TIPE2, a Novel Regulator of Immunity, Protects against Experimental Stroke*

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*Running title: TIPE2 and Cerebral Ischemia

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Keywords: TNFAIP8 family, inflammation, cerebral ischemia, NADPH oxidase

Background: TIPE2, a newly identified protein, is essential for maintaining immune homeostasis.

Results: Genetic ablation of the TIPE2 gene significantly increased the cerebral volume of infarction and neurological dysfunction in experimental stroke.

Conclusion: TIPE2 is involved in the pathogenesis of stroke.

Significance: TIPE2 plays an essential role for a signal transduction pathway that links inflammatory immune response to specific conditions after cerebral ischemia.

Abstract: The inflammatory responses accompanying stroke are recognized to contribute to secondary ischemic injury. TIPE2 is a very recently identified negative regulator of inflammation which maintains immune homeostasis. However, it is unknown whether TIPE2 is expressed in the brain and contributes to the regulation of cerebral diseases. The present study was to explore the potential roles of TIPE2 in cerebral ischemia/reperfusion injury. TIPE2⁻/⁻ mice were used to assess whether TIPE2 provides neuroprotection following cerebral ischemia reperfusion induced by middle cerebral artery occlusion (MCAO) and in vitro primary cerebral cell cultures were used to investigate the expression and regulation of TIPE2. Our results showed that genetic ablation of the TIPE2 gene significantly increased the cerebral volume of infarction and neurological dysfunction in mice subjected to MCAO. Flow cytometric analysis revealed more infiltrating macrophages, neutrophils, lymphocytes in the ischemic hemisphere of TIPE2⁻/⁻ mice. The response of inflammatory cytokines and chemokines were significantly increased in the brain of TIPE2⁻/⁻ mice after MCAO. We further observed that TIPE2 was highly induced in wild type (WT) mice after cerebral ischemia, and was mainly expressed in microglia/macrophages, but not neurons and astrocytes. Finally, we found that the regulation of TIPE2 expression was associated with NADPH oxidase activity. These findings for the first time demonstrate that TIPE2 is involved in the pathogenesis of stroke and suggest that TIPE2 plays an essential role for a signal transduction pathway that links inflammatory immune response to specific conditions after cerebral ischemia. Targeting TIPE2 may be a new therapeutic way for stroke treatment.

TIPE2 (TNF-α-inducible protein 8-like 2, or TNFAIP8L2), a newly identified protein, is essential for maintaining immune homeostasis (1). TIPE2 shares considerable sequence homology with
members of the tumor necrosis factor-α-inducible protein 8 (TNFAIP8) family, which are thought to regulate cellular and immune homeostasis. TNFAIP8, the first identified member in this family, is able to enhance cell survival and inhibit apoptosis (2,3). TIPE2 is a recently identified negative regulator of innate and adaptive immunity and preferentially expressed in lymphoid tissues (1,4). TIPE2 deletion in 129 strain of mice leads to multiorgan inflammation, splenomegaly, and premature death late in their lives. TIPE2-deficient cells are hyper-responsive to Toll-like receptor (TLR) activation indicating that TIPE2 is an essential regulator of TLRs-mediated innate immunity. Interestingly, although TIPE2 is not expressed in NIH 3T3 fibroblasts cell line, following stimulation with TNF-α, NIH 3T3 fibroblasts expressed detectable levels of TIPE2 mRNA, suggesting that TIPE2 may be expressed in other cell types to establish equilibrium during an inflammatory response (1,5). Besides TNFAIP8 and TIPE2, two additional uncharacterized members of the TNFAIP8 family may exist and are designated as TNFAIP8L1 (TIPE1) and TNFAIP8L3 (TIPE3) in the gene bank.

Stroke is one of the most common vascular diseases in the cerebral nervous system. The inflammatory responses accompanying stroke are recognized to contribute to secondary ischemic injury (6-8), which include induced microglial and astrocyte activity, increased production of cytokines, chemokines, adhesion molecules, metalloproteinases, and the infiltration of monocytes and leucocytes into injured cerebral regions. Recent studies have indicated that the activation of the immune system as a result of disturbances in the redox state of cells seems to contribute to the brain damage and cerebrovascular diseases in these conditions. However, to date it is unclear whether the TNFAIP8 family is expressed in the brain and contributes to the regulation of cerebral functions. Given the role of TIPE2 on the regulation of TLR function and the link between TLRs and ischemic cerebral injury, in the present study, we used an in vivo middle cerebral artery occlusion (MCAO) model and in vitro primary cerebral cell cultures to determine the expression and functions of TIPE2 in cerebral ischemia-induced injury.

EXPERIMENTAL PROCEDURES

Animals TIPE2-deficient (TIPE2−/−) mice were generated as described (1). All TIPE2−/− mice used in these studies had been backcrossed to the C57BL/6 genetic background for 10 generations, and were <10 weeks old. These mice did not develop spontaneous inflammatory diseases at this age. All procedures were pre-approved by Institutional Animal Care and Use Committee.

Model for transient focal cerebral ischemia Transient middle cerebral artery occlusion (MCAO) was induced in both TIPE2−/− and wild type C57BL/6 (WT) mice (22–25 g) as previously described (9). After 2 hours of MCAO, the filament was removed and blood flow was restored. Mice were killed after reperfusion at 12, 24, 48, 72 or 96 hours. The sham groups were subjected to the same procedure except for the occlusion of the MCA.

Infarct volume and neurological function assessment Stroke outcomes were assessed at 12, 24, 48 or 72 hours after reperfusion using both cerebral infarct volume and a 4-tiered neurological scoring system as described previously (10).

Primary cell cultures and treatments Primary microglia and astrocytes were isolated and cultured as described previously (11). The purity of microglia and astrocytes were evaluated by immunofluorescence staining using antibody against CD11b (BD Pharmingen, San Jose, CA) and glial fibrillary acidic protein (GFAP, Invitrogen, Carlsbad, CA) respectively. Primary microglial cells were pre-treated with apocynin (100 μM) for 30 min and then stimulated with LPS (0.1 μg/ml) or by the model of oxygen-glucose deprivation (OGD).

RNA extraction, RT-PCR and ELISA Total RNA was isolated from the brain or cells using TRIzol reagent (Invitrogen) as described previously (12). The mRNA levels were analyzed by RT-PCR or real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA). Proinflammatory cytokines were measured by ELISA as described (13).

Western blot analysis Western blotting was performed as described (14). Primary antibodies anti-TIPE2 and NOX2 (ProteinTech Group, Chicago, IL) were used in this study. To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein β-actin.
Immunofluorescence staining

Staining was performed on tissue sections or cultured cells as described (10). To determine the lineage of TIPE2-positive cells, additional staining of neurons was performed using anti-NeuN (Invitrogen, Carlsbad, CA), astrocyte was performed by anti-GFAP, while anti-CD11b was used to stain macrophages/microglia.

Cresyl violet and TUNEL staining

Cresyl violet staining was performed as described previously (15) and TUNEL staining was performed using an ApopTag peroxidase in situ apoptosis detection kit (EMD Millipore, Billerica, MA) according to the manufacturer's protocol.

Preparation of infiltrating cells and flow cytometry

The infiltrating cells in the brain were prepared according to the procedure described previously (16). Rat monoclonal antibodies specific for mouse surface markers were as follows (BD Pharmingen, CA): CD11b-FITC antibody recognizes monocytes/macrophages and microglia. CD45-PerCP antibody identifies leukocyte common antigen expressed in all leukocytes and at lower levels in resting microglia. PE-conjugated anti-CD3 is a T cell specific marker. The Ly6G-APC is a specific marker for neutrophil. Recovered cells were stained with fluorescently conjugated antibodies. Flow cytometric analysis was performed with a FACScan (Becton Dickinson). Data analyzed (Flowjo software; TreeStar) using isotype control antibodies to set quadrants before calculating the percentage of positive cells.

Measurements of NADPH oxidase activity

NADPH oxidase activity was determined by measurement of superoxide (O$_2^\cdot$-) production in brain tissue homogenates. Fluorescence spectrometry for O$_2^\cdot$- production was performed by using a modified DHE fluorescent spectrometric assay as described previously (17).

Statistics

All data are expressed as mean ± SEM. Statistical analysis was performed with a 2-way ANOVA followed by the Student-Newman-Keuls test. When only 2 groups were compared, a Student t test was used. Significance was placed at $P<0.05$.

RESULTS

Expression patterns of TNFAIP8 family in the brain

To determine the expression patterns of TNFAIP8 family in the brain, total RNA was extracted from tissues of C57BL/6 mice and RT-PCR was performed. As shown in Figure 1A, among brain (cortex and hippocampus), kidney, liver and spleen, TNFAIP8 was the most abundant in the kidney, and hardly detectable in the liver. TIPE1 was detected in these tissues with relatively high level in the liver. TIPE2 was rich in the spleen, present in the brain and liver and weak in the kidney and TIPE3 was abundant in the brain. Since the present study focuses on the role of TIPE2 in cerebral ischemia injury, we further confirmed the protein expression patterns of TIPE2 in these tissues by Western blot analysis as shown in Figure 1B.

TIPE2 deficiency exacerbates stroke outcomes

Neurological deficit score was used to evaluate the neurological function after MCAO. TIPE2$^{-/-}$ mice showed worse neurological dysfunctions as compared with WT mice at different time points after reperfusion (Figure 2A). To confirm that the ischemic insult was equivalent among all groups, relevant physiological parameters were assessed before and during MCAO. As an example in Table 1, physiological variables were not significantly different between WT and TIPE2$^{-/-}$ mice before MCAO, during MCAO, or at 24 hours after reperfusion. However, infarction volume at 24 hours after reperfusion was significantly larger in TIPE2$^{-/-}$ mice (42.1%±3.8%) compared with WT mice (31.5%±3.3%; n=8/group, $P=0.0016$; Figure 2B). The increase in infarct size in TIPE2$^{-/-}$ mice was associated with more severe neurological impairment (Figure 2A). To further assess the role of TIPE2 in ischemia-evoked neuronal apoptosis, HE staining was used to show morphological features of injured neurons in hippocampus and cerebral cortex (Figure 2C). Cresyl violet staining was used to examine the surviving cells of neurons (Figure 2D). It was found that ischemia/reperfusion induced severe neurons dead in TIPE2$^{-/-}$ mice. TUNEL staining was used to assess the apoptotic like neurons (Figure 2E), a significant number of TUNEL-positive neurons manifested as brownish staining in the nuclei in TIPE2$^{-/-}$ mice. Collectively, these results implicate that the lack of TIPE2 is detrimental to the brain during ischemia.

TIPE2 deficiency increases the levels of cytokines and alters cerebral inflammatory cell infiltration

To elucidate the roles of TIPE2 in vivo, we measured the production of cytokines in ischemic brains of
TIPE2<sup>-/-</sup> mice. It was found that the levels of proinflammatory cytokines including TNF-α, IL-1β and IL-6 in the TIPE2<sup>-/-</sup> group were significantly enhanced compared with ischemic brains of WT mice. The up-regulation of TNF-α peaked at 12 hours after reperfusion, the extent of the post-ischemic response was approximately as the same as IL-1β, but a delayed peak expression of IL-6 was observed at 24 hours after reperfusion. The expression of chemokine MCP-1 is also dramatically increased and reached the peak value at 24 hours after reperfusion (Figure 3A).

Leukocytes are major effectors of inflammatory damage after cerebral ischemia (18,19). To determine whether the deficiency of TIPE2 altered leukocyte composition in the brain after MCAO, numbers of infiltrating Gr1<sup>+</sup>neutrophils, CD11b<sup>+</sup>CD45 high macrophages and CD3<sup>+</sup>T cells were evaluated by flow cytometry after 2 hours of ischemia at different reperfusion time. It was found that the accumulation of these leukocyte subtypes was significantly greater in the TIPE2<sup>-/-</sup> group after reperfusion. (Figure 3B-D). Lack of TIPE2 significantly increased in the percentage of neutrophils, microglia/macrophages especially at the early reperfusion period. It seems that the threshold of the leukocytes lacking of TIPE2 was decreased. Infiltration patterns of neutrophil and macrophages were similar and reached the peak value at 48 hours after reperfusion. On the other hand, T cell infiltration had peaked at 72 hours. Overall, the absence of TIPE2 has a profound effect on the inflammatory response after reperfusion.

**Increased expression of TIPE2 after cerebral ischemia reperfusion** By real time RT-PCR analysis, we found that, among the TNFAIP8 family, TIPE2 mRNA levels were markedly enhanced in the brain from WT ischemia mice. TNFAIP8, TIPE1 and TIPE3 had no significant change compared with those from sham groups, although TNFAIP8 had a decrease tendency (Figure 4A). We further confirmed the upregulation of TIPE2 in post-ischemic brain by Western blot analysis (Figure 4B) and immunofluorescent analysis (Figure 4C). To identify cell types that express TIPE2 in the brain after ischemia reperfusion, dual immunofluorescent staining was performed. We identified neurons by staining for neuronal nuclear marker NeuN, astrocytes by GFAP, and activated microglia/macrophages by CD11b. It was found that TIPE2 is expressed in microglia of ischemic brain. However, our results did not reveal a colocalization of TIPE2 and GFAP (NeuN), despite an expression of GFAP (NeuN) and TIPE2 in direct vicinity, indicating that neither astrocytes nor neurons express TIPE2 before and after cerebral ischemia (Figure 4D). Collectively, microglia is a major resource of TIPE2 in regions vulnerable to ischemic insult.

**Enhanced the expression levels of cytokines in TIPE2-deficient microglia cells by OGD** To provide evidence to establish the relationship between TIPE2 expression in microglia and the altered immune response in the brain, we isolated microglia and astrocytes, and the purity was confirmed by staining with the specific markers. By immunofluorescent staining, we found that TIPE2 was expressed and reduced by OGD in microglia but not in astrocyte (Figure 5A). We then examined the expression of cytokines in isolated microglia from both WT and TIPE2<sup>-/-</sup> mice. It was found that TIPE2<sup>-/-</sup>-microglia expressed significantly more cytokines including IL-1β, TNF-α, IL-6 and MCP-1 than WT microglia by OGD (Figure 5B). Similar results were also obtained from knocking down TIPE2 expression in BV-2 cells (data not shown). Our results indicate that immune responses in TIPE2<sup>-/-</sup> microglia are augmented.

**TIPE2 expression is associated with NADPH oxidase activity in vivo** In the present study, we treated C57Bl/6 mice with apocynin, a well known NADPH oxidase inhibitor, at a dose of 3.0 mg/kg 30 min before reperfusion as reported (20). Cerebral ischemia induced NADPH oxidative subunit NOX2 expression (Figure 6A) and its activity was markedly attenuated by apocynin treatment (Figure 6B). Inhibition of NADPH oxidase activity significantly reduced infarct volume (Figure 6C) and neurological deficit score (Figure 6D). Interestingly, we further found that apocynin significantly induced more TIPE2 expression in ischemic WT mice (Figure 6E). Together with our preliminary studies showing that apocynin had no effect on TIPE2 expression in WT mice under the sham-operated condition, our results suggest that NADPH oxidase activity is involved in the regulation of TIPE2 expression after cerebral ischemia reperfusion.

**Apocynin attenuates TIPE2 down-regulation in response to LPS/OGD in primary microglial cells** WT microglia were treated with LPS, it was found
that LPS significantly reduced TIPE2 expression in a
time-dependent manner (Figure 7A). To further
elucidate the role of NADPH oxidase on the
regulation of TIPE2 expression, apocynin was used
in this study. As shown in Figure 7B and 7C, NOX2
expression was markedly enhanced by OGD and
apocynin inhibited OGD-induced NADPH oxidase
activity. Moreover, we found that OGD decreased
TIPE2 expression, which can be recovered by
apocynin (Figure 7D).

DISCUSSION

Increasing evidence indicates that the post-ischemic
inflammatory response plays a detrimental role in the
secondary progression of stroke injury (21,22).
Inflammatory cell recruitments and activation have
been implicated in the progression of cerebral
ischemia-reperfusion injury. Studies have
demonstrated that the infiltration of T cells into the
brain, as well as the cytokines IL-23 and IL-17, have
pivotal roles in the evolution of brain infarction and
accompanying neurological deficits (23) and T and
B-cell-deficient mice with experimental stroke have
reduced lesion size and inflammation (24). A major
conundrum in the immunology of stroke is how to
enhance the early immunoregulation that limits
excessive inflammation of cerebral nervous system.
This is the first report to characterize TIPE2, a newly
regulator of immunity, in the brain, and evaluate the
role of TIPE2 in cerebral ischemia reperfusion injury.
Our results showed that genetic ablation of the Tipe2
gene increased the cerebral volume of infarction,
neurological dysfunction, inflammatory cytokines
production and infiltration of inflammatory cells in
mice subjected to MCAO, suggesting a
neuroprotective role of TIPE2 through anti-
inflammatory mechanisms in the pathogenic
processes of stroke. We further found that TIPE2
was highly induced after cerebral ischemia and the
regulation of TIPE2 expression is associated with
NADPH oxidase activity. These data indicate that
TIPE2 is an immunoregulator to prevent deleterious
inflammatory responses after stroke.

A subfamily of proteins that contributes to cellular
homeostasis possesses a hexahelical bundle motif,
called the death effector domain (DED) (25).
Proteins with DED are key signal transducers
involved in cell death and inflammation (26-28).
TNFAIP8 family is normally considered as a new
subfamily of DED-containing proteins, although the
three dimensional structure of the TIPE2 DED-like
domain is distinct (4). Recent reports have
demonstrated that TIPE2 is an essential regulator of
inflammation and immune homeostasis (1,4). TIPE2
governs immune homeostasis by negatively
regulating signaling by T cell receptors and TLRs.
The most important findings of this study are that
TIPE2 deficiency increases the cerebral volume of
infarction in ischemic mice and TIPE2 is
significantly induced after cerebral ischemia,
indicating that TIPE2 is involved in the pathogenesis
of stroke. The main mechanism of TIPE2 deficiency
increasing the cerebral volume of infarction could be
accounted for inflammation. TIPE2 influences the
production of pro-inflammatory cytokines and
immune cell infiltration, which are required for the
detrimental post-ischemic inflammatory response
contribution to secondary brain damage.

Although these data clearly implicate TIPE2 as a
critical mediator of cerebral injury following stroke,
the source and distribution of TIPE2 in ischemic
brain remains to be further characterized. The brain
responds to ischemic injury with an acute and a
prolonged inflammatory phases, which are
characterized by rapid activation of resident cells
(mainly microglia), production of proinflammatory
mediators, and infiltration of various types of
inflammatory cells into the ischemic brain tissue (29).
These cellular events collaboratively contribute to
ischemic brain injury. It is not surprising that
infiltration of inflammatory cells into the ischemic
brain contributes to the upregulation of TIPE2
expression in the ischemic brain as we reported in
this study. We wondered whether TIPE2 is also
induced in cerebral resident cells after ischemia to
establish equilibrium during an inflammatory
response, we then detected the expression of TIPE2
in three major types of cerebral cells including
microglia, astrocytes and neurons. Microglial cells,
the resident macrophages of the brain, play a critical
role as resident immunocompetent and phagocytic
cells in the brain. Resident microglia are activated
within minutes of ischemia onset and produce a
plethora of proinflammatory mediators to exacerbate
tissue damage (29). In this study, we identified that
TIPE2 expression was expressed in microglia of ischemic cerebral cortex and hippocampus rather than in astrocytes and neurons, although astrocytes, like microglia, are also capable of secreting inflammatory factors. Convincingly, we isolated microglia from both WT and TIPE2−/− mice, it was found that TIPE2−/− microglia expressed significantly more cytokines under OGD condition, indicating that TIPE2 from microglia may play an important role in the regulation of immune responses in cerebral ischemia injury.

Recent studies have indicated that activation of the immune system as a result of disturbances in the redox state of cells seems to contribute to the brain damage (30-32). A number of studies have demonstrated that NADPH oxidase-derived reactive oxygen species (ROS) is central to cerebral ischemia-induced oxidative stress in the brain (33-36). Inhibition of NADPH oxidase activity is neuroprotective after ischemia reperfusion (20,34). In this study, we found that the expression of NADPH oxidase subunit NOX2 (gp91phox) and NADPH oxidase activity were dramatically increased after reperfusion. Therefore, it is important to determine whether the regulation of TIPE2 expression is associated with NADPH oxidase activity. Indeed, our results showed that inhibition of NADPH oxidase activity further increased TIPE2 expression under cerebral ischemia condition. These results suggest that neuroprotective effects of inhibition of NADPH oxidase activity after ischemia reperfusion may go through, at least in part, the regulation of TIPE2-related signaling pathways. In in vitro study, the expression of TIPE2 is significantly decreased and apocynin increased the expression of TIPE2 to protect against OGD in microglia. It is known that resting microglia are at a quiescent state and their ability to induce inflammation is low. Upon cerebral ischemia reperfusion injury, activation, recruitment and accumulation of inflammatory cells are significantly enhanced. Results reported here indicate that TIPE2 serves as a regulator of this process. TIPE2 is constitutively expressed with relative high levels in microglia cells to prevent their activation. Although OGD reduces TIPE2 levels in microglia leading to enhanced immune responses as in vitro studies indicated which is inconsistent with our in vivo study, the overall of higher expression of TIPE2 may be due to more inflammatory cells infiltrating to the ischemic penumbra. Inhibition of NADPH oxidase activity further induced TIPE2 expression in microglia to protect this damage. Thus, TIPE2 serves as a negative regulator of inflammation to keep immune homeostasis by mediating oxidative stress.

It should be noted that the present study did not further explore the regulatory mechanisms of TIPE2 expression by NADPH oxidase activity. Further studies need to be addressed these issues. Taken together, the present study investigates the expression and action of TIPE2 in the brain and addresses the role of TIPE2 in cerebral ischemia reperfusion injury. These findings for the first time demonstrate that TIPE2 is involved in the pathogenesis of stroke and suggest that TIPE2 may play an essential role for a signal transduction pathway that links inflammatory immune response to specific conditions after cerebral ischemia.

**FOOTNOTES**

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REFERENCES

FIGURE LEGENDS

Figure 1. Expression patterns of TNFAIP8 family in the brain. A. RT-PCR analysis of total RNAs extracted from freshly harvested organs of C57Bl/6 mice including brain (cortex and hippocampus), kidney, liver and spleen. B. Representative Western blot gel document and summarized data showing the protein levels of TIPE2 in the selected tissues (n=6).

Figure 2. TIPE2 deficiency exacerbates stroke outcomes. A. Neurological deficit scores in WT and TIPE2−/− mice after cerebral ischemia reperfusion. B. Representative photographs of TTC staining (upper panel) and calculated infarct volume showing increased cerebral volume at 24 hours of reperfusion after MCAO in wild-type (WT) and TIPE2−/− mice. C. Representative photomicrographs of HE staining in hippocampus and cerebral cortex after ischemia reperfusion, respectively. D. Representative photomicrographs of TUNEL staining counterstained with methyl green. For magnification×4, scale bars= 200μm; For magnification×40, scale bars= 20μm. *P<0.05, ** P<0.01 WT mice vs. TIPE2−/− mice (n=8).

Figure 3. TIPE2 deficiency increases the levels of cytokines and alters cerebral inflammatory cell infiltration. A. The levels of proinflammatory mediators including TNF-α, IL-1β, IL-6 and MCP-1 in ischemic brains as determined by ELISA at 12, 24, 48 or 72 hours after reperfusion. B. Leukocytes are major effectors of inflammatory damage after cerebral ischemia, number of infiltrating Gr1+neutrophils was evaluated in the ischemic brain by flow cytometric analysis. C. Numbers of infiltrating CD11b+CD45 high macrophages in the ischemic brain. D. Number of infiltrating CD3+T cells in the ischemic brain. * P<0.05, **P<0.01, ***P<0.001 WT vs. TIPE2−/− mice (n=8).

Figure 4. Increased the expression levels of TIPE2 in WT mice after cerebral ischemia reperfusion. A. Relative quantitation of mRNA levels in TNFAIP8 family by real-time RT-PCR analysis in ischemic brain. B. Western blotting analysis of TIPE2 protein levels in the brains from ischemic WT mice. C. Immunofluorescent staining showing the expression of TIPE2 in hippocampus and cortex from ischemic WT mice, respectively. HE staining images were accompanied by these parallel sections in order to see the histological details of the areas, scale bars= 50μm. D. Cellular localization of TIPE2 after cerebral ischemia reperfusion showing that TIPE2 is expressed in microglia rather than astrocytes and neurons of ischemic brain, scale bars= 20μm; *P<0.05, *** P < 0.001 ischemic WT mice vs. sham-operated WT mice (n=8).

Figure 5. Enhanced the expression of cytokines in TIPE2-deficient microglia cells by OGD. A. Immunofluorescent staining indicating that TIPE2 is expressed and reduced by OGD in microglia but not in astrocytes, scale bars= 20μm; B. The expression of cytokines including IL-1β, IL-6, TNF-α and MCP-1 in microglia isolated from WT and TIPE2−/− mice. **P<0.01, ***P<0.001, TIPE2−/−-microglia vs. WT-microglia group (n=8).

Figure 6. Effects of inhibition of NADPH oxidase activity on cerebral ischemia/ reperfusion injury and the expression of TIPE2. A. Western blotting analysis of NOX2 protein levels in the brains from ischemic WT mice. B. Summarized data showing the effect of NADPH oxidase inhibitor apocynin on superoxide production in ischemic brain. C. Calculated infarct volume in ischemic WT mice with apocynin treatment after cerebral ischemia reperfusion. D. Neurological deficit scores in WT mice with apocynin treatment after cerebral ischemia reperfusion. E. Representative Western blots and quantified data showing the relative protein levels of TIPE2 in cerebral cortex and hippocampus from ischemic WT mice with apocynin treatment. * P<0.05, **P<0.01 ischemic WT mice vs. sham-operated WT mice (n=8).

Figure 7. Apocynin attenuates TIPE2 down-regulation in response to LPS/OGD in microglia. A. Representative Western blots and quantified data showing that LPS significantly reduced TIPE2 expression in a time-dependent manner. *P<0.05, **P<0.01 LPS treated group vs. control group. B. Western blotting analysis of NOX2 protein levels in microglia under OGD condition. C. Summarized data showing the effect of NADPH oxidase inhibitor apocynin on superoxide production in microglia under OGD condition.
**P<0.01, OGD group compare with control group. *P<0.05 OGD group vs. OGD+Apo group. D. Representative Western blots and quantified data showing that OGD-reduced TIPE2 expression could be recovered by apocynin. ***P<0.001, OGD group vs. control group or OGD+Apo group (n=8).

TABLE 1. Comparison of physiological variables. Relevant physiological parameters were assessed before, during and after MCAO. The physiological variables were not significantly different between WT and TIPE2-/- mice before MCAO, during MCAO, or at 24 hours after reperfusion confirming that the ischemic insult was equivalent among all groups.

<table>
<thead>
<tr>
<th></th>
<th>WT mice</th>
<th>TIPE2-/- mice</th>
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<tbody>
<tr>
<td><strong>Before MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>455.13±5.72</td>
<td>459.36±6.12</td>
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<td>Arterial blood pH</td>
<td>738±0.10</td>
<td>7.40±0.08</td>
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<tr>
<td>SpO2, %</td>
<td>95.75±0.54</td>
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<td>Temperature, °C</td>
<td>36.47±0.21</td>
<td>36.41±0.11</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>215.25±5.22</td>
<td>213.16±5.13</td>
</tr>
<tr>
<td><strong>During MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>451.33±4.59</td>
<td>449.28±5.98</td>
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<tr>
<td>Arterial blood pH</td>
<td>7.38±0.27</td>
<td>7.41±0.31</td>
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<tr>
<td>SpO2, %</td>
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<tr>
<td>Temperature, °C</td>
<td>36.35±0.26</td>
<td>36.29±0.31</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>222.13±10.20</td>
<td>217.67±7.21</td>
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<tr>
<td><strong>After MCAO</strong></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>451.49±12.21</td>
<td>453.57±10.29</td>
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<td>SpO2, %</td>
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<tr>
<td>Temperature, °C</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>211.22±27.88</td>
<td>203.27±24.33</td>
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Values are mean±SEM, SpO2: oxygen saturation.
Figure 1
Figure 2

A

Neurological score

Before MCAO

0 24 48 72

WT

TIPE2^−/−

B

% Hemisphere volume

WT

TIPE2^−/−

1 cm

C

4× Hippocampus Cortex 40×

WT

TIPE2^−/−

D

Cresyl violet staining

WT

TIPE2^−/−

E

TUNEL staining

WT

TIPE2^−/−
Figure 3
Figure 4

A

Relative mRNA level (Normalized to sham group)

B

Relative TIPE2 protein level (Normalized to sham group)

Sham
IR(2h/12h)
IR(2h/24h)
IR(2h/48h)
IR(2h/72h)

TIPE1  TIPE2  TIPE3  TNF-αR8

-21 kDa

-45 kDa

Reperfusion time (h)

12  24  48  72

Sham

1

2

4

5

Reperfusion time (h)
Figure 5

A

<table>
<thead>
<tr>
<th>Control</th>
<th>OGD (2h/12h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>TIPE2</td>
</tr>
<tr>
<td>Control</td>
<td>OGD (2h/12h)</td>
</tr>
<tr>
<td>CD11b</td>
<td>TIPE2</td>
</tr>
</tbody>
</table>

B

- Relative IL-1β level
- Relative IL-6 level
- Relative TNF-α level
- Relative MCP-1 level

** and *** indicate statistical significance.
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>I/R(2h/24h)</td>
<td>![image]</td>
<td>![image]</td>
</tr>
</tbody>
</table>

Relative NOX2 protein level (Normalized to sham group)

B

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>I/R(2h/24h)</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>I/R+Apo</td>
<td>![image]</td>
<td>![image]</td>
</tr>
</tbody>
</table>

Relative NADPH oxidase activity (Normalized to sham group)

C

I/R(2h/24h) | I/R+Apo

% Hemisphere volume

D

I/R(2h/24h) | I/R+Apo

Neurological score

E

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>I/R(2h/24h)</td>
<td>![image]</td>
<td>![image]</td>
</tr>
<tr>
<td>I/R+Apo</td>
<td>![image]</td>
<td>![image]</td>
</tr>
</tbody>
</table>

Relative TIPE2 protein level (Normalized to sham group)
Figure 7

(A) TIPE2 and β-actin protein levels in Control, 3h, 6h, and 18h LPS (100ng/ml) groups. 

(B) NOX2 and β-actin protein levels in Control, OGD (2h/12h), and OGD+Apo groups. 

(C) Relative NADPH oxidase activity (normalized to control group) in Control, OGD (2h/12h), and OGD+Apo groups. 

(D) Relative TIPE2 protein level (normalized to control group) in Control, OGD (2h/12h), and OGD+Apo groups.
TIPE2, a novel regulator of immunity, protects against experimental stroke
Yan Zhang, Xinbing Wei, Lixia Liu, Suxia Liu, Ziyung Wang, Bin Zhang, Baoxia Fan, Fan Yang, Shanying Huang, Fan Jiang, Youhai H. Chen and Fan Yi

J. Biol. Chem. published online August 2, 2012

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