A Pentapeptide Monocyte Locomotion Inhibitory Factor Protects Brain Ischemia Injury by Targeting the eEF1A1/Endothelial Nitric Oxide Synthase Pathway

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Background and Purpose—Ischemic stroke is a major cause of death worldwide but lacks viable treatment or treatment targets. Monocyte locomotion inhibitory factor (MLIF) is a small heat-stable pentapeptide produced by Entamoeba histolytica in axenic culture, which is supposed to protect the brain from ischemic injury; the mechanism, however, remains unknown. In this study, we further investigated the mechanism underlying the protective role of MLIF in brain ischemia.

Methods—A middle cerebral artery occlusion model in rats was used for detecting the effect of MLIF in the brain ischemia in vivo. To identify targets of MLIF in brain endothelial cells, we performed immunoprecipitation of biotin-conjugated MLIF and mass spectrometry.

Results—MLIF can protect the brain from ischemic injury in vivo, yielding decreased ischemic volume, prolonged survival, and improved neurological outcome. In vitro studies showed that MLIF displayed protective effects through inhibition of expression of pathological inflammatory adhesion molecules and enhancing endothelial nitric oxide synthase expression and nitric oxide release in the cerebrovascular endothelium. The target screening experiments demonstrated binding of MLIF to the ribosomal protein translation elongation factor eEF1A1. MLIF enhanced endothelial nitric oxide synthase expression through stabilization of endothelial nitric oxide synthase mRNA, and eEF1A1 was shown to be necessary for this enhanced expression. Knockdown of eEF1A1 or inhibition of endothelial nitric oxide synthase attenuated MLIF-mediated inhibition of adhesion molecule expression.

Conclusions—in this study, we identified a new potential pharmacologically targetable mechanism underlying MLIF’s protective effects in brain ischemia through the eEF1A1/endothelial nitric oxide synthase pathway. (Stroke. 2012;43:2764-2773.)

Key Words: brain ischemia ■ eNOS ■ pentapeptide ■ eEF1A1 ■ monocyte locomotion inhibitory factor

Stroke is the third leading cause of death in industrialized countries and the third common cause of death in China. Stroke is also the most frequent cause of permanent disability in adults worldwide. Many medical conditions can be the risk factors of stroke, including high blood pressure, high blood cholesterol, heart disease, diabetes, and obesity. Among all types of stroke, >80% are ischemic.1 The most common cause of ischemic stroke is the sudden occlusion of a blood vessel by a thrombus or embolism, resulting in an almost immediate loss of oxygen and glucose to the cerebral tissue.2 Although different mechanisms are involved in the pathogenesis of stroke, increasing evidence shows that ischemic injury and inflammation account for its pathogenic progression.3,4 In the response of the ischemic/reperfusion injury, increase in oxygen-free radicals and cytokines in the cerebral tissue is induced, and consequently, the expression of adhesion molecules on the endothelial cell surface is induced, including intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), and selectins, which mediate adhesion of leukocytes to endothelia in the periphery of the infarct and trigger the inflammatory cascade to cerebral damage.5–9

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Monocyte locomotion inhibitory factor (MLIF), a heat-stable pentapeptide (Met-Gln-Cys-Asn-Ser) produced by Entamoeba histolytica in axenic culture, is first found to be an anti-inflammatory functional peptide.\textsuperscript{10} MLIF can inhibit the locomotion of human monocytes in vivo. The synthetic MLIF had the same anti-inflammatory features as the native MLIF in inhibition of the locomotion of human microparticles, and of the respiratory burst in human microparticles and neutrophil polymorphonuclear leukocyte, and depression of delayed hypersensitivity skin reactions to DNCB in guinea pigs. It is also reported that MLIF can inhibit the expression of interleukin-1β, interferon-γ, interleukin-5, and interleukin-6 and increase the expression of interleukin-10 in macrophages.\textsuperscript{11–16}

The anti-inflammatory effect of MLIF led us to the initial interest to test whether it can act inflammatory response in brain ischemia. We\textsuperscript{17} and other laboratories\textsuperscript{18} have found MLIF can remarkably protect brain ischemic injury with decreased ischemic volume changes. In this study, we explored a further study that made a deep evaluation on its therapeutic window and prognosis on the multiple administrations. We found MLIF can protect acute brain ischemia with administration of MLIF within 1 hour after occlusion in a middle cerebral artery occlusion (MCAO) model. The in vitro studies showed that the protective roles of MLIF on cerebrovascular endothelial cells through inhibiting oxidized low-density lipoprotein (LDL) and hypoxia induced ICAM-1 and VCAM-1 expression, at the same time as enhancing endothelial nitric oxide synthase (eNOS) expression and nitric oxide releasing. These phenotypes of protective roles further led us to use a pulldown assay and biological mass spectrum identification to explore the target of MLIF, in which we confirmed MLIF binds to the ribosomal protein translation elongation factor eEF1A. Targeting eEF1A1 and thus enhancing eNOS expression contribute to the protective action of MLIF in cerebrovascular endothelium. The eEF1A1 knockdown or eNOS inhibition both attenuated the inhibitory the effect of MLIF on oxidized LDL-induced adhesion molecule expression. Therefore, our finding may present a new therapeutic drug candidate for targeting the eEF1A1/eNOS pathway and protecting cerebrovascular endothelium injury from brain ischemia.

Materials and Methods

Animals and Reagents

Male Sprague-Dawley rats provided by the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) were housed in controlled conditions and received a standard rat chow and tap water ad libitum. All the animals used in this work received humane care in compliance with institutional animal care guidelines, and the research protocol was approved by the Local Institutional Committee. MLIF and biotinylating MLIF were synthesized by the Chinese Peptide Company (Hangzhou, China). Anti-ICAM-1, VCAM-1, eNOS, and antirabbit phycoerythrin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), antibody for rabbit eEF1A was purchased from Cell Signaling Technology Biotechnology (CST, Danvers, MA), antibody for FLAG was purchased from Abmart (Arlington, MA), and secondary antibodies were purchased from Kangchen (Shanghai, China).

Cell Culture

Mouse microvascular endothelial cells (bEnd.3), THP1 cells, and AD293 cells were obtained from the American Type Culture Collection (Manassas, VA). All the cells were cultured in endotoxin-free Dulbecco modified Eagle medium with 10% fetal calf serum (PAA Laboratories GmbH).

Middle Cerebral Artery Occlusion

Male Wistar rats received MCAO operation. The MCAO ischemia was induced by placing an 11-mm silicone-coated 8-0 filament from the left common carotid artery into the internal carotid artery for 2 hours. After 24 hours, we evaluated the neurological deficit score based on the detection of hemiparesis and abnormal posture. After operation, the infarct size was measured by the 2,3,5-triphenyltetrazolium chloride staining assay. Brain slices were scanned and the areas were determined by planimetry of computer images (Image-Pro plus 6.0; Bethesda, MD). The ischemic percentage was calculated by the necrotic brain weight than the whole brain.

Western Blotting

Cells were lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail; protein concentrations of the extracts were measured with BCA assay (Pierce) and equalized with the extraction reagent. An equal amount of the extracts was analyzed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes for blotting with antibodies. Relative protein levels were normalized with the glyceraldehyde-3-phosphate dehydrogenase gray value in Western blot.

Immunoprecipitation

The lysate of bEnd.3 cells was stirred calmly with biotinylating MLIF (bio-MLIF) and 20 μg streptavidin-agarose (Invitrogen) at 4°C for approximately 40 minutes to capture binding proteins. The streptavidin-agarose was completely removed by centrifugation in a microcentrifuge at 12 000 rpm for 1 minute. The agarose was washed 4 times. The washed agarose was then resuspended in a 20-μl sodium dodecyl sulfate sample buffer solution to elute the bound proteins. The supernatant was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. Each protein studied in this study was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Voyager DESTR mass spectrometer, Applied Biosystems) after in-gel digestion, and protein identification searches were performed by the MASCOT search engine (Matrix Science).

Expression of FLAG Tagged Proteins

The full length eEF1A1 and its domains were amplified by polymerase chain reaction and cloned in pcDNA3.1(-) vector (Invitrogen). AD293 cells were transfected with FLAG-eEF1A1, FLAG–eEF1A1-D1, FLAG–eEF1A1-D2, FLAG–eEF1A1-D3, FLAG–eEF1A1-D1D2, or FLAG–eEF1A1-D2D3 plasmids, respectively. The extracts from cultured AD293 cells were immuno-noblotted with FLAG antibody.

Confocal Microscopy

The bEnd.3 cells were cultured on coverslips and fixed in 4% paraformaldehyde. Slides loaded with antibody-stained cells were added FITC-MLIF and viewed with a Leica SP5 confocal microscope. The images were acquired using the LAS-AF software (Leica).

Assay of Luciferase Reporter Gene Expression

Fire luciferase cDNA fused with eNOS mRNA 3'–UTR (420nt, 3906–4325) was cloned in pGL3 vector. AD293 cells were cotransfected with the mixture of indicated luciferase reporter plasmid, pRL-TK-RENilla-luciferase plasmid, and indicated pGL3-eNOS UTR plasmid. After 24 hours, luciferase activity in the cells was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Data are normalized for transfection efficiency by dividing Firefly luciferase activity with that of Renilla luciferase.
Nitric Oxide Production Measurement
To assess nitric oxide (NO) production, we measured the final stable productions of NO metabolism, nitrite (NO$_2^-$), and nitrate (NO$_3^-$) based on the Griess reaction. The bEnd.3 cell supernatant (100 mL) was mixed with 100 mL Griess reagent (Sigma, St Louis, MO). The absorbance of the reaction product was determined at 540 nm and the total nitrite concentration was calculated from a standard curve derived from the reaction of NaNO$_2$ under assay conditions.

Suppression by RNAi
The following small interfering RNA (siRNA) for eEF1A1-siRNA were obtained from Invitrogen (eEF1A1-siRNA: 5'-GCA CCA UGAAGC UUGAAGC UUU GUG UGA AGC T-3' eEF1A1-siRNA-A; 5'-GUG CUA ACA UGC CUU GGU UCAAGG G-3' eEF1A1-siRNA-B; control siRNA oligonucleotide: 5'-CAG AGA GGA GGAAGG GAG ACG CAG G-3'). The bEnd.3 cells were transfected with each siRNA or control siRNA (20 nmol/L) by using the HiPerFect Transfection Reagent (Qiagen, Shanghai, China). After 2 days of transfection, cells were lysed. The knockdown effect of eEF1A1 was analyzed by Western blot.

Monocyte Adhesion
To test effects of MLIF on adhesion of monocytes to endothelial cells, bEnd.3 cells were grown to confluence on 96-well culture plates and then treated with oxidized LDL before the adhesion assays. Ten minutes before the adhesion assay, endothelial cells were washed with phosphate-buffered saline. Cultured THP-1, a human monocyte cell line, were washed and diluted to a final concentration of 10^6 cells/mL. THP-1 cells were added to each well of endothelial cells and were allowed to coincide with the endothelial monolayer for 1 hour. Each well was turned appropriate degrees at 15 minutes to allow uniform distribution of the THP-1 cells across the endothelial monolayer. No adherent mononuclear cells were carefully removed by 2 washes with preheated phosphate-buffered saline. Adherent cells were counted by microscopy using a computer-aided image analysis system (ImageAnalyst; Automatix Corp, Billerica, MA). The adhesion rate was calculated by adherent mononuclear cells than the whole mononuclear cells.

Statistical Analysis
Statistical significance was determined by Student t test with a value of $P<0.05$ considered to be statistically significant ($^*P<0.05$; $^{**}P<0.01$).

Results
MLIF Protects Brain Ischemic Injury in a Rat MCAO Model In Vivo
To investigate the in vivo effect of MLIF, we explored the well-established brain ischemic model, MCAO, for investi-
gation on the role of MLIF in brain ischemic injury condition. In this model, brain transient ischemia was induced by placing an 11-mm silicone-coated 8-0 filament into the internal carotid artery for 2 hours from the left common carotid artery in male Sprague-Dawley rats. As shown in Figure 1A, we first evaluated effect of MLIF at difference time courses, in which 1 mg/kg MLIF or control saline was injected intravenously at indicated time after occlusion. Among 114 rats successfully induced to be brain ischemia, we found that administration of MLIF at 30 or 60 minutes after occlusion can significantly reduce brain infarct volume in the MCAO rat (Figure 1A). Then, we chose 30 minutes after occlusion to treat the MCAO rats with MLIF injection at different dosages with evaluation of infarct volume and neurological behavior. The results showed that 1 mg/kg MLIF can significantly protect brain ischemic injury with decreased infarct volume and improved neurological behavior (Figure 1B). These data strongly suggested the therapy effect of MLIF for acute brain ischemia in this MCAO model.

To investigate the long-term effect of MLIF, we administered MLIF for MCAO rats at 4 multiple treatment time points at 0, 4, 24, and 48 hours after occlusion and then monitored their survival for 14 days. Although the survival rate did not exert a significant difference, the median survival time was prolonged in the MLIF treatment group. As shown in online-only Supplement Data Figure I, the median survival time of the control MCAO group was 4 days, but in the MLIF treatment group, the median survival time extended to 10 days. Importantly, the neurological behavior was significantly improved by MLIF multiple treatments at 14 days (online-only Data Supplement Figure I). Taken together, our results in the rat MCAO model suggested the in vivo protective roles of MLIF in brain ischemic injury with the released symptoms on ischemic volume and concomitant neurological deficit changes.

MLIF Attenuates an Inflammatory Process by Inhibiting Adhesion Molecule Expression in Brain Microvascular Endothelial Cells

The brain microvascular endothelial cell-derived adhesion molecule expression, including ICAM-1 and VCAM-1, has been identified to be the pivotal inflammatory marker and

**Figure 2.** MLIF treatment antagonizes expression of inflammatory adhesion molecules in response of brain ischemia and vascular endothelium dysfunction. A, MLIF treatment inhibits adhesion molecules VCAM-1 and ICAM-1 in the cortical extracts from MCAO rats as in B. Protein expression was determined by Western blot (left) with data showing normalized expression fold was quantified (right). Mean±SD. **P<0.01. B, MLIF inhibits oxLDL-induced VCAM-1 and ICAM-1 expression in brain vascular endothelial cells (bEnd.3). After being stimulated with 10^{-5} M oxLDL for 2 hours, the bEnd.3 cells were treated with MLIF (5 mg/mL and 50 mg/mL) for 6 hours. The mRNA levels determined by qRT-PCR (left). Protein expressions of ICAM-1 and VCAM-1 were determined by Western blot (right). n=3. Mean±SD. **P<0.01 versus oxLDL only treatment group. C, MLIF inhibits hypoxia-induced ICAM-1 and VCAM-1 in brain vascular endothelial cells (bEnd.3). After stimulated with hypoxia (5% CO\textsubscript{2} and 95% N\textsubscript{2}) for 6 hours, the bEnd.3 cells were treated with MLIF for 24 hours. Protein expressions of ICAM-1 and VCAM-1 were determined by Western blot. Similar results were obtained in 3 independent experiments. D, MLIF inhibits oxLDL-induced adhesion of monocytes to brain vascular endothelium in vitro. After being treated with MLIF (5 mg/L and 50 mg/L) for 1 hour, the bEnd.3 cells were stimulated with 10^{-5} M oxLDL for 24 hours. After being washed with PBS, cultured THP-1 cells were added to the stimulated bEnd.3 cells and coincubated with the endothelial monolayer for 1 hour. Non-adherent mononuclear cells were removed, and adherent cells were counted by microscopy using a computer-aided image analysis system (n=3). The adhesion rate was calculated by adherent mononuclear cells normalized to the whole mononuclear cells. Mean±SD. **P<0.01. MLIF indicates monocyte locomotion inhibitory factor; VCAM-1, vascular adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; MCAO, middle cerebral artery occlusion; oxLDL, oxidized low-density lipoprotein; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline.
important therapeutic target for brain ischemia. Adhesion molecules express in the endothelium at the initial ischemic injury with hypoxic condition or active substance induction such as oxidized LDL and mediate infiltration of leukocytes into brain parenchyma, which crucially aggravate the inflammatory process. Because MLIF was found to be an anti-inflammatory peptide, we first investigate whether MLIFs protect brain ischemia through inhibition of an inflammatory response. To test this function, we homogenized the ischemic brain from MCAO rats like in Figure 1B and determined the protein expression level of ICAM-1 and VCAM-1 in the cortical extracts. As shown in Figure 2A, 1 mg/kg MLIF greatly inhibits the expression of these adhesion molecules, which indicated a direct anti-inflammatory role of MLIF for brain ischemia.

To further evaluate the mechanism of MLIF in the brain vascular endothelium to confirm its regulatory effect on adhesion molecules, we first investigate whether MLIFs protect brain ischemia through inhibition of an inflammatory response. To test this function, we homogenized the ischemic brain from MCAO rats like in Figure 1B and determined the protein expression level of ICAM-1 and VCAM-1 in the cortical extracts. As shown in Figure 2A, 1 mg/kg MLIF greatly inhibits the expression of these adhesion molecules, which indicated a direct anti-inflammatory role of MLIF for brain ischemia.

To further evaluate the mechanism of MLIF in the brain vascular endothelium to confirm its regulatory effect on adhesion molecules, we determined the effect of MLIF on oxidized LDL or hypoxia-induced adhesion molecule expressions in a brain microvascular endothelial cell line, bEnd.3 cells. As shown in Figure 2B, the oxidized LDL-induced ICAM-1 or VCAM-1 can be significantly inhibited by MLIF in dose-dependent manner, which is confirmed by Western blot and quantitative reverse transcriptase-polymerase chain reaction in protein and mRNA level separately. To mimic brain ischemic condition, we also treated bEnd.3 cells with a hypoxia condition. Consistently, the hypoxia-induced ICAM-1 and VCAM-1 expressions can be remarkably inhibited by MLIF in dose-dependent manner (Figure 2C).

These adhesion molecules derived from brain endothelial cells can mediate infiltration of leukocytes into the brain and initiate the brain inflammation. Therefore, we further countered the adhesion rate of monocyte THP-1 cells to the bEnd.3 cell-formed cultured endothelium. As shown in Figure 2D, the increased adhesion rate after bEnd.3 cells induced by oxidized LDL was significantly inhibited by MLIF, which is consistent with the regulatory ability of MLIF on adhesion molecule expressions. These findings suggested MLIF can attenuate the inflammatory process in brain endothelium through inhibition of adhesion molecules in brain vascular endothelial cells.

**MLIF Binds to eEF1A1 in Brain Microvascular Endothelial Cells**

The remarkable protective effect of MLIF for brain ischemic injury and the anti-inflammatory role of MLIF for brain vascular endothelium led us to further explore the possible pharmacodynamic target. We first synthesized the biotin-
conjugated MLIF peptide (bio-MLIF) to interact with the cell lysate of the brain microvascular endothelial bEnd.3 cells. Then we used the streptavidin-agarose beads to capture the MLIF-binding proteins. We used a serial affinity purification to eliminate the circumstance interference. The streptavidin-agarose was first mixed with protein exact, and then supernatant was transferred to a tube mixed with the same amount of streptavdin-agarose, and so on. Therefore, most of the target proteins were captured by the first IP1 mixture, and little of the target proteins was captured by the following mixtures (Figure 3A). As shown in Figure 3B, the binding band at near 50 kDa emerged in MLIF-binding IP1 mixture and was decreased in the following IP mixtures. After in-gel digestion, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification, and MASCOT search engine database analysis, the 50-kDa protein was identified to be the ribosomal protein translation elongation factor eEF1A1 (Figure 3C). Then, we transferred the IP samples to the membrane to make Western blot confirmation by antieEF1A1 antibody. As shown in Figure 3D, the 50 kDa protein band was identified to be eEF1A1 in Western blot, which confirmed the binding of MLIF with eEF1A1.

There are 3 functional domains in eEF1A1 protein including domain I, domain II, and domain III. To better understand the molecular mechanism, we further localized the MLIF-binding site in eEF1A1 protein by pulldown assay of bio-MLIF and FLAG fusion proteins bearing the full-length eEF1A1 and different domains (Figure 4A). The bio-MLIF has been shown to bind specifically to eEF1A1 and domain I (Figure 4B). To confirm the binding of MLIF with eEF1A1, we also explored immunostaining by using the rabbit antieEF1A1 and antirabbit phycoerythrin antibodies. As shown in Figure 4C, the colocalization of fluorescein isothiocyanate-MLIF with eEF1A1 detected by confocal microscope strongly suggested that MLIF directly targets eEF1A1. After transfection with FLAG-eEF1A1 plasmid and stained with anti-FLAG phycoerythrin antibodies, we also confirmed the colocalization of MLIF and recombinant eEF1A1 and domain I of eEF1A1. These data suggested domain I of eEF1A1 is the binding target of eEF1A1.

Interactions With eEF1A1 Contributes to eNOS Upregulation by MLIF

After confirmation of the interaction of MLIF with eEF1A1, we next identified the target function of eEF1A1 for the cerebrovascular system, because it is not well investigated. Fortunately, one recent report demonstrated that eEF1A1 mediates tumor necrosis factor-α-induced decrease in eNOS mRNA stability through binding of eNOS 3′-UTR.20 Thus, we investigated whether MLIF protects brain ischemic injury through the eEF1A1/eNOS pathway. We first determined the effect of MLIF for eNOS mRNA stability by using the luciferase reporter system encoded with eNOS 3′-UTR sequence. As shown in Figure 5A, MLIF can dose-dependently promote stability of eNOS 3′-UTR. To confirm the regulation in protein level, we also determined eNOS level in endothelial cells treated by MLIF. As shown in Figure 5B, in cultured brain vascular endothelial cells as well as the cortical extracts from MCAO rats, MLIF can directly enhance eNOS expression level in a dose-dependent manner.

In the stress condition such as oxidized LDL stimulation or hypoxia, endothelium-derived eNOS expression is often injured which initiates vascular inflammation with endothelial dysfunction. Therefore, we tested the effect of MLIF on the regulation of eNOS in hypoxia and oxidized LDL-treated endothelial cells. As shown in Figure 5C, MLIF treatment...
Figure 5. MLIF enhances eNOS expression in brain endothelial cells through targeting eEF1A1. A, MLIF enhances eNOS mRNA stability. Luciferase activity of the AD293 cells extracts transfected with eNOS 3'UTR luciferase reporter plasmid and pTK-Renilla-luciferase in the presence or absence of MLIF (0.5 mg/mL, 5 mg/mL, and 50 mg/mL; n=6). *P<0.05, **P<0.01 versus control group. B, eNOS expression was enhanced in response of MLIF treatment. Left, After the bEnd.3 cells were treated with MLIF (5 mg/mL and 50 mg/mL) for 6 hours, immunoblots were used for eNOS detection from cultured bEnd.3 cells. Normalized expression fold was quantified shown in the table. Mean±SD. **P<0.01 versus mock control. Right, MLIF treatment upregulates eNOS expression in the cortical extracts from MCAO rats as in Figure 1B. Protein expression was determined by Western blot and its normalized expression fold was quantified shown in the table. Mean±SD. **P<0.01. C, MLIF upregulates eNOS expression in response of stress including hypoxia and oxLDL stimulation. Left, After stimulated with hypoxia (5% CO2 and 95% N2) for 6 hours, the bEnd.3 cells were treated with MLIF (5 mg/mL and 50 mg/mL) for 24 hours and immunoblots for eNOS of the extracts from cultured bEnd.3 cells. Middle, After stimulated with 10^{-5} M oxLDL for 2 hours, the bEnd.3 cells were treated with MLIF (5 mg/mL and 50 mg/mL) for 6 hours and immunoblots for eNOS of the extracts from cultured bEnd.3 cells. Normalized expression fold was quantified shown in the table. Mean±SD. **P<0.01. Right, NO production in supernatants from bEnd.3 cells induced by oxLDL in the presence or absence of MLIF (5 mg/mL and 50 mg/mL) were measured using Griess method (n=3). Data are presented as mean±SD. **P<0.01 versus oxLDL group. D, The eEF1A1 is responsible for upregulation of eNOS by MLIF in brain endothelial cells. Left, Immunoblots for eEF1A1 of the extracts from cultured bEnd.3 cells transfected with eEF1A1-siRNA or control siRNA for 40 hours and stimulated with 10^{-5} M oxLDL for 2 hours, the bEnd.3 cells were treated with MLIF (50 mg/mL) for 6 hours and immunoblots for eNOS of the extracts from cultured bEnd.3 cells (n=3). **P<0.01 versus control siRNA group. Right, MLIFs enhance NO production through eEF1A1. NO production in supernatants from bEnd.3 cells transfected with eEF1A1-siRNA or control siRNA for 40 hours and stimulated with 10^{-5} M oxLDL for 2 hours, the bEnd.3 cells were treated with MLIF (50 mg/mL) for 6 hours and immunoblots for eNOS of the extracts from cultured bEnd.3 cells (n=6). Data are presented as mean±SD. **P<0.01 versus oxLDL group. MLIF indicates monocyte locomotion inhibitory factor; eNOS, endothelial nitric oxide synthase; MCAO, middle cerebral artery occlusion; oxLDL, oxidized low-density lipoprotein; NO, nitric oxide; siRNA, small interfering RNA.
significantly enhanced eNOS expression both in hypoxia and oxidized LDL stimulation. Importantly, the NO production was also increased by MLIF in oxidized LDL-treated brain vascular endothelial cells. This indicated that MLIF can upregulate eNOS and enhance NO production in hypoxia or oxidized LDL-stimulated endothelial cells with pathological conditions, which may protect the dysfunction of brain endothelium.

To further substantiate the role of eEF1A1 interaction for MLIF-induced eNOS upregulation, we synthesized eEF1A1 siRNA, which showed remarkable ability for eEF1A1 knockdown. As shown in Figure 5D, the upregulation ability of eNOS in bEnd.3 cells was significantly attenuated by eEF1A1 knockdown. Consistent with the eNOS expression, the NO production that enhanced by MLIF was also blocked by eEF1A1 knockdown. These data indicated that targeting eEF1A1 is required for MLIF-induced eNOS upregulation in brain endothelial cells.

The eEF1A1/eNOS Pathway Is Responsible for the Anti-Inflammatory Effect of MLIF

On the basis of MLIF targeting the eEF1A1/eNOS pathway, we next used eEF1A1 knockdown or eNOS inhibitor to investigate the role of the eEF1A1/eNOS pathway on the protective effect of MLIF. We used N\textsuperscript{5}-nitro-L-arginine methyl ester, the inhibitor of eNOS, in pretreatment of brain vascular endothelial bEnd.3 cells. The inhibitory effect of MLIF on ICAM-1/VCAM-1 expression was greatly attenuated by N\textsuperscript{5}-nitro-L-arginine methyl ester pretreatment, which indicated eNOS upregulation is required for effect of MLIF (Figure 6A–B).

After eEF1A1 knockdown by siRNA, the bEnd.3 cells were treated with oxidized LDL and ICAM-1/VCAM-1 expression was determined by Western blot. As shown in Figure 6C, the inhibitory effect of MLIF on oxidized LDL-induced ICAM-1/VCAM-1 expression was blocked by eEF1A1 knockdown, which indicated that eEF1A1 is required for protective roles of MLIF in brain microvascular endothelial cells. Taken together, our data suggested that the eEF1A1/eNOS pathway is responsible for the inhibitory effect of MLIF on inflammatory adhesion molecule ICAM-1/VCAM-1 expression.

Discussion

In this study, we present the evidence to find a potential medical valuable pentapeptide, MLIF, can protect brain ischemic injury with an anti-inflammatory role in cerebrovascular endothelium. The protection of MLIF in vitro is demonstrated to inhibit oxidized LDL and hypoxia-induced ICAM-1 and VCAM-1 expression, at the same time as enhancing eNOS expression in cultured cerebrovascular endothelial cells. For the target scanning, we explored biotin-conjugated MLIF pulldown assay and biological mass spectrum identification, which confirmed MLIF binds to the ribosomal protein translation elongation factor eEF1A1, especially at domain I of eEF1A1. Targeting eEF1A1 and thus enhancing eNOS expression contribute to the protective action of MLIF in cerebrovascular endothelium. The eEF1A1
knockdown or eNOS inhibition both attenuated the inhibitory effect of MLIF on oxidized LDL-induced adhesion molecules expression. Thus, we found a new pentapeptide, MLIF, which can protect brain ischemia injury through targeting the eEF1A1/eNOS pathway (Figure 6D).

MLIF was first found to be an anti-inflammatory active peptide in axenic culture of E. histolytica. The anti-inflammatory effect of MLIF led us to test whether it can protect inflammatory response in brain ischemia. During the pathological process after the onset of ischemia, the expressions of adhesion molecules including ICAM-1 and VCAM-1 were increased greatly, which mediates a pivotal role in ischemic injury by promoting leukocytes adhering to the vascular endothelium and crucially aggravating the inflammatory response. It is reported that anti-ICAM-1 antibody reduces ischemic damage in the transient MCAO rat. As expected, the data first validated our hypothesis that MLIF can protect brain injury in cerebrovascular ischemia by an anti-inflammatory role with antagonism on ICAM-1/VCAM-1 expressions.

However, what is the mechanism of this protective role and what is the target of this anti-inflammatory action of MLIF, especially in cerebrovascular endothelium? These questions have never been mentioned in previous studies. On the basis of the remarkable protective effect of MLIF for brain MCAO injury and the inhibitory effect on ICAM-1/VCAM-1 expressions in brain microvascular endothelial cells, we further explored the pulldown assay and biological mass spectrum method to search the potential target, in which we confirmed that eEF1A1 was the intracellular binding protein of MLIF. Our data also proved that eEF1A1 is required for MLIF-induced upregulation of eNOS. Thus, we demonstrated that MLIF protects brain ischemic injury through the eEF1A1/eNOS pathway.

The regulation of eNOS plays the key role in blood vessels and regulates almost all kinds of vascular functions. In many pathological conditions, the abnormal modulation of eNOS has been shown to cause several vascular diseases. In ischemia, NO produced by eNOS can inhibit platelet aggregation and adhesion, prevent leukocyte adhesion to the endothelium, inhibit smooth muscle proliferation, and stimulate endothelial cell regeneration. It is reported that administration of N\textsuperscript{G}-nitro-L-arginine methyl ester, NO synthase inhibitors, can cause increased blood pressure and vasoconstriction and enhance leukocytes rolling and adherence. In the present study, we demonstrate that MLIF can upregulate eNOS expression, which is also required for the inhibitory effect on the hypoxia or oxidized LDL-induced adhesion molecule expression, indicating a new therapeutic role for protecting cerebrovascular endothelium injury in response to brain ischemia.

The regulation of eNOS is reported at multiple levels, including gene transcription, posttranscriptional, and posttranslational mechanisms and protein–protein interactions. It is also reported that the modulation of eNOS mRNA stability plays an important role in eNOS regulation, especially in response to pathological stimuli, including oxidized LDL and cytokine tumor necrosis factor-\alpha. Importantly, eNOS mRNA stability was found to be regulated by statins, which are widely used in clinical cardiovascular and cerebrovascular diseases. However, the regulatory mechanism of eNOS mRNA stability remains unclear. Kosmidou et al reported statins could enhance eNOS mRNA polyadenylation and thus increase mRNA stability. Recently, eEF1A1 was identified as a cytosolic protein that binds to the eNOS \textsuperscript{3′}-UTR in endothelial cells in response to tumor necrosis factor-\alpha stimulation, which provides a new therapeutic target that protects endothelium. Fortunately, our pulldown assay showed that MLIF can directly interact with eEF1A1, which contributed to MLIF-induced eNOS upregulation. Thus, our finding may present a new therapeutic drug candidate for enhancing eNOS mRNA stability and protecting cerebrovascular endothelium injury from brain ischemia.

In summary, our study proved a new therapeutic pentapeptide, MLIF, which can protect brain ischemia injury through targeting the eEF1A1/eNOS pathway. Further studies may be carried on in preclinical investigations to evaluate the medical use of MLIF for brain ischemia.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL

Supplementary Figures for

A pentapeptide MLIF protects brain ischemia injury via targeting eEF1A1/eNOS pathway

Yuefan Zhang¹, Jun chen¹, Fan Li¹, Dong Li¹, Qinhui Xiong¹, Yang Lin¹, Dazhi Zhang², Xiao-Fan Wang³, Pengyuan Yang¹,³ and Yao-Cheng Rui¹.

S1. MLIF multiple treatments improve prognosis of MCAO rats.

The rats with cerebral infarction were treated with 4 times iv injection of MLIF or control saline buffer at 0, 4, 24, 48 h after occlusion. The rats (total n labeled in each group) were observed for 14 days with record of survival rate (A), and neurological behavior score were evaluated in survival ones (n labeled in each group) at the 14th day (B). Mean±SD. **P < 0.01.
S2. MLIF treatment exerts minimal effects on eNOS phosphorylation in mouse brain vascular endothelial cells (bEnd.3).

After the bEnd.3 cells were treated with 50mg/ml MLIF for indicated time points, immunoblots was used for detecting p-eNOS from cell lysis of bEnd.3 cells. GAPDH was used for the protein loading control.