Recombinant Milk Fat Globule–EGF Factor-8 Reduces Oxidative Stress via Integrin β3/Nuclear Factor Erythroid 2–Related Factor 2/Heme Oxygenase Pathway in Subarachnoid Hemorrhage Rats

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Background and Purpose—Milk fat globule–EGF factor-8 (MFG-E8) has been reported to be neuroprotective in ischemic stroke. However, the effects of MFG-E8 in early brain injury after subarachnoid hemorrhage (SAH) have not been investigated. We investigated the role of MFG-E8 in early brain injury and the potential mechanisms in antioxidation after SAH.

Methods—Two dosages (1 μg and 3.3 μg) of recombinant human MFG-E8 were injected intracerebroventricularly at 1.5 hours after SAH. SAH grades, neurological scores, and brain water content were measured at 24 and 72 hours. For mechanistic study, MFG-E8 siRNA, integrin β3 siRNA, and heme oxygenase (HO) inhibitor SnPP IX were used for intervention. The oxidative stress and expression of MFG-E8, integrin β3, HO-1, extracellular signal-regulated kinase, and nuclear factor erythroid 2–related factor 2 were measured by Western blots 24 hours after SAH.

Results—The expression of MFG-E8 and HO-1 increased and peaked 24 hours after SAH. Administration of recombinant human MFG-E8 decreased brain water content and improved neurological functions both at 24 hours and at 72 hours after SAH. Recombinant human MFG-E8 reduced oxidative stress and enhanced the expression of extracellular signal-regulated kinase, nuclear factor erythroid 2–related factor 2, and HO-1; and the effects were abolished by integrin β3 siRNA and HO inhibitor SnPP IX.

Conclusions—Recombinant MFG-E8 attenuated oxidative stress that may be mediated by integrin β3/nuclear factor erythroid 2–related factor 2/HO pathway after SAH. Recombinant MFG-E8 may serve as an alternative treatment to ameliorate early brain injury for SAH patients. (Stroke. 2014;45:3691-3697.)

Key Words: heme oxygenase-1 ■ MFG-E8 protein, rat ■ oxidative stress ■ subarachnoid hemorrhage

Aneurysmal subarachnoid hemorrhage (SAH) is one of the most life-threatening diseases with high mortality and disability rates. Early brain injury has been reported as the primary cause of mortality in SAH patients and has been considered as a primary target for treatment. One of the key factors involved in the pathogenesis of early brain injury is oxidative stress, which is caused by free radicals, including excess production of reactive oxygen species and reactive nitrogen species. Therefore, an antioxidative strategy has attracted attention in the treatment of SAH.

Milk fat globule–EGF factor-8 (MFG-E8) is a multifunctional glycoprotein originally identified as part of the milk fat globule membrane. MFG-E8 seems to be instrumental in cell-cell interactions and has been involved in diverse physiological and pathophysiological functions, including fertilization, angiogenesis, and phagocytosis of apoptotic cells. More recent studies have shown that MFG-E8 regulates adaptive immune responses and inflammation. In the central nervous system, administration of recombinant human MFG-E8 (rhMFG-E8) has neuroprotection against cerebral ischemia through suppression of inflammation and apoptosis after brain ischemia. rhMFG-E8 also exhibits significant antioxidative effects through increasing heme oxygenase-1 (HO-1) in the Alzheimer disease model. However, the effects of MFG-E8 in early brain injury after SAH have not been investigated. In the present work, we addressed the role of MFG-E8 in SAH. We showed for the first time that rhMFG-E8 attenuated early brain injury through suppression of the oxidative stress involving integrin β3–dependent pathway.

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Methods
All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

SAH Model and Experimental Protocol
Two hundred ten male (280–320 g) Sprague-Dawley rats (Indianapolis, IN) were used. The endovascular perforation model of SAH in rats was performed as reported previously. Briefly, with 3% isoflurane anesthesia, a sharpened 4-0 monofilament nylon suture was inserted rostrally into the right internal carotid artery from the external carotid artery stump and perforated the bifurcation of the anterior and middle cerebral arteries. Sham-operated rats underwent the same procedures, except the suture was withdrawn without puncture.

Two dosages (1 μg and 3.3 μg) of rhMFGE8 were injected intracerebroventricularly at 1.5 hours after SAH. SAH grades, neurological scores, and brain water content were measured at 24 and 72 hours. To study mechanisms, MFGE8 small interfering RNA (siRNA), integrin β3 siRNA, and HO inhibitor SnPP IX were used for intervention. The oxidative stress and expression of MFGE8, integrin β3, HO-1, extracellular signal-regulated kinase (ERK), and nuclear factor erythroid 2–related factor 2 (Nrf2) were measured by Western blots at 24 hours after SAH.

Intracerebroventricular Drug Administration
Intracerebroventricular drug administration was performed as previously described. Briefly, rats were placed in a stereotaxic apparatus under 2.5% isoflurane anesthesia. The needle of a 10-μL Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was inserted through a burr hole into the right lateral ventricles at the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of the skull. rhMFGE8 (Sigma) and SnPP IX (Santa Cruz Biotechnology, 3.3 μg) were injected, respectively, at 1.5 hours after SAH induction by a pump at a rate of 0.5 μL/min, respectively. MFGE8 siRNA,
integrin β3 siRNA, and scrambled siRNA (500 pmol/3 μL, Santa Cruz Biotechnology) were injected at 2 days before SAH induction by a pump at a rate of 0.5 μL/min.

SAH Grade
The severity of SAH was blindly evaluated using the SAH grading scale at the time of euthanasia, as previously reported. Rats with mild SAH (SAH grades ≤7 at 24 hours and SAH grades ≤5 at day 7) were excluded from the study. A total of 16 animals were excluded as a result of mild SAH (SAH, 2; SAH+vehicle, 3; SAH+10 μg/kg rhMFGE8, 2; SAH+3 μg/kg rhMFGE8, 1; SAH+MFGE8 siRNA, 2; SAH+Scramble siRNA, 2; SAH+rhMFGE8+Scramble siRNA, 1; SAH+rhMFGE8+SnPP IX, 1; SAH+ rhMFGE8+integrin β3 siRNA, 2).

Neurological Score
Neurological scores were evaluated at 1 hour before euthanization by a blinded observer according to the 21-point scoring system described by Garcia et al., with modifications.

Brain Water Content
Brains were removed at 24 or 72 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight) and once more after drying in 105°C for 72 hours. The percentage of water content was calculated as (wet weight−dry weight)/wet weight.

Immunofluorescence Staining
Double-fluorescence labeling was performed as previously described. Sections were incubated overnight at 4°C with goat anti-MFGE8 (Santa Cruz Biotechnology), rabbit anti-ionized calcium-binding adaptor molecule 1 (Abcam), goat antignial fibrillary acidic protein (Santa Cruz Biotechnology), and mouse anti-neuronal nuclei (EMD Millipore) primary antibodies. The expression of HO-1 was detected by fluorescence labeling with goat anti-HO-1 (Santa Cruz Biotechnology). Appropriate fluorescence dye–conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) were applied in the dark for 1 hour at 21°C. For negative controls, the primary antibodies were omitted and the same staining procedures were performed. The sections were visualized with a fluorescence microscope, and the photomicrographs were saved and merged with Image Pro Plus software (Olympus, Melville, NY).

Western Blot
The brain samples were collected at 24 hours after SAH. Western blotting was performed as described previously. Primary antibodies used were MFGF8 (Santa Cruz Biotechnology), HO-1, Nrf2 (Santa Cruz Biotechnology), 4-hydroxynonenal (4-HNE, Abcam Biotech Company), Nitrotyrosine (Santa Cruz Biotechnology), integrin β3, ERK, and phosphorylated ERK (p-ERK).

Statistical Analysis
Data are expressed as a means±SEM. All other data were analyzed by 1-way analysis of variance followed by the Tukey post hoc test. P value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism for Windows.

Results
The Expression of MFGE8 and HO-1 Increased and Peaked at 24 Hours After SAH
After SAH, there were clear blood clots around basal cistern, and the SAH grades decreased gradually as time passed. There were significant differences of SAH grades between sham and SAH animals up to day 7 (Figure 1A). Rather, the neurological deficits relived gradually and the animals showed no obvious difference on the neurological scores at 7 days after SAH (Figure 1B).

Western blots showed that MFGE8 was seldom expressed in sham animals and increased from 6 hours after SAH. The increase in MFGE8 reached peak at 24 hours lasting to day 7.
The protein expression of HO-1 also increased after SAH and peaked at 24 hours, and almost back to baseline at 7 days after SAH (Figure 1D).

Double immunostaining of MFGE8 with ionized calcium-binding adaptor molecule 1 (marker for microglia), glial fibrillary acidic protein (marker for astrocyte), and neuronal nuclei (marker for neuron) showed that MFGE8 is intensely expressed in the brain after SAH and highly colocalized with ionized calcium-binding adaptor molecule 1 at 24 hours (Figure 2). MFGE8 is not colocalized with glial fibrillary acidic protein and neuronal nuclei after SAH (Figure 2A). In brain tissue, the expression of HO-1 was low in shamed animals and remarkably increased at 24 hours after SAH (Figure 2B).

**Administration of rhMFGE8 Decreased Brain Water Content and Improved Neurological Functions After SAH**

Two dosages of rhMFGE8 (1 μg and 3.3 μg) were administrated intracerebroventricularly 1.5 hours after SAH. Brain water content and neurological scores were measured. The data showed that both high dosage and low dosage decreased the brain water content at 24 hours (Figure 3A and 3C), but only the high dosage improved the neurological deficits at 72 hours after SAH (Figure 3B and 3D). The results indicated that the high dosage is more effective, so we chose the high dosage for the following mechanism studies.

**Knockdown Integrin β3 and Inhibition of HO Abolished the Beneficial Effects of rhMFGE8 at 24 Hours After SAH**

To investigate the potential mechanisms of recombinant MFGE8, we administrated integrin β3 siRNA or HO inhibitor SnPP IX with rhMFGE8 treatment. Both integrin β3 siRNA and SnPP IX significantly increased brain water content (Figure 4A; \( P<0.05 \) versus SAH+rhMFGE8) and decreased the neurological scores at 24 hours after SAH (Figure 4B; \( P<0.05 \) versus SAH+rhMFGE8).

**Administration of rhMFGE8Enhanced the Expression of HO-1 and Decreased Oxidative Stress at 24 Hours After SAH**

Administration of rhMFGE8 significantly increased the protein level of MFGE8 in the brain, and MFGE8 siRNA effectively decreased it at 24 hours after SAH (Figure 5A). Consistent with MFGE8, the protein level of HO-1 was intensely increased by rhMFGE8 administration (Figure 5B). After SAH, oxidative stress had been generated and Western blots revealed a significant increase in 4-HNE (Figure 5C) and nitrotyrosine (Figure 5D). Administration of rhMFGE8 suppressed the expression of 4-HNE and nitrotyrosine and MFGE8 siRNA prevented the effects of rhMFGE8 (Figure 5). HO inhibitor SnPP IX did not change the protein level of HO-1 (Figure 1A in the online-only Data Supplement) but increased the expression of 4-HNE and nitrotyrosine in rhMFGE8-treated animals (Figure IB and IC in the online-only Data Supplement).

**MFGE8 Decreased HO-1 Dependent on Integrin β3/ERK/Nrf2 Pathway**

At 24 hours after SAH, administration of rhMFGE8 has no significant effects on the protein level of its receptor integrin β3 (Figure 6A). Integrinβ3 siRNA knocked down the receptor efficiently (Figure 6A). rhMFGE8 treatment increased p-ERK, Nrf2, and HO-1 compared with vehicle group (Figures 6B–6D). Integrin β3 siRNA remarkably abolished the up-regulation of p-ERK, Nrf2, and HO-1 after rhMFGE8 administration (Figure 6B–6D).

**Discussion**

Early brain injury, which occurs within 72 hours after cerebral aneurysm rupture, has been considered a new target for improving the outcome after SAH. Recent studies reported that oxidative stress was involved in the pathogenesis of early brain injury and antioxidative treatment can be one of the therapeutic candidates for early brain injury after experimental SAH or in a clinical setting. In this study, our goals were to test first whether rhMFGE8 administration suppressed oxidative stress and improved the outcome after SAH; and second, to investigate the potential mechanisms of rhMFGE8 in antioxidation. The results showed that MFGE8 was increased in the early stage of SAH, and its expression was well consistent with the expression of HO-1, which is an essential cellular defense molecular of oxidative stress. Administration of rhMFGE8 significantly decreased brain edema and improved neurological deficits. rhMFGE8 up-regulated the expression...
MFGE8 plays important roles in several biological processes, including apoptotic cell clearance, angiogenesis, and anti-inflammation. Recent studies showed MFGE8-mediated potential therapeutic benefits in several models of central nervous system diseases. Li et al showed that MFGE8 was released from microglia and increased microglial neuroprotective activity against oligomeric amyloid β-induced neuronal cell death. This neuroprotection was mediated through integrin receptor and activation of Nrf2/HO-1 pathway. In permanent middle cerebral artery occlusion rats, endogenous brain MFGE8 levels were decreased after cerebral ischemia, and exogenous rhMFGE8 was reported to reduce the infarct size and improve neurological function through suppression of inflammation and apoptosis. In agreement with this study, MFGE8 knockout mice had significantly larger infarct size and showed a marked increase in the expression of proinflammatory mediators interleukin-1β and tumor necrosis factor-α in the ischemic brain, which might be dependent on its receptor integrin β3. These results clearly indicated that endogenous MFGE8 is required for the neuroprotection against neurodegenerative disease and ischemic cerebral damage. In our study, we found that the level of MFGE8 was increased in SAH rats and highly expressed in microglia. Administration of rhMFGE8 improved the neurological deficits and reduced brain edema via suppressing oxidative stress, whereas knockdown MFGE8 by siRNA exacerbated the outcomes and intensified oxidative stress. Our results were consistent with the previous studies to support the beneficial effects of MFGE8 in animal stroke models.

Oxidative stress became emerged as a key player in the development and progression of many pathological conditions including stroke. HO-1 is an essential cellular defense system against oxidant-induced injury during inflammatory processes. HO-1 offers protection by catalyzing the first and rate-limiting step in the oxidative degradation of heme.
(Fe-protoporphyrin-IX) to carbon monoxide, ferrous iron (Fe2+), and biliverdin. Studies have revealed that HO-1 played an important role in neuroprotection in ischemic stroke. In this study, we found that rhMFGE8 decreased the expressions of 4-HNE and nitrotyrosine, which are markers of lipid peroxidation (4-HNE) and protein nitrification (nitrotyrosine), when compared with the vehicle group. We further observed that the levels of HO-1 in ischemic brain tissue were up-regulated by rhMFGE8 after treatment, and HO inhibitor SnPP IX removed the antioxidation effects of rhMFGE8. Therefore, we conceptualize that rhMFGE8 improved the outcomes after SAH, possibly by suppressing oxidative stress via increasing HO-1.

How did rhMFGE8 decrease the expression of HO-1 and reduce oxidative stress after SAH? It is well-known that integrin is a putative receptor of MFGE8 in various cell types. In vascular smooth muscle cells, Wang et al demonstrated that MFGE8 facilitation of the cell cycle was mediated by integrins/ERK signaling. In diabetic mice, MFGE8 was found to coordinate fatty acid uptake through integrin avβ3 and integrin avβ5-dependent phosphorylation of Akt by phosphatidylinositol-3 kinase and mammalian target of rapamycin complex 2. Recently, Deroide et al showed that MFGE8 attenuated inflammation in ischemic cerebral injury through integrin β3-dependent inhibition of NLRP3 inflammasome. However, the precise mechanisms underlying its role in antioxidation after SAH are poorly understood. Increasing evidence suggests that HO-1 is up-regulated through the Nrf2 cascade. In the Alzheimer disease model, MFGE8 was released from microglia, activated Nrf2/HO-1 pathway through integrin receptor, and suppressed oxidative stress. In our experiment, we found that the expression of HO-1 was coincident with that of MFGE8 after SAH. Treatment with rhMFGE8 significantly increased the expression of p-ERK, Nrf2, and HO-1 at 24 hours after SAH and reduced oxidative stress; knockdown integrin β3 by siRNA deteriorated the neurological scores, increased brain edema, and decreased the expression of p-ERK, Nrf2, and HO-1. With the relevant cumulative findings, herein we concluded that MFGE8 improved the outcome of SAH via antioxidation, which may depend on integrin β3/ERK/HO pathway.

In conclusion, our results demonstrate, for the first time, that MFGE8 signaling is linked to modulation of oxidative stress after SAH. Administration of rhMFGE8 improved neurological deficits and attenuated brain edema through decreasing oxidative stress; silencing MFGE8 or its receptor integrin β3, or blocking the activity of HO-1 abolished the protective effects of rhMFGE8. MFGE8-attenuated oxidative stress may depend on integrin β3/ERK/HO pathway. Thus, targeting MFGE8 could be a novel strategy to decrease oxidative stress and ameliorate early brain injury after SAH.
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Disclosures
None.

References
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Supplementary figure 1. HO-1 inhibitor SnPP IX has no effects on the expression of HO-1 (A), and increased the expression of 4-HNE (B) and nitrotyrosine (C) in rhMFGE8 treated animals. n=6 for each group. \( *p<0.05 \) vs. Sham; \( #p<0.05 \) vs. SAH+vehicle.