR2, TLR4, and IRAK1 immunoreactive vessels were fibrin/fibrinogen positive and that VELCADE alone or in combination with tPA substantially reduced the number of fibrin/fibrinogen-positive vessels (Figure 2A–2M). These data suggest that stroke-induced fibrin/fibrinogen deposition on cerebral vessels could trigger TLR expression. To further examine this possibility, we performed in vitro experiments using primary HBECs. Western blot analysis shows that incubation of the HBECs with fibrin and fibrinogen increased IRAK1, but fibrin-elevated IRAK1 at a concentration 10× less than the dose of fibrinogen (Figure 2N), suggesting that fibrin and fibrinogen activate TLR signaling on cerebral endothelial cells. We, therefore, selected fibrin at 1.5 µg/mL for subsequent in vitro experiments. VELCADE suppressed fibrin-elevated IRAK1 (Figure 2O) but failed to reduce fibrin-elevated TLR4 levels in HBECs (Figure II in the online-only Data Supplement).

Aforementioned in vitro data indicate that VELCADE blocks IRAK1 but not TLR4 on the endothelial cells. miR-146a targets IRAK1.24 We then asked whether miR-146a is involved in fibrin-induced activation of the TLR signaling pathway and whether VELCADE affects miR-146a levels. HBECs were treated with fibrin in the presence and absence of VELCADE. RT-PCR analysis revealed that fibrin alone significantly reduced mature miR-146a and primary miR-146a levels, whereas VELCADE abolished fibrin-induced reduction of mature miR-146a but failed to reverse fibrin-induced reduction of primary miR-146a in HBECs (Figure 3A and 3B). To examine the cause effects of miR-146a on IRAK1 expression, the endothelial cells were transfected with miR-146a mimic or miR-146a inhibitor. Using real-time RT-PCR analysis, we first examined the efficacy of transfection and found that transfection of the miR-146a mimic elevated miR-146a levels by 3-fold (Figure 3C), whereas miR-146 inhibitor reduced endogenous miR-146a by 52% in naïve HBECs (Figure III in the online-only Data Supplement). Western blot analysis shows that an increase in exogenous miR-146a attenuated IRAK1 protein levels by 50% (Figure 3D). Interestingly, attenuation of endogenous miR-146a by the miR-146a inhibitor significantly increased IRAK1 and p-IRAK1 protein levels compared with the negative control miRNA (Figure 3F and 3G). Addition of fibrin or fibrin along with VELCADE did not significantly alter both the IRAK1 levels in HBECs transfected with the miR-146a inhibitor (Figure 3F and 3G). Collectively, our data suggest that endogenous miR-146a represses endothelial IRAK1, the reduction of fibrin-increased IRAK1 in HBECs by VELCADE is mediated by miR-146a, and the derepression of endogenous miR-146a increases IRAK1, leading to concealing the effect of fibrin and VELCADE on IRAK1 in HBECs.

To verify our in vitro findings, we measured miR-146a levels on cerebral endothelial cells isolated from brain...
coronal sections by laser capture microdissection. Real-time RT-PCR analysis shows the presence of platelet endothelial cell adhesion molecule-1 mRNA (Figure IV in the online-only Data Supplement), but not GFAP transcripts in the cells (data not shown), indicating that these cells are cerebral endothelial cells. Rats subjected to the combination treatment with VELCADE and tPA exhibited significantly increased miR-146a compared with saline-treated rats (Figure 3E). Monotherapy with VELCADE did not significantly change miR-146a expression compared with saline-treated rats (Figure 3E). These data suggest that miR-146a is involved in the effect of VELCADE on the TLR signaling pathway.

**Treatment Effects on Inflammatory Responses**

Activation of the TLR signaling triggers NF-κB activation through recruitment of IRAK family members, which initiates an inflammatory cascade via proinflammatory mediator production. We examined the effect of VELCADE on NF-κB activation, and proinflammatory gene expression, ICAM-1 and MMP-9, on cerebral vessels, and parenchymal neutrophil infiltration. tPA monotherapy significantly increased the number of NF-κB immunoreactive vessels compared with saline-treated rats (Figure 4E). In contrast, treatment with VELCADE alone and in combination with tPA significantly reduced the number of NF-κB immunoreactive vessels compared with saline- or tPA monotherapy–treated rats (Figure 4E). Concurrently, whereas rats treated with tPA alone exhibited a robust increase in endothelial ICAM-1 and MMP-9 gene expression, treatment with VELCADE alone significantly reduced endothelial ICAM-1 and MMP-9 gene expression compared with saline-treated rats (Figure 4G and 4H). In addition, VELCADE in combination with tPA completely blocked tPA-induced upregulation of endothelial ICAM-1 and MMP-9 gene expression, which was associated with a significant reduction of the parenchymal neutrophil infiltration compared with rats treated with saline or tPA.
Thus, our data suggest that VELCADE alone and in combination with tPA reduces NF-κB activation and inflammatory response.

Infarct Volume and Functional Outcome
We then examined the neuroprotective effect of VELCADE by the measurement of infarct volume and functional outcome in ischemic rats treated with VELCADE at a dose of 0.1 mg/kg, because this concentration has minimal toxic effects and produces a comparable level of proteasome inhibition as its clinical usage in cancer patients. Treatment with VELCADE significantly reduced lesion volume and modified Neurological Severity Score score compared with saline-treated rats 7 days after stroke onset. In addition, ischemic rats treated with combination of VELCADE and tPA exhibited a significant improvement of neurological outcome as early as 1 day after stroke and had significantly reduced ischemic lesion volume compared with rats treated with monotherapies of VELCADE, tPA, and saline 7 days after stroke (Figure 5). These data indicate that VELCADE is beneficial in aged rats after stroke and that the combination treatment with VELCADE and tPA attains synergistic neuroprotective effects.

Discussion
The present study demonstrated that stroke acutely activated the TLR signaling pathway on cerebral vasculature in aged rats. Treatment with VELCADE either alone or in combination with tPA substantially attenuated activation of TLR signaling, which was associated with amelioration of proinflammatory responses and ischemic brain injury. In vitro, fibrin upregulated IRAK1 and TLR4 on primary cerebral endothelial cells, whereas VELCADE suppressed fibrin-elevated IRAK1, which was associated with upregulation of miR-146a. Our data suggest that the cerebral vascular TLR signaling pathway plays an important role in the pathogenesis of stroke in aged rat, whereas the neuroprotective effect of VELCADE is at least partly mediated via inactivation of TLR signaling pathway.

In cerebral endothelial cells, a recent in vitro study indicated that the activation of TLRs provokes blood–brain barrier permeability triggered by oxidative stress. In addition, preconditioning with various TLR ligands, such as Pam3CSK4 and lipopolysaccharide, induce tolerance to cerebral ischemia via attenuation of blood–brain barrier disruption and microvascular perfusion deficits. These data implicate the importance of TLR signaling in regulating cerebral vasculature homeostasis after stroke. In the present study, a robust increase in TLR2 and TLR4 immunoreactivity in cerebral vessels 24 hours after stroke suggest an acute upregulation of TLR2 and TLR4 on the cerebral vasculature after stroke. Moreover, as TLRs exert their effects by signaling through downstream adaptors, we found that the expression of TLRs on the cerebral vessels was closely associated with the...
upregulation of IRAK1 after stroke. Thus, our data provide direct evidence indicating that cerebral ischemia evokes the activation of TLR signaling on cerebral vasculature in the aged brain. Previous studies indicated that cerebral ischemia evokes the activation of TLR2 and TLR4 in neurons and glia, which orchestrates the activation of proinflammatory and proapoptotic pathways, and subsequently contributes to ischemic brain damage.8,10,11,29 The discrepancy of cellular sources of TLR2 and TLR4 in the ischemic brain between the present study and published data may be attributable to differences among species (mouse versus rat), age (young adult versus aged), and models (transient versus embolic MCA occlusion). Nevertheless, the present results along with the published data indicate that the TLR signaling pathway plays an important role in acute stroke development.

In the central nervous system, several endogenous ligands of TLRs have been identified.30 Among these, TLR signaling has been shown to respond to fibrin/fibrinogen stimulation which induces proinflammatory responses.23 However, the interaction between fibrin/fibrinogen and TLRs signaling has not been established in the ischemic brain. The present study shows that stroke-induced activation of TLRs occurred at the site of vascular fibrin/fibrinogen accumulation. In addition, incubation of the cerebral endothelial cells with fibrin increased IRAK1 and TLR4 expression. Thus, we contend that stroke triggers vascular fibrin accumulation to function as an endogenous ligand, which evokes the activation of TLR signaling on the cerebral endothelial cells. As an important pathological characteristic of vascular dysfunction, the acute accumulation of fibrin has been tightly intertwined with inflammatory response, as well as blood–brain barrier disruption after ischemic insult.31,32 The activation of TLRs may exacerbate these detrimental vascular events and, therefore, plays a causative role in ischemic brain damage.

The activation of IRAK1 is a key event involved in downstream signaling of TLRs.22 Interestingly, in the present study, whereas treatment with VELCADE alone and in combination with tPA both exhibited a moderate reduction of TLR expression, the combination treatment resulted in a further reduction of IRAK1 expression compared with VELCADE monotherapy. In addition, our in vitro data show that VELCADE suppressed fibrin-elevated IRAK1 but failed to reduce TLR4 levels on endothelial cells. These data imply that additional regulatory mechanisms may be involved in VELCADE-mediated TLR signaling via targeting the IRAK1. In this context, miR-146a is reported to be an important regulator of TLR signaling through IRAK1 repression.24,33 The profound effects of VELCADE and tPA on TLR signaling prompted us to investigate the involvement of endothelial miR-146a after stroke. Here we show, for the first time, that miR-146a was upregulated on the cerebral vasculature in aged rats after stroke. The combination treatment with VELCADE and tPA further substantially increased miR-146a levels on the cerebral vessels. As a number of experiments demonstrate that elevated miR-146a levels block TLR signaling pathway through targeting IRAK1 and tumor necrosis factor receptor–associated factor 6 genes,24,33 we found that the elevated miR-146a levels after the combination treatment was inversely associated with a robust reduction of IRAK1 expression. A direct effect of VELCADE on miR-146a was demonstrated by our in vitro data showing that VELCADE upregulated miR-146a on cerebral endothelial cells, which completely abolished fibrin-elevated IRAK1 proteins. Moreover, transfection of miR-146a mimics decreased IRAK1 expression, whereas attenuation of endogenous miR-146a in cerebral endothelial cells by the miR-146 inhibitor substantially increased IRAK1, suggesting that miR-146a represses IRAK1 in cerebral endothelial cells. These data are consistent with in vivo findings from miR-146a–knockout mice showing that miR-146a directly targets IRAK1 and knockout of miR-146a derepresses IRAK1.34 IRAK1 is one of the signal transducers in activation of the NF-κB pathway.35 By repressing IRAK1, miR-146a acts as a negative regulator of the NF-κB pathway.35 We speculate that miR-146a upregulated by VELCADE represses IRAK1, which results in attenuation of NF-κB activation, consequently leading to diminishing

Figure 5. Stroke outcome. A, The hematoxylin and eosin–stained coronal sections from representative rats treated with saline, tissue plasminogen activator (tPA), VELCADE, and the combination of VELCADE and tPA. B, Infarct volume assessed 7 days after stroke onset. C, The modified neurological severity score measured on day 1 and 7 after stroke onset.
inflammatory responses (Figure 6 for a simplified schematic diagram illustrating the effects of VELCADE on miR-146a and TLR signaling pathway).

miRNA genes are transcribed into pri-miRNA that generate pre-miRNA by a Drosha. The pre-miRNA is then cleaved to produce the mature by a Dicer. Our data suggest that fibrin suppresses the biosynthesis of miR-146a at both pri-miRNA and mature miRNA levels, whereas VELCADE likely acts on mature miR-146a because VECALDE significantly increased the miR-146a levels in fibrin-treated cerebral endothelial cells but failed to reverse fibrin-induced reduction of primary miR-146a. Currently, we do not know mechanisms underlying upregulation of mature miR-146a by VELCADE. Nevertheless, it is noteworthy that proteasome inhibitors stabilize Smads and the tumor-suppressor protein p53, all of which has been shown to interact with miRNA processing enzymes, such as Drosha and Dicer and, thereby, regulates miRNA biogenesis at the posttranscriptional level.

Interestingly, although in vivo monotherapy of VELCADE significantly reduced TLR2, TLR4, and IRAK1 expression and ischemic lesion volume, it did not substantially elevate miR-146a levels on cerebral vasculature. These data suggest that VELCADE may directly modulate TLR signaling and exerts the neuroprotective effect independent of miR-146a regulation. Proteasome inhibitors target the ubiquitin-proteasome pathway, which mediates a variety of cellular functions through multiple mechanisms. Given the large variety of substrates in the ubiquitin-proteasome pathway, we propose that the upregulation of miR-146a after the combination treatment represents another potential mechanism for turning down the stroke-induced activation of TLR signaling, which in addition to the direct effects of VELCADE on TLR signaling, may offer greater neuroprotective effects in the treatment of stroke. Further in vivo experiments are warranted to address a cause effect of miR-146a on VELCADE-mediated neuroprotection in stroke.

In conclusion, we have demonstrated that the treatment of acute stroke with VELCADE alone and in combination with tPA facilitates a multilevel inhibition of cerebral vascular TLR signaling in the ischemic brain, leading to attenuation of ischemic brain damage. miR-146a functions as a negative regulator of endothelial TLR signaling and likely participates in VELCADE- and tPA-mediated neuroprotection through targeting IRAK1.

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Disclosures

None.

References


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Supplement Material

Supplemental Methods:

**Laser Capture Microdissection (LCM).** Briefly, frozen brain coronal sections (8 μm) were immersed in acetone for 2 min of fixation and air-dried for 30s. After a brief rinse with 0.1% diethylpyrocarbonate-treated phosphate buffered saline (PBS), sections were stained with an antibody against von Willebrand factor (vWF, 1:50 dilution) for 10 min followed by a Cy3-conjugated secondary antibody (DAKO, 1:50 dilution) for 10 min. After a brief rinse with PBS, sections were air-dried under laminar flow for 10 min and immediately used for LCM. vWF-positive cells at the ischemic boundary area and homologous area of the contralateral hemisphere were excised with a Leica AS LMD System (Leica Microsystems Inc). Approximately 1000 endothelial cells from each animal were collected into a lysis buffer containing QIAzol reagent (Qiagen) in Eppendorf tubes and were stored at -80°C before miRNA isolation.

**Quantification of mature and primary miRNAs by real-time RT-PCR.** For evaluation of miR-146a levels, total RNAs from HBECs or endothelial cells isolated by LCM were extracted using miRNeasy Mini Kit (Qiagen). 15 μL Reverse transcription reactions consisted of 1-10 ng total RNA, 1x stem-loop RT specific primer, 1x reaction buffer, 0.25 mM each of dNTPs, 3.33 U/μl Multiscribe RT enzyme, 0.25 U/μl RNase inhibitor, and RNase-free water were incubated at 16°C for 30min, 42°C for 30min, 85°C for 5 min, and then held at 4°C until use in TaqMan real-time PCR. TaqMan real-time PCR reactions of miR-146a consisted of 1× TaqMan Universal PCR Master Mix No AmpErase UNG, 1× TaqMan miRNA assay, 1.33 μL of undiluted Reverse transcription product (cDNA), and nuclease free water at a total volume of 20 μL.
For evaluation of pri-miR-146a levels, RNAs extracted from the endothelial cells were reverse-transcribed using High Capacity RNA-To-cDNA Kit (Applied Biosystem). TaqMan® Pri-miRNA assays were performed. 20 μL Reverse transcription reactions consisted of 10-100 ng Total RNA, 1 μL of 20× RT Enzyme Mix and excessive RNAase free water. Reverse transcription reactions were incubated at 37°C for 60min, 94°C for 5min and then stored at 4°C until use in TaqMan Pri-miRNA assays. 20 μL TaqMan real-time PCR reactions were composed of 10 μL of 2× TaqMan Universal PCR Master Mix No AmpErase UNG, 1μL of 20× TaqMan Pri-miRNA assay, 1.33 μL of undiluted cDNA, and extra nuclease free water.

Each TaqMan assay was done in triplicate for each sample tested. Relative quantities were calculated using the $2^{-\Delta\Delta CT}$ method with U6 snRNA TaqMan miRNA control assay (Applied Biosystem) as the endogenous normalization control. Representative results are shown as fold expression relative to contralateral homologues area on the endothelial cells isolated by LCM, or to the control experimental conditions on primary human brain microvascular endothelial cells (HBECs). Reactions were run with the Standard 7000 default cycling protocol without the 50°C incubation stage, with reactions incubated at 95°C 10 min, followed by 40 cycles of 95°C 15 sec, 60°C 1 min. Fluorescence readings were collected during the 60°C step.
Supplemental Figures:

I. Co-localization of TLR2 with specific markers for neurons (NeuN), astrocytes (GFAP), and pericytes (PDGFRβ).
II. Western blot analysis and quantitative data of TLR4 protein levels on HBECs treated with fibrin in the presence and absence of VELCADE.
III. Real-time RT-PC analysis of miR-146a expression in HBECs transfected with miR-146a inhibitor (Anti-miR-146a) and a negative control miRNA inhibitor (Anti-miR-neg).
IV. RT-PCR amplification plots of PECAM-1 (A) and GFAP (B) in cells isolated by laser capture microdissection (LCM). Panel C shows the quantitative data of PECAM-1 mRNA levels on cells isolated from ipsilateral and contralateral hemisphere by LCM.