Acute White Matter Injury After Experimental Subarachnoid Hemorrhage
Potential Role of Lipocalin 2

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Background and Purpose—White matter injury occurs after subarachnoid hemorrhage (SAH) and has not been well studied. In this study, we investigated acute white matter injury in a mouse SAH model and the role of lipocalin 2 (LCN2) in that injury.

Methods—SAH was induced by endovascular perforation in wild-type (WT) or LCN2 knockout (LCN2−/−) mice. Sham WT mice underwent the same procedure without perforation. MRI was performed 24 hours after SAH and the volumes of the T2-hyperintensity in white matter were measured. Immunohistochemistry was performed to determine white matter injury.

Results—Mortality rates and SAH severity were not significantly different between WT and LCN2−/− animals. T2-hyperintensity in the white matter was observed in all WT animals at 24 hours after SAH (6.1±2.7 versus 0.06±0.07 mm³ in sham; P<0.001), and the volume of T2-hyperintensity tended to correlate with SAH severity (r=0.30; P=0.055). In WT animals with SAH, numerous LCN2-positive cells were observed in white matter. In contrast, LCN2−/− animals scarcely developed white matter T2-hyperintensity after SAH (0.5±0.5 mm³; P<0.001, versus WT). Markers of axonal damage and myelin degradation were increased in white matter after SAH in WT compared with those in LCN2−/− animals (P<0.05).

Conclusions—SAH results in an acute white matter injury at 24 hours in mice, and LCN2 plays an important role in SAH-induced white matter injury.

Key Words: mice ■ subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disorder with a high mortality and morbidity rate. Early brain injury is a major component of SAH. Approximately 50% human brain tissue is white matter, but acute white matter injury has been scarcely studied in experimental SAH. There is growing evidence that iron has a role in various forms of cerebral hemorrhage, including SAH. Lipocalin 2 (LCN2) is an iron transport protein that recently has been implicated in brain injury. The current study investigates acute white matter injury after SAH induced by endovascular perforation in mice and the potential role of LCN2 in that injury.

Materials and Methods
All animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 25 male wild-type (WT) C57/BL6 mice (22–30 g; Charles River Laboratories) and 11 male LCN2 knockout (LCN2−/−) mice (University of Michigan Breeding Core, gift from Dr Xiaoli Chen, University of Minnesota) were used. SAH was induced using an endovascular perforation technique as previously described (WT, n=19; LCN2−/−, n=6). Sham control mice underwent the same surgical procedure without perforation (WT, n=6; LCN2−/−, n=5). MRI was performed 24 hours after SAH in a 7.0-T Varian MR scanner with acquisition of T2 fast spin-echo and T2* gradient-echo sequences using a field of view of 20×20 mm, matrix of 256×256 mm, and 25 coronal slices (0.5-mm thick). To calculate the volume, the area of white matter T2-hyperintensity was measured in all slices and multiplied by section thickness. Ventricular volume was measured as previously described.

After MRI, mice were euthanized and SAH severity assessed using a modified grading system. For immunohistochemistry, the forebrain was embedded and sliced into 10-μm-thick coronal sections (Sham, n=4; WT+SAH, n=6; LCN2−/−+SAH, n=5). LCN2 (dilution 1:200; R&D Systems), glial fibrillary acidic protein (1:400; Millipore), Iba-1 (1:200; Wako), NG2 (1:200; Millipore), β-amyloid precursor protein (β-APP, 1:1000; Invitrogen), and degraded myelin basic protein (DMBP, 1:2000; Millipore) antibodies were used. NG2 is an oligodendrocyte precursor cell marker, NG2 expression correlated with axonal degeneration and oligodendrocyte death. β-APP and DMBP identify damaged axons and degraded myelin, respectively. For quantification, 4 slides from each brain with each slide containing 3 fields from white matter tracts (corpus callosum, external capsule, or fimbriae) were digitized using a BX-51 Olympus microscope (×40 objective). The number of NG2-positive cells was counted. β-APP and DMBP immunoreactivity were scored 0 to 3 (none-extensive) as described previously, and the score summed >12 fields. Image analysis was performed by blinded investigator using ImageJ software.

Data are expressed as means±SD. Statistical differences among groups were analyzed using 1-way ANOVA, Spearman rank correlation test, and Mann–Whitney U test. A Bonferroni correction was used for multiple comparisons. P<0.05 was considered statistically significant.
Results
Mortality rates were 26% (5/19) and 17% (1/6) at 24 hours after endovascular perforation in WT and LCN2−/− animals, respectively (P>0.05). No sham animals died (n=6 for WT; n=5 for LCN2−/− animals). One WT animal that developed a large hemispheric infarction after SAH was excluded from further investigation. The SAH severity score were not different between WT and LCN2−/− animals (9±3 and 8±4, respectively; P>0.05). White matter T2-hyperintensity was observed in all WT animals undergoing endovascular perforation (Figure 1A). The hyperintensity volumes were 6.1±2.7 and 0.06±0.07 mm³ in SAH (n=13) and sham mice (n=6), respectively (P<0.001; Figure 1A). Ventricular volume was significantly greater in SAH mice than sham mice (16.1±2.7 and 8.9±2.2 mm³, respectively; P<0.001; Figure 1A). The volume of white matter T2-hyperintensity tended to correlate with SAH severity (r=0.30; P=0.055; Figure 1B), but not ventricular volume (r=0.12; P=0.249; Figure 1C).

Numerous LCN2-positive cells were observed in the white matter after SAH (Figure 2A). Double labeling showed that LCN2-positive cells were mainly astrocytes (Figure 2B). In contrast to WT animals, LCN2−/− animals developed little white matter T2-hyperintensity at 24 hours after SAH (0.5±0.5 mm³; n = 5; P<0.001, versus WT animals; Figure 3A). Ventricular volume after SAH in LCN2−/− animals was similar to those in WT animals (15.9±3.3 mm³; P=0.99). White matter T2-hyperintensity and ventricular volume of LCN2−/− sham animals (0.07±0.04 and 11.1±3.1 mm³, respectively; n=5) did not significantly differ from those of WT animals (P>0.05 for each). NG2, β-APP, and DMBP expression was significantly increased in white matter of WT animals with SAH compared with LCN2−/− animals (P<0.01 for NG2, and P<0.05 for β-APP and DMBP; Figure 3B and 3C).

Discussion
The present study contained 3 major findings: (1) experimental SAH induced by endovascular perforation caused acute white matter injury; (2) prominent LCN2 expressions were observed in white matter after SAH; and (3) LCN2−/− animals had less acute white matter injury.

The clinical relevance and causative mechanism of early brain injury in white matter after SAH is still uncertain.9 White matter injury has not been well studied in experimental SAH models. In the present study, MRI demonstrated that all WT animals undergoing endovascular perforation developed white matter T2-hyperintensity. As well as this hyperintensity, immunohistchemistry clearly demonstrated that markers

Figure 1. Coronal T2 images after 24 hours in sham and subarachnoid hemorrhage (SAH) mice. Animals with SAH developed marked white matter hyperintensity. The volume of T2-hyperintensity in white matter (WM) and ventricular volume at 24 hours after endovascular perforation or sham procedure (A). Correlations between white matter T2-hyperintensity and SAH score (B), and ventricular volume and white matter T2-hyperintensity (C). Values are means±SD; ***P<0.001; n=6 for sham and n=13 for SAH.

Figure 2. Magnified view of coronal T2 images and lipocalin 2 (LCN2) immunohistochemistry in corpus callosum of sham and subarachnoid hemorrhage (SAH) animals at 24 hours (A). Boxes show areas examined at higher power in the lower micrographs (scale bars=200 and 50 μm). LCN2, glial fibrillary acidic protein (GFAP) and Iba-1 immunoreactivity, and the colocalization of LCN2 with GFAP or Iba-1 by double labeling (B; scale bar=50 μm).
of axonal damage (NG2 and β-APP) or myelin degradation (DMBP) were prominently expressed in white matter. Hence, these lesions were considered as acute white matter injury. This suggests that a mouse SAH model induced by endovascular perforation is usable for the experimental research of SAH-induced white matter injury.

In this study, white matter LCN2 expression was markedly increased after SAH and acute white matter injury was less in LCN2−/− animals. LCN2 is a mediator of iron uptake and recent studies found that LCN2 plays a detrimental role after acute injury in the central nervous system. LCN2 was also upregulated in a rodent intracerebral hemorrhage model, an upregulation reduced by the iron chelator deferoxamine. The detailed role of LCN2 in SAH still remains to be elucidated fully, for example, it is not clear whether LCN2 through LCN2 receptor causes white matter damage. There is evidence that the LCN2 receptor is involved in LCN2-induced apoptosis. Our results do suggest, though, that LCN2 plays an important role for acute white matter injury after SAH.

Conclusions
SAH results in LCN2 expression and acute white matter injury, and the latter is less in LCN2−/− mice. Future studies should determine the mechanisms of LCN2-mediated white matter injury after SAH.

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Disclosures
None.

References
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