Cystatin C Is a Crucial Endogenous Protective Determinant Against Stroke

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Background and Purpose—Endogenous neuroprotection can be induced by ischemic and nonischemic preconditioning. However, not all subjects that undergo preconditioning exhibit similar favorable outcome. This study is to explore the molecules responsible for this phenomenon and find new therapeutic targets for stroke.

Methods—Adult male Sprague–Dawley rats were subjected to transient middle cerebral artery occlusion. High-throughput proteomic technique, isobaric tag for relative and absolute quantification, was used to screen differentially expressed proteins in the rats that developed ischemic tolerance from hyperbaric oxygen (HBO) preconditioning. The proteomic results were verified by Western blot and ELISA. Then, short interfering RNA and gene knockout rats were used to further determine the pivotal role of candidate proteins in HBO preconditioning–induced endogenous neuroprotection. Finally, lysosomal permeability was tested to elaborate the mechanism underlying this intrinsic neuroprotective effect.

Results—Nine proteins differentially expressed in the serum of rats, which acquired benefits from HBO preconditioning, were screened and identified. Western blot and ELISA revealed that cystatin C (CysC) and mannose-binding lectin protein C were uniquely changed in rats with smaller infarction after HBO preconditioning and cerebral ischemia. Knockdown and knockout of CysC abolished HBO-induced neuroprotection. Moreover, HBO-induced endogenous CysC elevation preserved lysosomal membrane integrity after stroke in wild-type rats but not in CysC siRNA infusion or $CysC^{-/-}$ rats. Most importantly, exogenous CysC also induced neuroprotection against ischemic/reperfusion injury.

Conclusions—CysC is a crucial determinant contributing to endogenous neuroprotection. It is also a novel candidate for stroke treatment through maintaining lysosomal membrane integrity.

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Key Words: cystatin C ■ infarction ■ lysosomes ■ neuroprotection ■ permeability

Stroke is responsible for 9.5% of total mortality each year and ranks as the second leading cause of death worldwide.[1,2] About 800 000 and 2 000 000 people had stroke in the United States and China each year, respectively.[3] Considerable efforts have been devoted to exploring novel protective measures against ischemic stroke. However, in the past 2 decades, only tissue plasminogen activator has been proved and recommended as an effective treatment. However, the application is with strict limitations, including a narrow time window and risk of inducing intracranial hemorrhage. Therefore, the research direction for stroke treatment needs to be rerouted.

The brain has a remarkable endogenous self-protective capacity, which can be induced by ischemic preconditioning.[4] Previous reports have demonstrated that endogenous neuroprotection can also be induced by nonischemic preconditioning. Repeated hyperbaric oxygen (HBO) preconditioning is one of them.[5] More importantly, our previous clinical trial showed that the elevation of neurological injury markers, S100β and neuron-specific enolase, was attenuated by repeated HBO preconditioning in patients undergoing on-pump coronary artery bypass graft surgery,[6] indicating a cerebral protective effect of HBO preconditioning in clinical settings. However, not all animals or patients who received HBO preconditioning exhibited a favorable outcome after cerebral injury. It is reasonable to deduce that some intrinsic molecules are responsible for the differential response of individuals after HBO preconditioning because of differences in genetic and epigenetic. Therefore, identifying the crucial beneficial molecules may help to understand the endogenous neuroprotective mechanism and provide new insights into stroke therapy.

To find out the crucial determinants of endogenous neuroprotection, proteomic technique was used to screen out differentially expressed proteins in the serum of rats that acquired benefit from HBO preconditioning followed by ischemic

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insult. Among these proteins, the change of cystatin C (CysC) was further verified in the brain. Then, the role of CysC in ischemic tolerance induced by HBO preconditioning was evaluated through gene manipulation. Moreover, because CysC is a cysteine protease inhibitor that modulates lysosomal function, the critical role of CysC in maintaining lysosomal permeability was evaluated in the ischemic cortex.

Materials and Methods

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of The Fourth Military Medical University.

Animals

A total of 246 wild-type male Sprague–Dawley rats and 26 CysC knockout rats weighing 280 to 300 g were used in the experiment. Using young, male rats is a quality control strategy to reduce sex and age influences on molecular changes after HBO preconditioning. Sprague–Dawley rats were provided by the Experimental Animal Center of The Fourth Military Medical University. A breeding colony of CysC knockout rat was generated using a site-specific gene modification technique based on transcriptional activator–like effector nucleases. The animals were housed in a controlled environment (25±2ºC, 50% humidity, and 12-hour light–dark cycle from 8 AM to 8 PM) and allowed free access to food and water.

HBO Preconditioning and Serum Collection

The rats were randomly assigned to control or HBO group by completely randomization design. The rats in the HBO group were exposed to HBO (2.5 atmosphere absolute, 100% O_2, 1 hour per day) for 5 consecutive days. The control rats were put into the hyperbaric chamber with normobaric air. Serum was collected from the femoral artery at 6 hours after the last session of HBO preconditioning but before ischemia. Samples were allowed to clot for 2 hours at 4°C followed by 3000*g* centrifugation for 15 minutes at 4°C and then stored at −80°C before use.

Transient Focal Cerebral Ischemia and Cerebral Blood Flow Measurement

Transient focal cerebral ischemia was induced by right middle cerebral artery occlusion (MCAO) using an intraluminal filament as described previously.[7] Regional cerebral blood flow (rCBF) was monitored using PF 5000 Laser Doppler Perfusion Monitoring Unit (PeriFlux 5000; Perimed AB). The intervention was considered successful if rCBF sharply decreased to ≤30% of the baseline level after MCAO and increased back to ≥70% of the baseline level within 10 minutes after reperfusion.[8] Throughout surgery, the temporal temperature was maintained at 37±1°C by a thermostatic pad.

Neurological Evaluation, Magnetic Resonance Image, and 2,3,5-Triphenyltetrazolium Chloride Staining

Neurological deficit scores (Garcia score) were assessed as previously reported by an observer blinded to the grouping information.[9] After neurological assessment, the rats were subjected to brain magnetic resonance image (MRI) scanning or euthanized for brain infarction volume assessment with 2,3,5-triphenyltetrazolium chloride staining as previously described.[5,10]

Serum Preparation, Grouping, and Analysis by Isobaric Tag for Relative and Absolute Quantification

The collected serum was retrospectively pooled into 3 groups (n=8 in each group) according to the calculated infarct volume ratio: control group (rats without HBO preconditioning); HBO-protected (HBO-P) group (rats underwent HBO preconditioning with an infarct volume ratio < [Mean-SD] that of the control group); and HBO-unprotected (HBO-U) group (rats underwent HBO preconditioning with an infarct volume ratio ≥ [Mean-SD] that of the control group). The sample labeling and isobaric tag for relative and absolute quantification techniques for serum protein screening are shown in Methods in the online-only Data Supplement.

Western Blot Analysis

Western blot was performed as previously described.[11] The designated region of cerebral cortexes (from bregma −2.0 to +3.0 mm, presumably the penumbra region after middle cerebral ischemia) was harvested as previously described.[12] The following primary antibodies were used: rabbit polyclonal anti-CysC (Epitomics Inc), mouse monoclonal anti–mannose-binding lectin protein C (anti-MBLC; Abcam), and mouse monoclonal anti-β-tubulin (Huaan Biotech). Immunoreactive bands were visualized using enhanced chemiluminescence horseradish peroxidase substrate (Millipore Corporation). The optical density was determined using Image Lab 4.1 software.

ELISA

The concentrations of serum CysC and MBLC were measured using a quantitative competitive sandwich ELISA kit (Westang Biotechnology) according to the manufacturer's protocols. All standards and samples were tested in duplicate wells.

Intracerebroventricular Injection of CysC siRNA and Exogenous CysC

Twenty microliters of CysC siRNA (20 μmol/L) or scramble siRNA was delivered into the right lateral ventricle 3 days before MCAO as previously reported.[5] The indicated concentration of exogenous CysC was delivered 30 minutes after reperfusion. Details of intracerebroventricular injection are in Methods in the online-only Data Supplement.

Double Immunofluorescence Staining

The rats were euthanized at 6 or 24 hours after the last session of HBO preconditioning. Immunofluorescent staining protocol is described in Methods in the online-only Data Supplement. The following antibodies were used: rabbit polyclonal anti-CysC (Epitomics, Inc), rabbit polyclonal anti-cathepsin B (Abcam), mouse monoclonal anti-NeuN (Abcam), and mouse monoclonal anti–glial fibrillary acidic protein (Sigma–Aldrich). DAPI (4',6-diamidino-2-phenylindole; Sigma–Aldrich) was used for nuclei staining. Fluorescent signals in the right cortex 2 mm from the midline (presumed penumbra region if MCAO injury was induced) were detected using confocal laser scanning microscopy (FV1000; Olympus).

Immunoelectron Microscopy (Immunogold-Silver Cytochemistry)

The rats were euthanized 24 hours after MCAO for immunogold-silver staining of cathepsin B. Electronic micrographs were captured using a Gatan digital camera and analyzed using its software (832 SC1000, Gatan). Details are shown in Methods in the online-only Data Supplement.

Statistical Analysis

SPSS 19.0 was used to conduct statistical analysis. All the data except neurological score were presented as mean±SE of mean and analyzed using a 2-tailed Student *t* test if 2 groups were compared. One-way ANOVA followed by the Bonferroni post hoc test was used for comparisons of >2 groups. The neurological scores were expressed as median with range and analyzed using the Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Differences were considered significant if the *P* value was <0.05.

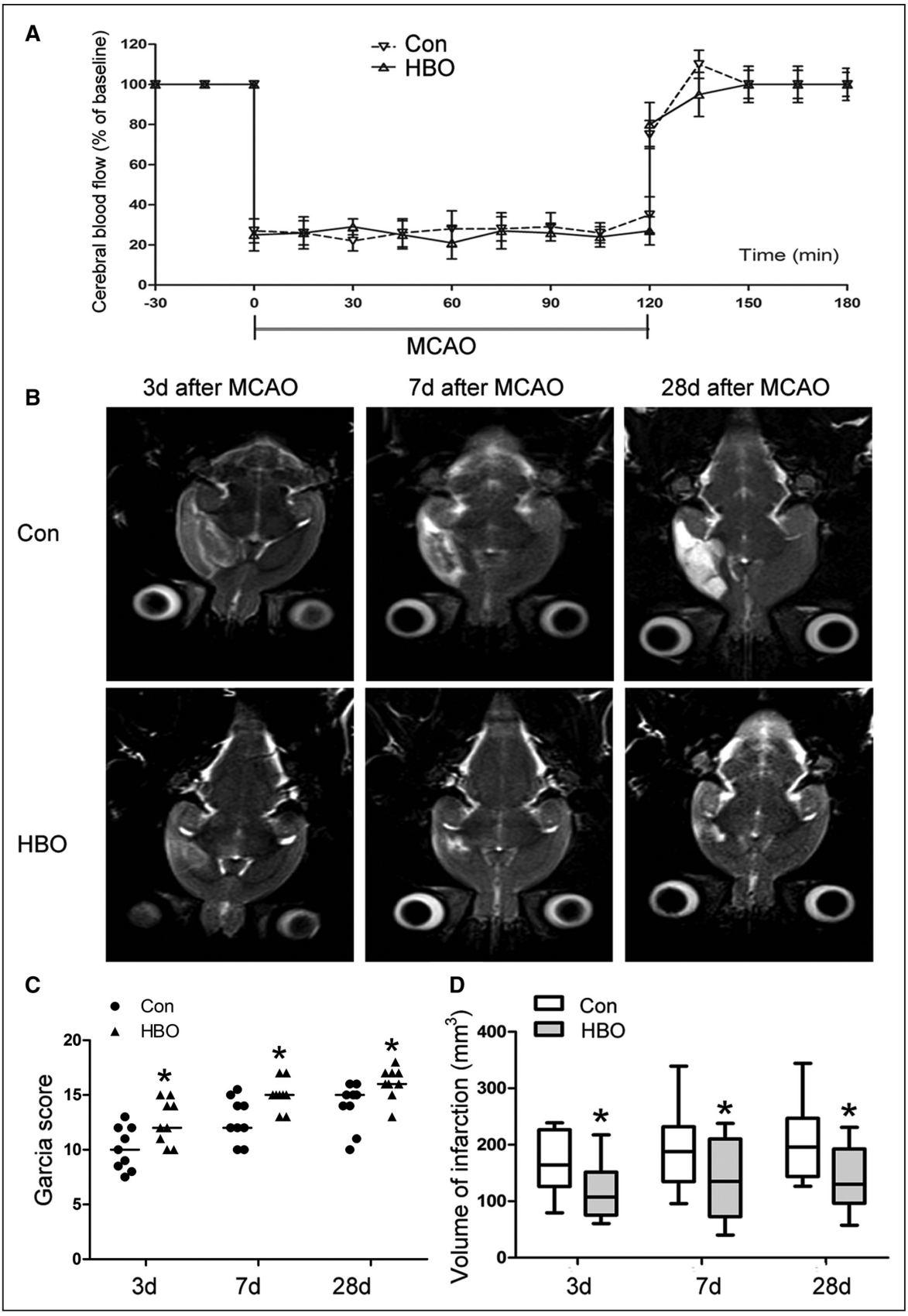

Figure 1. Hyperbaric oxygen (HBO) preconditioning–induced long-lasting neuroprotection. **A**, Regional cerebral blood flow in the ischemic hemisphere of rats during middle cerebral artery occlusion (MCAO). **B**, Representative magnetic resonance imaging (MRI) scanning images at 3, 7, and 28 d after reperfusion in each group. High-signal area circled represents edema and infarct areas. **C**, Neurological deficit scores. **D**, Infarct volume ratio. n=9 in each group. *P<0.05 vs control group.

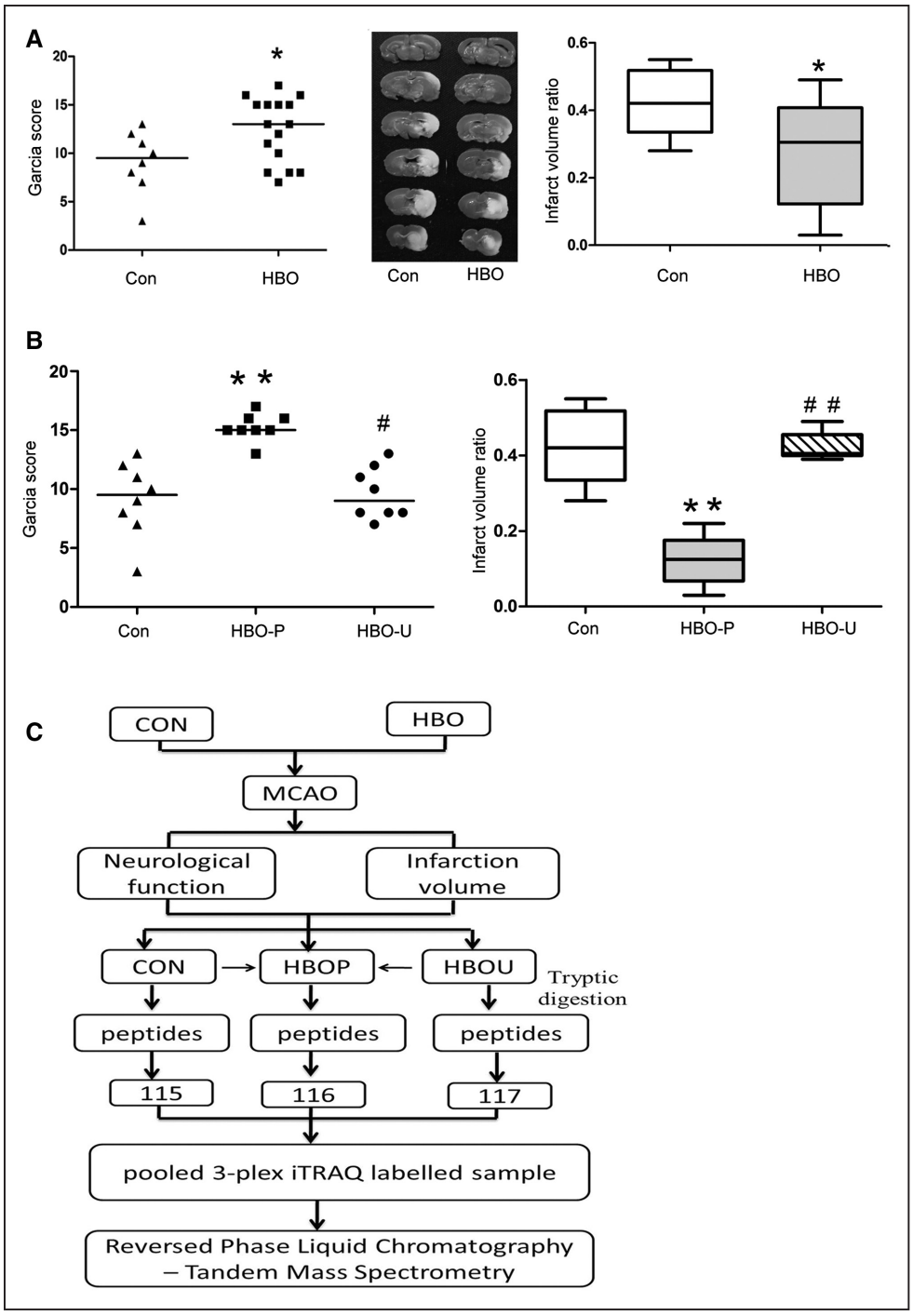

Figure 2. Experimental design for the isobaric tag for relative and absolute quantification/liquid chromatography coupled with tandem mass spectrometry. **A**, Neurological deficit and infarct volume of rats at 3 d after middle cerebral artery occlusion (MCAO; n=8 for control and n=16 for hyperbaric oxygen [HBO]) in the proteomic study. **B**, Neurological deficit evaluation and infarct volume ratio after regrouping. **C**, Experimental scheme for the proteomic study. **P*<0.05 and ***P*<0.01 vs control group; #*P*<0.05 and ##*P*<0.01 vs HBO-P group. n=8 in each group. HBO-P indicates rats in the HBO group that showed protective effect of HBO preconditioning; and HBO-U, rats in the HBO group that showed no benefit from HBO preconditioning.

Results

In our study, 15 wild-type Sprague–Dawley rats were excluded because they did not meet the rCBF criteria during ischemia. And 16 wild-type rats died after MCAO before final assessment. Two CysC knockout rats died after MCAO.

HBO Preconditioning–Induced Long-Lasting Neuroprotection Against Cerebral Ischemia in Rats

When the middle cerebral artery was occluded, rCBF in the middle cerebral artery supply territory immediately decreased to <30% of the baseline (Figure 1A). After 2 hours, rCBF returned back to ≥70% of the baseline within 10 minutes

after reperfusion. Magnetic resonance image and neurological behavioral evaluation demonstrated that at 3, 7, and 28 days after stroke onset, the subjects in the HBO preconditioning group exhibited smaller infarct volume (Figure 1B and 1D) and less severe neurological deficits (Figure 1C) compared with the rats in the control group. These results demonstrated for the first time that neuroprotection induced by HBO preconditioning was sustainable for at least 28 days after stroke. Knowing that ischemic tolerance persists beyond 3 days, 3 days after cerebral reperfusion was chosen as the end point assessment for further study.

Proteomic Analysis Screened Out 9 Differentially Expressed Proteins in the Serum of Rats That Benefited From HBO Preconditioning Against Cerebral Ischemic Injury

To screen proteins differentially expressed in animals that acquired neuroprotection from HBO preconditioning, blood sample from the femoral artery was collected from all subjects at 6 hours after the last HBO or sham session. After another 18 hours, all rats were subjected to MCAO. Three days after reperfusion, HBO preconditioning significantly improved neurobehavioral recovery and reduced infarct volume ratio (Figure 2A). However, neuroprotection was not manifested in every individual rat that accepted HBO preconditioning. A retrospective subgrouping method according to the infarct volume ratio in the HBO preconditioning group was used. Therefore, serum collected from the rats subjected to HBO preconditioning was divided into HBO-P and HBO-U groups for further proteomic screening (Figure 2B; n=8 in each group).

Serum from control, HBO-P, and HBO-U groups were analyzed using the isobaric tag for relative and absolute quantification technique (Figure 2C). A total of 559 proteins were identified with 95% confidence intervals. Twenty-five proteins were differentially expressed between the HBO-P and control groups, and 18 proteins between the HBO-P and HBO-U groups. Nine proteins overlapped in these 2 comparisons, as shown in Table. Serum levels of CysC, Dickkopf-related protein 3, and platelet factor 4 increased, whereas the levels of MBLC, corticosteroid-binding globulin, RAB2, protein Z, and macrophage stimulating 1 decreased in the HBO-P group. The α-2-macroglobulin in the HBO-P group was lower than that in the control group, but higher than that in the HBO-U group. Representative liquid chromatography coupled with tandem mass spectrometry fragmentation spectrum is shown in Figure IA (CysC) and IB (MBLC) in the online-only Data Supplement.

Verification of Serum CysC and MBLC Changes

All the overlapped 9 proteins in Table were verified using Western blot and ELISA in another set of rats. First, the neurological outcome of the animals was assessed, and the infarct volume ratio was used as the criterion for subgrouping HBO-preconditioned rats (Figure IC and ID in the online-only Data Supplement). Only changes of serum CysC and MBLC expression were found to be in accordance with proteomic findings (Figure 3A). The serum CysC level of the HBO-P group was significantly higher, whereas the serum level of MBLC was significantly lower than that in the other 2 groups. ELISA also showed similar expression patterns of CysC and MBLC (Figure 3B). Besides, serum CysC concentration was negatively correlated with the infarct volume ratio after cerebral ischemic reperfusion (correlation coefficient, =−0.66; 95% confidence interval, −0.85 to −0.32; $P<0.05$; Figure 3C), whereas serum MBLC concentration was positively correlated with the infarct volume ratio (correlation coefficient, 0.74; 95% confidence interval, 0.46 to 0.89; $P<0.05$; Figure 3C). These results indicated that CysC and MBLC were candidate molecules that might contribute to the neuroprotection induced by HBO preconditioning.

Upregulation of Endogenous CysC in the Brain-Induced Neuroprotection

In this study, Western blot and immunofluorescence did not detect positive staining for MBLC in the brain (data not shown). Therefore, a subsequent study investigated the neuroprotective effect of endogenous CysC. Western blot for the cerebral cortex tissue showed that CysC expression was upregulated at 6 and 24 hours after HBO preconditioning (Figure 4A). Immunofluorescence revealed more CysC-positive staining neurons and astrocytes after HBO preconditioning in comparison with that in the control group (Figure 4B).

To determine whether increased expression of endogenous CysC in the brain was responsible for the neuroprotection induced by HBO preconditioning, intracerebroventricular injection of

Table. Differentially Expressed Proteins in Comparisons of HBO-P Versus Control Group and HBO-U Versus Control Group

Accession Number	Protein Name	Con:HBO-P	*P* Value	HBO-U:HBO-P	*P* Value
sp|P14841|CYTC_RAT	Cystatin C	0.6368	0.0164	0.3565	0.0461
tr|B1H219|B1H219_RAT	Dkk3 protein	0.5495	0.0323	0.5598	0.0327
sp|P06765|PLF4_RAT	Platelet factor 4	0.6368	0.0425	0.5861	0.0377
sp|P08661|MBL2_RAT	Mannose-binding protein C	2.1084	0.0414	1.6444	0.0158
sp|P06238|A2MG_RAT	α-2-macroglobulin	2.1086	0	0.5754	0.0218
sp|P31211|CBG_RAT	Corticosteroid-binding globulin	2.3335	0.0094	2.1677	0.0053
tr|Q6MGC5|Q6MGC5_RAT	RAB2, member RAS oncogene family-like	5.3456	0.0146	4.2855	0.0167
tr|P70521|P70521_RAT	Macrophage stimulating 1 (Hepatocyte growth factor like)	2.0324	0.0025	1.8197	0.0171
tr|G3V8KB|G3V3K8_RAT	Protein Z, vitamin K–dependent plasma glycoprotein	1.9588	0.0384	1.4997	0.0500

Dkk3 indicates Dickkopf-related protein 3; HBO-P, hyperbaric oxygen protected; and HBO-U, hyperbaric oxygen unprotected.

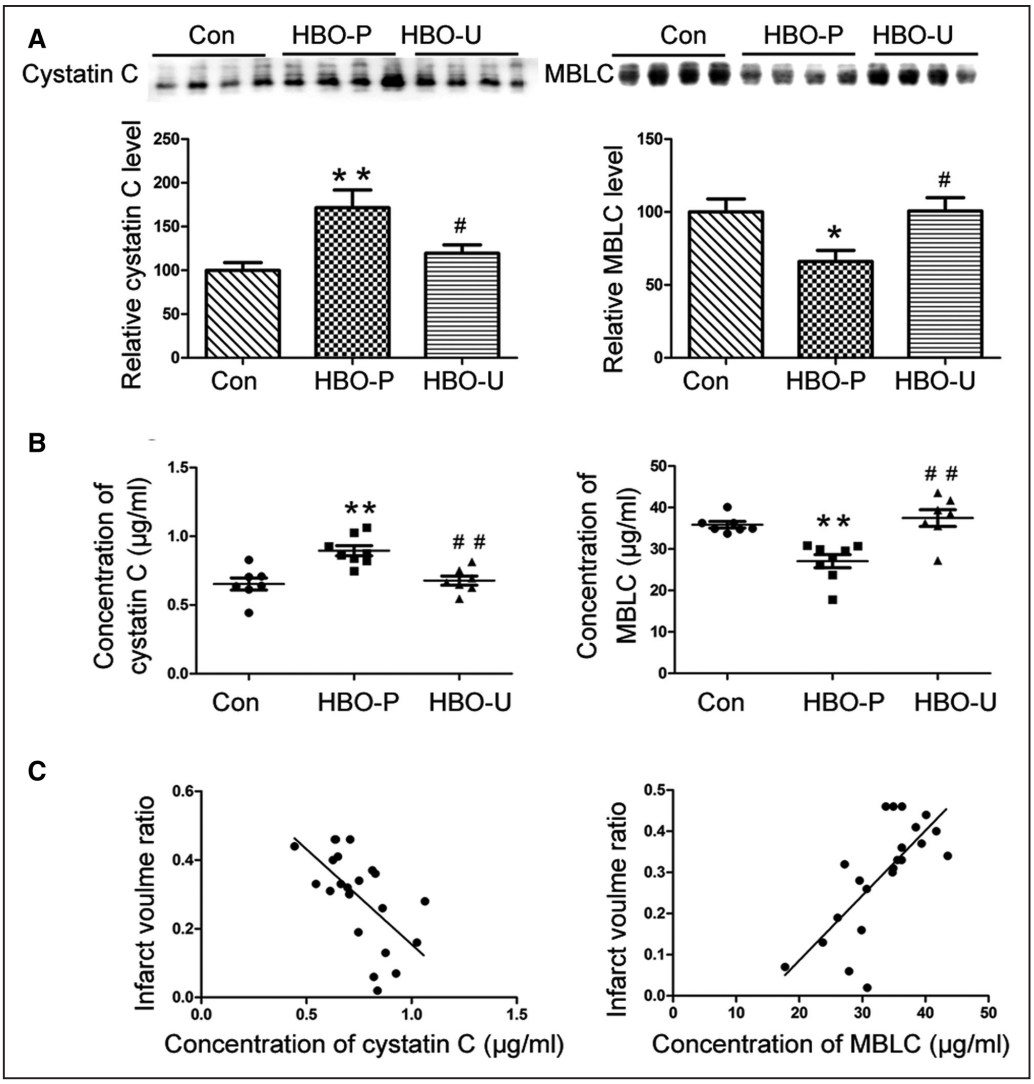

Figure 3. Verification for cystatin C (CysC) and mannose-binding lectin protein C (MBLC) expression in serum. **A**, Western blotting results of CysC and MBLC in the serum of rats (n=7 in control and HBO-U groups and n=8 in HBO-P group). **B**, Quantification of CysC and MBLC using ELISA. **P*<0.05 and ***P*<0.01 vs control group; #*P*<0.05, ##*P*<0.01 vs HBO-P group. **C**, Correlation analysis for serum CysC and MBLC levels with infarct volume ratio from rats in all 3 groups. Correlation coefficient for CysC, =–0.66; 95% CI (–0.85 to –0.32); *P*<0.01; and correlation coefficient for MBLC, 0.74; 95% CI, 0.46 to 0.89; *P*<0.01. CI indicates confidence interval; HBO-P, hyperbaric oxygen protected; and HBO-U, hyperbaric oxygen unprotected.

CysC siRNA was used to knock down the expression of CysC in the brain of rats. The silencing efficacy of CysC siRNA was confirmed by Western blot at 3 days after injection (Figure IIA and IIB in the online-only Data Supplement). Knockdown of CysC abolished HBO-induced reduction of cerebral damage after MCAO, whereas the control scramble siRNA had no such effect (Figure IIC in the online-only Data Supplement).

To corroborate the critical role of endogenous CysC in mediating neuroprotection, CysC knockout rats were generated. Within the coding DNA sequence of CysC, base pairs between 95 and 99 were systematically deleted (Figure 4C). Deletion of CysC in the brain of knockout rats was confirmed by Western blot (Figure 4C). CysC knockout rats subjected to MCAO exhibited no neurobehavioral improvement and infarction volume reduction after HBO preconditioning (Figure 4D). These results indicated that CysC was a critical endogenous determinant for the neuroprotection induced by HBO preconditioning.

Exogenous CysC Significantly Reduced Cerebral Ischemic Injury

This study also explored whether exogenous CysC was sufficient to induce protection against cerebral ischemic injury. Exogenous CysC (10, 20, and 40 μg/kg) was administered intracerebroventricularly at 30 minutes after reperfusion, of which 20 and 40 μg/kg CysC significantly reduced neurological deficit and infarct volume ratio evaluated at 3 days after reperfusion (Figure III in the online-only Data Supplement).

Upregulation of CysC-Mediated Neuroprotection Through Maintaining Lysosomal Membrane Integrity

CysC is an endogenous protease inhibitor of cathepsins B, H, K, L, and S, which are mainly distributed in the intact lysosome.[13,14] Under pathological conditions, such as stroke,

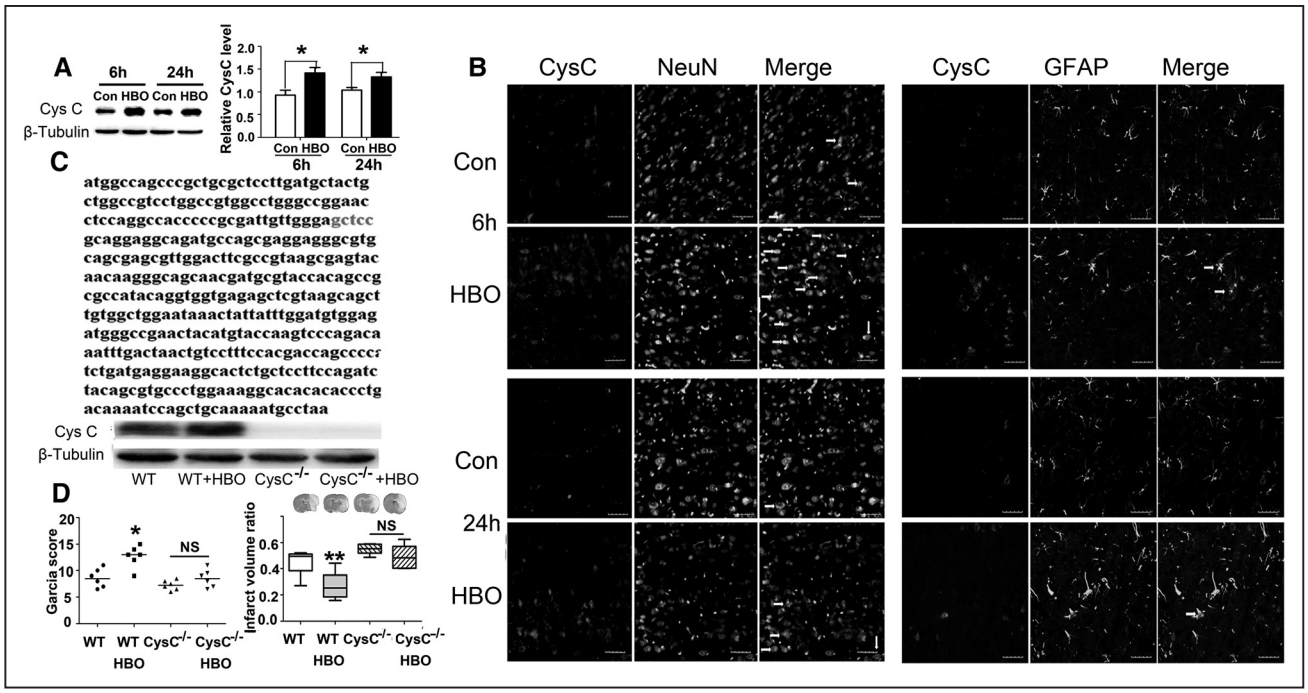

Figure 4. Cystatin C (CysC) increased in the brain after hyperbaric oxygen (HBO) preconditioning and mediated neuroprotection induced by HBO preconditioning. **A**, CysC expression in the cerebral cortex of rats was upregulated after HBO preconditioning (n=6 in each group). **P*<0.05. **B**, Elevation of CysC is found in both neurons (NeuN) and astrocytes (glial fibrillary acidic protein [GFAP]) of the cerebral cortex in HBO-preconditioned animals (n=3 in each group). **C**, Deleted base pairs (red) between 95 and 99 in the DNA coding sequence for CysC and CysC protein expression in CysC$^{-/-}$ rats (n=3 in each group). **D**, Neurological outcomes and infarct volume ratio of rats at 3 d after middle cerebral artery occlusion (MCAO) in wild-type (WT) and CysC knockout rats (n=6 in each group). **P*<0.05 and ***P*<0.01 vs WT.

leakage of cathepsins from lysosomes damages organelles, including the lysosome itself,[13,14] which causes more release of proteases and in turn exacerbates the injury. Therefore, minimizing the damage of lysosomes can be crucial mechanism underlying CysC-induced neural tissue preservation during ischemic reperfusion injury.

Immunofluorescence staining revealed that cathepsin B was diffusely distributed throughout the cytoplasm and nucleus in neurons at 24 hours after reperfusion in MCAO rats, whereas HBO preconditioning alleviated cathepsin B diffusion. Knockdown of CysC with siRNA reversed HBO preconditioning–induced preservation in lysosomal membrane integrity (Figure 5A). To further corroborate the critical role of endogenous CysC induced by HBO preconditioning in protecting lysosomes against ischemic injury, immunoelectron microscopy was used to evaluate subcellular localization of cathepsin B in wild-type and CysC knockout rats. Under normal conditions, cathepsin B is mainly localized within the lysosomes where immunogold-silver particles are found in clusters within a single-membrane structure (Figure 5B, arrow heads). In the neurons of ischemic penumbra in wild-type rats without HBO preconditioning, other than within the lysosomes, immunogold-silver staining of cathepsin B was also found diffusely localized in both cytosol and nuclei, an indication of lysosomal disruption. HBO preconditioning significantly attenuated the damage of lysosomes caused by ischemic injury in wild-type rats, as demonstrated by fewer immunogold-silver particles in the cytosol and nuclei. In CysC knockout rats, cathepsin B also leaked from the lysosomes, although HBO preconditioning could not inhibit this diffusion in CysC knockout rats. Taken together, these results suggested that leakage of the enzyme from the lysosomes after ischemia–reperfusion injury was effectively alleviated by HBO preconditioning through upregulation of CysC.

Discussion

The brain has a remarkable ability to repair itself. Under circumstances of injury, intrinsic prosurvival pathways are activated. Several strategies were known to preactivate or strengthen this endogenous protection, such as HBO preconditioning, which has been widely reported to be a prominent strategy against cerebral ischemia.[5–7] However, how HBO preconditioning induces neuroprotection is far from clear. Investigating how the brain triggers and implements endogenous neuroprotection may facilitate HBO preconditioning into clinical practice. In this study, a proteomic screening for molecules that specifically changed in animals that acquired benefit from HBO preconditioning provided new insights into discovering which molecules were pivotal. The results showed that elevated CysC was a crucial determinant for HBO-induced neuroprotection.

CysC is a 13.3 kDa secreted cysteine protease inhibitor[15] that exists broadly in mammalian body fluids and tissues.[16] Notably, CysC is highly abundant in the central nervous system.[17] The concentration of CysC in the cerebrospinal fluid is >5× higher than that in blood, highlighting the critical role of CysC in the brain.[18] Recent studies also demonstrated that CysC could prevent oxidative injury.[19] In contrast, a higher level of serum CysC is considered as a predictor of higher

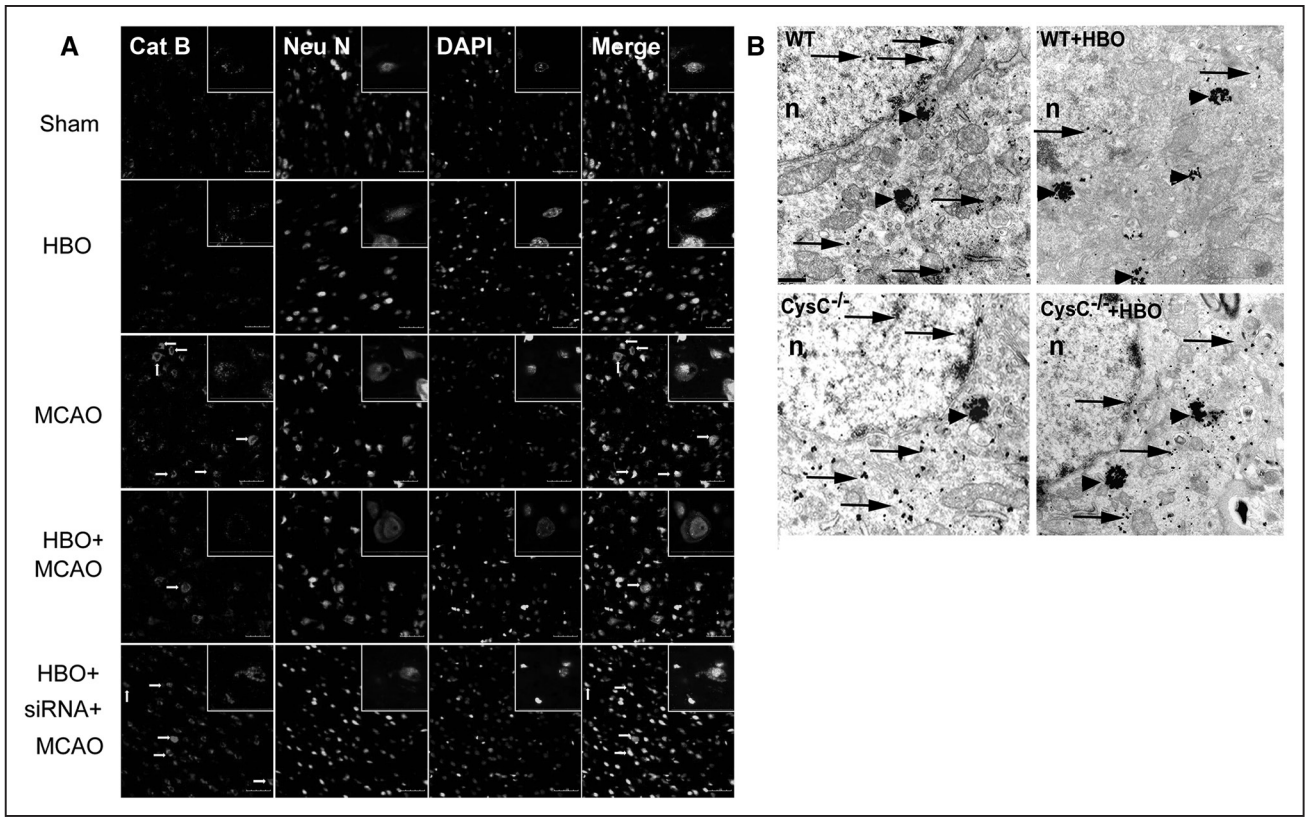

Figure 5. Upregulation of cystatin C (CysC) induced by hyperbaric oxygen (HBO) preconditioning–mediated neuroprotection through maintaining lysosomal membrane integrity. **A**, Immunofluorescent staining of cathepsin B (Cat B; red) with NeuN (green) in the ischemic penumbra at 24 h after middle cerebral artery occlusion (MCAO). Diffused cytoplasmic and nuclear cathepsin B immunoreactivity indicates lysosomal leakage. HBO preconditioning reduced this leakage in neurons. CysC siRNA reversed the reduction of cathepsin B diffusion induced by HBO. Scale bar=50 μm. **B**, Representative immune-electron micrographs of neurons from the ischemic penumbra of rats at 24 h after MCAO. Arrowheads indicate intact lysosomes where immunogold-silver particles (cathepsin B) are in clusters within a single-membrane structure. Diffusion of cytoplasmic and nuclear cathepsin B (arrows) indicates lysosomal leakage. HBO preconditioning reduced cathepsin B leakage in wild-type rats, but not in CysC knockout rats. Scale bar=0.5 μm. n=3 in each group. n in the immune-electron micrographs indicates Nucleus.

risk for stroke, as well as poorer outcome.[20] To reconcile these views, gene manipulation techniques, including siRNA and construction of CysC knockout rats, were used for further investigation.

With CysC knocked down or knocked out, neuroprotection induced by HBO preconditioning was abolished, whereas in vivo application of exogenous CysC significantly reduced ischemic neuronal damage. It was previously reported that CysC was neuroprotective in an in vitro model of oxidative stress,[21] which was in accordance with our findings. These results demonstrated that CysC was a key determinant for HBO preconditioning–induced protection against stroke. Interestingly, unlike a previous report that showed CysC knockout exacerbated neurological damage induced by ischemia in mice,[19] a tendency, but not significant deterioration (P=0.35), was observed in CysC knockout rats compared with wild-type rats in this study. Given that different species (mice versus rats) were used, it was possible that gene deletion would result in different compensatory response during development that could affect the outcome of neurological injury. Another consideration was that the previous study used 40 minutes of MCAO, whereas this study used 120 minutes of MCAO, which could cause a rather intense brain damage where a significant increase in the infarct in CysC$^{-/-}$ rats might be covered by the ceiling effect.

It is well known that lysosomes contain many proteases, nucleases, esterases, polysaccharidases, and glycosidases.[22] These enzymes are involved in the breakdown and degradation of cell proteins and recycling of amino acids, which are pivotal in maintaining cellular homeostasis. However, oxidative stress can trigger lysosomal rupture or permeability changes, which induce leakage of hydrolases into the cytosol.[23] Among the hydrolases, cathepsins B, L, and D are abundant in neurons, and cell death procedures, including apoptosis and necrosis, are preceded by cytoplasmic release of lysosomal cathepsins B and D.[24] Moreover, cytosolic cathepsin B can damage the lysosomes, forming a vicious circle resulting in further leakage of hydrolases into the cytosol and more intensive cell damage.[13] This study proved for the first time that endogenous CysC is pivotal in mediating HBO preconditioning–induced improvement in lysosomal membrane integrity after cerebral ischemia. This protective effect of HBO preconditioning was absent in rats that received CysC siRNA injection. Immunoelectron micrographs also showed that in CysC$^{-/-}$ rats that underwent MCAO, HBO preconditioning failed to reduce cathepsin B leakage from lysosomes into cytosol and nucleus.

However, why HBO could induce differential expression of CysC needs further exploration. For each individual, the response to HBO stimulation may be different because of various genetic, epigenetic, or signal transduction changes. A clinical study showed that CysC gene polymorphism, a G-to-A transition in exon 1, could result in reduced CysC expression and higher risk of Alzheimer's disease.[25] Besides, a CpG island sits in the promoter region of CysC gene, suggesting that CysC could potentially be silenced by DNA hypermethylation.[26]

In summary, for the first time, this study characterized CysC and MBLC as key molecules for HBO preconditioning to successfully induce ischemic tolerance. Furthermore, it was proved that CysC was a pivotal endogenous determinant for the neuroprotective effect of HBO preconditioning by preserving lysosomal integrity. Although further validation and evaluation in clinical settings are needed, the present findings showed that CysC was an endogenous neuroprotectant that might be of great value for stroke treatment.

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Disclosures

None.

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