Basic Sciences

Alternatively-Spliced Extra Domain A of Fibronectin Promotes Acute Inflammation and Brain Injury After Cerebral Ischemia in Mice

Mohammad Moshahid Khan, PhD; Chintan Gandhi, PhD; Neelam Chauhan, PhD; Jeff W. Stevens, PhD; David G. Motto, MD, PhD; Steven R. Lentz, MD, PhD; Anil K. Chauhan, PhD

Background and Purpose—The fibronectin isoform containing the alternatively spliced extra domain A (EDA
-FN) is normally absent from the circulation, but plasma levels of EDA+FN can become markedly elevated in several human pathological conditions associated with inflammation including ischemic stroke. It remains unknown whether EDA+FN contributes to stroke pathogenesis or is simply an associative marker. Several in vitro studies suggest that EDA+FN can activate Toll-like receptor 4, an innate immune receptor that triggers proinflammatory responses. We undertook a genetic approach in mice to investigate the ability of EDA+FN to mediate inflammatory brain damage in a focal cerebral ischemia/reperfusion injury model.

Methods—We used genetically modified EDA+/- mice, which constitutively express EDA+FN. Extent of injury, neurological outcome, and inflammatory mechanisms were assessed after 1-hour cerebral ischemia/23-hour reperfusion injury and compared with wild-type mice.

Results—We found that EDA+/- mice developed significantly larger infarcts and severe neurological deficits that were associated with significant increased neutrophil and macrophage infiltration as quantitated by immunohistochemistry. Additionally, we found upregulation of nuclear factor-kB, cyclo-oxygenase-2, and inflammatory cytokines tumor necrosis factor-α, interleukin-1β, and interleukin-6 in the EDA+/- mice compared with wild-type mice. Interestingly, increased brain injury and neurological deficits were largely abrogated in EDA+/- mice by treatment with a specific Toll-like receptor 4 inhibitor.

Conclusions—These findings provide the first evidence that EDA+FN promotes inflammatory brain injury after ischemic stroke and suggest that the elevated levels of plasma EDA+FN observed in chronic inflammatory conditions could worsen injury and outcome in patients after acute stroke. (Stroke. 2012;43:1376-1382.)

Key Words: cerebral ischemia • fibronectin • inflammation • mice

Ischemic stroke is the third leading cause of morbidity and mortality worldwide and remains a major challenge to public health. Elucidation of novel cellular and molecular pathways that influence the pathogenesis of ischemic stroke could lead to development of new therapeutic approaches and greater insight into disease pathophysiology.

Recently, the dimeric glycoprotein fibronectin (FN) has emerged as a key factor contributing to the pathogenesis of several diseases associated with thrombosis and inflammation (extensively reviewed). FN is present in plasma and in tissue extracellular matrix. FN is a ligand for many members of the integrin family, and the various domains of FN interact with thrombosis-related proteins including collagen, fibrin, and heparin (Supplemental Figure I; http://stroke.ahajournals.org). FN protein diversity is generated by alternative splicing of the primary transcript at 3 sites: extra domain A (EDA), extra domain B (EDB), and the Type III homologies connecting segment (Supplemental Figure I). Two major isoforms of FN exist in humans and mice: (1) plasma FN, which is synthesized by hepatocyte and does not contain the alternatively-spliced EDA or extra domain B domains; and (2) cellular FN, which is synthesized by cells such as fibroblasts, endothelial cells, and smooth muscle cells. Cellular FN can contain either of the EDA or EDB domains independently or both domains in different proportions. The alternative splicing of EDA and EDB is independent and conserved across species, including mice, rats, chickens, and humans. Alternative splicing of the Type III homologies connecting segment generates additional FN isoforms that are species-specific.
Fibronectin-containing the alternatively-spliced extra domain A (EDA\(^{+/-}\)-FN) is normally absent from the plasma of humans and mice\(^{4,5}\) but high plasma levels of EDA\(^{+/-}\)-FN have been found in patients with chronic inflammation and ischemic stroke.\(^{6-9}\) However, it remains unclear in humans whether these elevated levels of EDA\(^{+/-}\)-FN are actively contributing to disease pathogenesis or rather simply serving as an associated marker. Several recent findings led us to hypothesize that EDA\(^{+/-}\)-FN may have the ability to exacerbate tissue damage and neurological outcome in acute ischemic stroke, a pathophysiological process that is mediated by both thrombotic and inflammatory components. First, recently we have demonstrated that EDA\(^{+/-}\)-FN accelerates thrombosis in ferric chloride-injured mesenteric arterioles, suggesting that EDA\(^{+/-}\)-FN is prothrombotic.\(^{10}\) Second, the EDA domain of FN contains binding sites for integrins \(\alpha_4\beta_1\) and \(\alpha_9\beta_1\) present on leukocytes and endothelial cells,\(^{11}\) suggesting the EDA\(^{+/-}\)-FN may influence inflammatory processes. Third, several in vitro studies demonstrate that the EDA domain of FN can activate Toll-like receptor 4 (TLR4), an innate immune receptor that triggers proinflammatory responses.\(^{12-14}\)

In this study, we compared EDA\(^{+/-}\) and wild-type (WT) mice to test the hypothesis that EDA\(^{+/-}\)-FN aggravates ischemia/reperfusion brain injury and examined the mechanistic role of TLR4 in this process. The EDA\(^{+/-}\) strain constitutively expresses EDA\(^{+/-}\)-FN in the plasma and tissues.\(^{3}\) WT mice contain the WT FN allele. Under normal conditions, plasma FN in WT mice lacks the EDA domain; however, EDA\(^{+/-}\)-FN can appear in the plasma during conditions of chronic inflammation such as atherosclerosis.\(^{4}\) In the present study, we show that EDA\(^{+/-}\)-FN promotes ischemia/reperfusion brain injury through a TLR4-dependent mechanism.

**Methods**

An expanded version of the method section is available in the Online Data Supplemental section.

**Mice**

EDA\(^{+/-}\), EDA\(^{+/-}\)/wt mice have been described and characterized previously.\(^{5}\) Both strains have been backcrossed >15 generations to C57BL/6J background. Control mice were heterozygous littermates or age-matched WT C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). All mice used were males between 8 and 10 weeks of age. All experiments were approved by the University of Iowa Animal Care and Use Committee.

**Cerebral Ischemia and Reperfusion Injury**

Transient focal cerebral ischemia was induced by 60 minutes of occlusion of the right middle cerebral artery with a 7.0 siliconized filament followed by either 5 hours or 23 hours of reperfusion. Mice were anesthetized with 1% to 1.5% isoflurane mixed with medical air. Body temperature was maintained at 37±1.0°C using a heating pad. Laser Doppler flowmetry (Perimed Instruments) was used to confirm induction of ischemia and reperfusion (Supplemental Table I). Physiological parameters, including \(\text{pH}, \text{pO}_2, \text{and pCO}_2\) (Supplemental Table II), were analyzed using a blood analyzer (Radiometer). Mean arterial blood pressure was determined by a noninvasive blood pressure system (CODA Monitor; Kent Scientific). Body temperature was measured by an infrared thermometer (Kent Scientific). Before euthanasia, mice were evaluated for motor deficits by an observer blinded to the identity of the groups. The motor deficit was scored on a 4-point neurological scale as described\(^{12}\): 0, no observable neurological deficit (normal); 1, failure to extend the left forepaw on lifting the whole body by the tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, leaning to the contralateral side at rest (severe); and 4, no spontaneous motor activity. For morphometric measurement, 8 coronal serial sections were cut at 1-mm intervals from the frontal pole using a mouse Brain Matrix (Roboz Surgical Instrument). Coronal sections were stained with 2% triphenyl-2,3,4-tetrazolium-chloride for 15 minutes at 37°C. Sections were scanned, digitalized, and infarct areas were measured blindly using Nikon NIS element software. To correct for brain swelling due to edema after ischemia, the corrected total infarct volume (\(\%\)) was calculated as follows: corrected infarct volume (\(\%\)) = ((volume of contralateral hemisphere − volume of ipsilateral hemisphere − volume of infarct))/volume of contralateral hemisphere×100.

**Statistics**

Results are reported as the mean±SEM. Statistical significance of the difference between means was assessed by unpaired Student t-test (for comparison of 2 groups) or by analysis of variance followed by Bonferroni multiple comparison test. Analysis of variance on ranks was applied to test for significant differences in the neuroscore. Treatment and genotype effects were analyzed by 2-way analysis of variance followed by Holm-Sidak multiple comparison tests. Values of \(P<0.05\) were considered significant.

**Results**

**Exacerbated Brain Injury and Worsened Neurological Outcome After Ischemic Stroke in Mice Expressing EDA\(^{+/-}\)-FN**

To test the hypothesis that EDA\(^{+/-}\)-FN exacerbates brain injury after ischemic stroke, we compared infarct volume and neurological outcome in the EDA\(^{+/-}\), EDA\(^{+/-}\)/wt, and WT mice after 60 minutes ischemia/23 hours reperfusion injury. EDA\(^{+/-}\) and EDA\(^{+/-}\)/wt mice had significantly increased in infarct volume (EDA\(^{+/-}\) mice, mean ±SEM: 37.3%±4.1%; EDA\(^{+/-}\)/wt mice, mean±SEM: 36.1%±3.9%, \(P<0.01\), analysis of variance) in the ischemic brain hemisphere compared with WT mice (mean±SEM: 22.3%±3.4%; Figure 1A–B). The increased infarct volume in the EDA\(^{+/-}\) and EDA\(^{+/-}\)/wt mice was associated with severe neurological deficits compared with WT mice (\(P<0.01\); Figure 1C). Exacerbated infarct volume in the EDA\(^{+/-}\) and EDA\(^{+/-}\)/wt mice was not associated with increased mortality when compared with WT mice (not shown). Laser Doppler measurements (Supplemental Table I) and physiological parameters (Supplemental Table II) were similar among groups during and after ischemia. Together these findings demonstrate that EDA\(^{+/-}\)-FN exacerbates brain damage and worsens neurological outcome after ischemic stroke.

**Enhanced Brain Tissue Inflammation in Mice Expressing EDA\(^{+/-}\)-FN**

To determine whether EDA\(^{+/-}\)-FN-exacerbated brain injury is associated with increased inflammation, we measured myeloperoxidase (MPO) activity in the supernatant fractions of brain homogenates prepared from the infarcted and surrounding areas in the EDA\(^{+/-}\) and WT mice after 60 minutes ischemia/23 hours reperfusion injury. EDA\(^{+/-}\) mice demonstrated significantly increased MPO activity in the ischemic hemisphere compared with WT mice (Figure 2A), suggesting enhanced inflammation in the damaged tissue. No differences...
in MPO activity were observed in the uninjured contralateral hemisphere of WT and EDA+/+ mice (Figure 2A), suggesting that EDA-FN promotes inflammation only in the setting of injury. In concordance with the increased tissue MPO activity, neutrophils and macrophages were significantly elevated in EDA+/+ mice compared with WT (P<0.01; Figure 2B–C) but not in the contralateral hemisphere (not shown). These findings demonstrate that EDA-FN exacerbates tissue inflammation after ischemic stroke.

Upregulation of Nuclear Factor-κB, Cyclo-Oxygenase2, and Proinflammatory Cytokines Expression After 60 Minutes Ischemia/23 Hours Reperfusion Injury in Mice Expressing EDA-FN

The EDA domain of FN has been shown to trigger gene expression of nuclear factor (NF)-κB in vitro.12 We measured NF-κB p65 (a marker of NF-κB activation) in nuclear extracts by Western blotting (Figure 3A). EDA+/+

Figure 1. Fibronectin isoform containing the alternatively spliced extra domain A (EDA-FN) promotes brain injury and worsens neurological outcome during cerebral ischemia. A, Representative triphenyl-2,3,4-tetrazolium-chloride-stained serial coronal brain sections from 1 mouse of each genotype after 60 minutes of ischemia/23 hours of reperfusion injury. B, Corrected mean infarct volumes (%) of each genotype (N=11–13/group). C, Neurological score from each genotype before euthanasia sacrifice (N=11–13/group). Data are medium±SD. Analysis of variance on ranks was used to test for significant differences.

Figure 2. Fibronectin isoform containing the alternatively spliced extra domain A (EDA-FN) enhances inflammatory response during cerebral ischemia. A, Myeloperoxidase (MPO) activity from each genotype quantified by enzyme-linked immunosorbent assay from the ipsilateral (infarct and surrounding region) or contralateral hemispheres (N=7/group). B, The left panel shows representative coronal brain sections stained for neutrophil marker (NIMP-positive cells are stained as brown) and counterstained with hematoxylin (blue) from 1 mouse of each genotype. The right panel shows quantification of NIMP-stained cells in the infarct region. Scale bar=20 μm. C, The left panel shows representative coronal brain sections stained for macrophages (Mac-3-positive cells are stained as brown) and counterstained with hematoxylin (blue) from 1 mouse of each genotype. The right panel shows quantification of Mac-3-stained cells in the infarct region. Scale bar=20 μm. Quantification was done as described in the “Methods” section. Nine coronal sections per mouse (separated by 100 μm from the frontal pole) were analyzed from 4 mice of each genotype (N=36 sections/genotype).
mice demonstrated significantly higher levels of NF-κB p65 compared with WT mice. We next investigated expression of the inflammatory enzyme cyclo-oxygenase-2, which is an NF-κB-responsive gene, by Western blotting of cytoplasmic fractions (Figure 3B). Similar to NF-κB p65, we found significantly higher protein levels of cyclo-oxygenase-2 in injured tissue in the EDA+/− mice compared with WT mice. In addition to cyclo-oxygenase-2, NF-κB regulates the coordinated expression of many genes, including genes encoding proinflammatory cytokines that amplify and perpetuate the inflammatory response.16 We therefore investigated the effect of EDA−/−FN on proinflammatory cytokine production (Figure 3C). EDA+/− mice demonstrated significantly elevated levels of interleukin (IL)-6, IL-1β, and tumor necrosis factor-α compared with WT in the supernatant fractions of brain homogenates prepared from the infarcted and surrounding areas. No differences in levels of IL-6, IL-1β, and tumor necrosis factor-α were observed in the uninjured contralateral hemisphere of WT and EDA+/− mice or at baseline in sham-operated EDA+/+ animals (not shown).

**EDA+−FNs Promote Early Tissue Inflammation After 60 Minutes Ischemia/5 Hours Reperfusion Injury**

Increased markers of inflammation at the 24-hour time point may merely represent a late response to increased lesion size in the EDA+/− mice. To examine the inflammatory response at an earlier time point, WT and EDA+/− mice were subjected to 60 minutes ischemia/5 hours reperfusion injury. EDA+/− mice demonstrated significantly increased MPO activity and neutrophil influx in the ischemic hemisphere compared with WT mice (Figure 4A–B). No differences in MPO activity were observed in the uninjured contralateral hemisphere of WT and EDA+/− mice (Figure 4A). Next, we investigated the effect of EDA+/−FN on proinflammatory cytokine production in the ischemic hemisphere. EDA+/− mice demonstrated significantly elevated levels of inflammatory cytokines, including IL-6, IL-1β, and tumor necrosis factor-α compared with WT (Figure 4C) in the supernatant fractions of brain homogenates prepared from the infarcted and surrounding areas. Together these results suggest that the increased inflammatory response observed in EDA+/− mice is not simply a late consequence of increased lesion size at the 24-hour time point.

**Brain Injury by EDA+−FN Occurs Through a TLR4-Dependent Mechanism**

To investigate the molecular mechanism by which EDA−/−FN exacerbates brain injury, we targeted TLR4, a proinflammatory receptor that has previously shown to interact with the EDA domain of FN in vitro.12–14 Mice were injected intravenously with the specific TLR4 inhibitor CLI-095 (1 mg/kg) or vehicle 30 minutes before 60 minutes ischemia/23 hours reperfusion injury. TLR4 inhibitor CLI-095 at this dose has been previously demonstrated to specifically suppress TLR4 signaling and downstream cytokine production in several murine models.14,17,18 Administration of CLI-095 to WT mice resulted in a modest but statistically significant reduction of infarct volume (P<0.05; Figure 5A–B) and improvement of neurological outcome (P<0.01; Figure 5C). Interestingly, the same treatment in EDA+/− mice resulted in an even more robust reduction of infarct volume (P<0.001; Figure 5A–B) and improvement of neurological outcome (P<0.01; Figure 5C), protecting nearly to the extent of injury seen in CLI-095-treated WT mice. There was a 2-fold reduction of infarct volume in CLI-095-treated EDA+/− mice compared with CLI-095-treated WT mice (P<0.0001; Figure 5B). Two-way analysis of variance showed that the interaction of genotype and treatment with the TLR4 inhibitor CLI-095 was significant (P=0.018, F=6.149). This observation suggests that a majority of the effect introduced by EDA+−FN is occurring through a TLR4-dependent mechanism.

**Discussion**

Several human pathological conditions including ischemic stroke are associated with elevated plasma levels of EDA+−FN, an endogenous splice variant of FN, which is normally absent from the circulation.6–9,19 The significance of the
elevated EDA⁺-FN levels in circulation observed in ischemic stroke and other chronic inflammatory conditions is not known. In this study, we show that EDA⁺-FN mice constitutively expressing EDA⁺-FN exhibit exacerbated brain injury, worsened neurological outcome, and enhanced postischemic inflammation, demonstrating an active functional role for EDA⁺-FN in disease pathogenesis.

We demonstrated that EDA⁺-FN promotes the recruitment of inflammatory cells (neutrophil and macrophage) in the infarcted tissue after reperfusion injury. This observation is consistent with neutrophil influx correlating positively with enhanced ischemic damage, as documented by other studies. We next demonstrated enhanced activation of transcription factor NF-κB in the ischemic hemisphere of mice constitutively expressing EDA⁺-FN. NF-κB activation is known to promote brain injury during focal cerebral ischemia through a cell death-promoting mechanism. Interestingly, the EDA domain of FN has been shown to trigger gene
expression of NF-κB in in vitro. Activation of NF-κB also induces proinflammatory genes encoding enzymes, cytokines, and other adhesion molecules, all of which are known to promote inflammatory tissue injury. Consisting with this mechanism, we detected enhanced expression of cyclo-oxygenase-2, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 that generate signals for the recruitment of inflammatory cells resulting in aggravated brain tissue injury.

Next we investigated the molecular mechanism by which EDA+–FN exacerbates inflammation after brain injury. Using a specific TLR4 inhibitor that has been shown to be effective in mice (CLI-095), we found that inhibition of TLR4 signaling in the EDA+/− mice resulted in a robust reduction of infarct volume and improvement of neurological outcome (Figure 5). We propose that EDA+–FN interacts with TLR4 to upregulate the NF-κB pathway, which mediates brain injury after ischemic stroke (Figure 6). In line with our study, previously it was demonstrated that the EDA domain of FN is able to activate TLR4 signaling in vitro. In this study, Okamura et al transfected HEK 293 cells, which do not express endogenous TLR4, with a TLR4 plasmid and demonstrated that the EDA domain of FN stimulates NF-κB through TLR4 activation. However, WT mice treated with the same inhibitor also exhibited a modest but statistically significant reduction of infarct volume, presumably because of the presence of other endogenous ligands for TLR4. For example, heat shock proteins released from necrotic cells, fibrinogen, and fibrin deposited during vascular injury have been reported to activate TLR4 and generate an inflammatory response. Interestingly, CLI-095-treated EDA+/− mice exhibited a reduction of infarct volume down equivalent to the size seen in CLI-095-treated WT mice. This result strongly suggests that the majority of the effect introduced by EDA+–FN on brain injury occurs through a TLR4-dependent mechanism. Multiple cell types that express TLR4 might contribute to the inflammatory response to EDA+–FN in the setting of acute brain injury. In line with our studies, recently it was shown that the recombinant isolated fibronectin EDA domain is able to promote neutrophil migration both in vitro and in vivo through a process dependent on TLR4 signaling in neutrophils. Thus, our data together with these findings suggest that EDA+–FN may aggravate brain injury after ischemia through a TLR4-dependent mechanism involving either neutrophils or macrophages independently or both through an NF-κB signaling pathway. Although ours and other studies indicate that EDA+–FN/TLR4 signaling is likely a significant mediator of postischemic inflammation, it remains possible that some of the proinflammatory effects of EDA+–FN are TLR4-independent, perhaps mediated by binding sites for leukocyte integrins α4β1 and α5β1 in the EDA domain. Additional future studies will be required to determine if disruption of EDA+–FN–integrin interactions in vivo prevents inflammatory cells recruitment and subsequent tissue damage.

A potential limitation of this study is that EDA+/+ mice have lower plasma levels of total FN compared with WT mice, presumably due to decreased secretion. The effect of EDA+–FN on brain injury is unlikely to be due to decreased levels of FN in plasma or brain tissue, because EDA+/−/− mice, which have normal levels of FN but contain EDA–FN, also exhibit exacerbated brain injury similar to EDA+/+ mice (Figure 1).

Conclusions

In summary, our study unveils a previously unknown role of EDA+–FN in promoting inflammation and brain injury in mice after ischemia/reperfusion. The mechanism is likely related in part to TLR4 signaling as illustrated in Figure 6. Our findings suggest that the elevated levels of plasma EDA+–FN observed in chronic inflammatory conditions could worsen injury and outcome in patients after acute ischemic injury. These findings shed new light on the pathophysiology of ischemic stroke and suggest new potential therapeutic approaches to this important cause of human morbidity and mortality.

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Disclosures

None.

References

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Myeloperoxidase (MPO) activity assay
To measure the MPO activity, contralateral (control) and ischemic (infarcted and surrounding areas) regions were homogenized and extracted in 1% cetyltrimethylammonium bromide (Sigma-Aldrich) in 50 mM KPO₄ buffer, pH 7.0. The resultant suspensions were sonicated for 30 s with 3 cycles of freeze-thaw in liquid nitrogen. Subsequently, the suspensions were centrifuged at 16,000 X g for 15 min and supernatant fractions were used for measurement of MPO activity and protein estimation. 100 μl of tetramethylbenzidine (TMB) solution (Sigma-Aldrich) was added to 40 μl of supernatant in a 96-well plate in duplicates. The reaction was stopped with 100 μl 1N HCl after 10 minutes and read at 450 nm. MPO activity was calculated using an MPO standard (Sigma-Aldrich) and the resultant activity was normalized as MPO units/mg of protein.

Immunohistochemical analysis
Immunostaining of neutrophils and macrophages was slightly modified and done as described.¹ Briefly, 9.0 μm coronal sections of fresh frozen tissue were incubated with blocking reagent followed by primary antibody (rat anti-mouse neutrophil marker (NIMP) or rat anti-mouse Mac-3) or rat Ig (control) in the presence of 5% goat serum overnight at 4°C followed by biotin-conjugated goat anti-rat Ig, avidin-biotin complex, and 3,3’-diaminobenzidine as substrate. Slides were counterstained with hematoxylin, dehydrated, and examined under a light microscope (Zeiss). Immunoreactive cells (brown staining) were counted in five different cerebral regions (3 within parietal cortex and 2 within basal ganglia) across different stereotactic levels. The ratio of immunoreactive cells per total number of cells in the defined infarcted area was used to calculate
the fraction of immuno-positive cells. A mean was calculated from the five different cerebral regions to represent each section. Nine coronal sections, separated by 100 μm from the frontal pole, per mouse were analyzed.

Western Blot Analysis
Brain cortical tissue was collected from the infarcted and surrounding areas. Cytosolic and nuclear extracts were prepared as described.\(^2\) Samples of nuclear and cytoplasmic fractions containing equal amounts of protein (40 μg) were separated in 10 % SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane (Hybond- P) and incubated overnight at 4°C with specific primary rabbit polyclonal antibody against NF-κB p65 (1:2000, Rockland, PA, USA), or a goat polyclonal antibody against COX-2 (1:200, Santa Cruz, USA) followed by appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). Proteins recognized by the antibody were visualized by enhanced chemiluminescence Femto kit (Thermo Scientific) according to manufacturer instructions. All blots were stripped and re-incubated with primary antibody specific to β-actin (Sigma) as a loading control. Intensity of the bands was measured by densitometry and quantified using NIH-Image J software.

ELISA assay for TNF-α, IL-1β, IL-6
To prepare homogenates brain cortical tissue was collected from the infarcted and surrounding areas and lysed in tissue lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 2% Triton X-100). Supernatants from brain homogenates were used for determination of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) with commercially available mouse ELISA kits (R&D Systems) according to the manufacturer’s instructions.
TLR4 inhibitor

TLR4 specific inhibitor CLI-095 (Invivogen, San Diego, USA) was prepared according to the manufacturer’s instructions. Mice were injected intravenously with CLI-095 (1mg/Kg) or vehicle 30 minutes prior to 60 min ischemia/ 23 h reperfusion injury. Prior to sacrifice mice were evaluated for neurological deficits as a functional outcome as described above.

References

### Supplementary Tables

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<th>Groups</th>
<th>Ischemia LDF (%)</th>
<th>Reperfusion LDF (%)</th>
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<tr>
<td>WT</td>
<td>13 ± 2</td>
<td>79 ± 5</td>
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<tr>
<td>EDA&lt;sup&gt;++&lt;/sup&gt;</td>
<td>14 ± 1</td>
<td>82 ± 4</td>
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<td>16 ± 3</td>
<td>83 ± 7</td>
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<tr>
<td>WT (Treated with TLR4 inhibitor)</td>
<td>16 ± 2</td>
<td>80 ± 7</td>
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<tr>
<td>EDA&lt;sup&gt;++&lt;/sup&gt; (Treated with TLR4 inhibitor)</td>
<td>16 ± 3</td>
<td>89 ± 8</td>
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**Table 1.** Laser Doppler Flowmetry (LDF) was similar among groups during and after ischemia. Values are expressed as mean ± SEM. N= 8-10 mice/group

<table>
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<th>Variables</th>
<th>WT Before</th>
<th>WT After</th>
<th>EDA&lt;sup&gt;++&lt;/sup&gt; Before</th>
<th>EDA&lt;sup&gt;++&lt;/sup&gt; After</th>
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<td>60 ± 7</td>
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<td>Body temperature</td>
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<td>36.3 ± 0.2</td>
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**Table 2.** Physiological parameters. 50 µl of blood was withdrawn before and after ischemia for blood gases determination. Values are expressed as mean ± SD. Before: before ischemia; After: 60 min after ischemia; MABP: Mean Arterial Blood Pressure under anesthetized conditions. Physiological parameters were similar among groups during the procedure. N= 4 mice/group.
**Figure 1. Murine FN schematic structure and binding sites.** Various domains of FN that interact with fibrin, collagen and heparin are indicated. The alternative spliced sites EDB, EDA and IIICS domains are shown with black boxes. Integrins binding site are indicated.