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Original Research

Lactylation of PLBD1 Facilitates Brain Injury Induced by Ischemic Stroke

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Abstract

Background: Ischemic stroke is a prevalent global condition and its associated brain damage poses a significant threat to patient survival and outcomes. The underlying mechanisms of ischemic stroke-induced brain injury remain elusive, necessitating further investigation. **Methods**: Ischemic stroke models were established using middle cerebral artery occlusion (MCAO) in animals and oxygen-glucose deprivation and reperfusion (OGD-R) in cells. Phospholipase B domain-containing protein 1 (PLBD1) expression in these models was assessed *via* western blotting analysis, reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR), and cell immunofluorescence. A comprehensive evaluation, incorporating cellular lactate dehydrogenase (LDH) release assays, glycolysis metabolism kits, RT-qPCR, western blotting, triphenyl tetrazolium chloride (TTC) staining, neurological scoring, brain tissue water content measurement, and creatine kinase-MB (CK-MB) analysis, was conducted to determine the impact of PLBD1 on brain injury. Potential lactylation sites in PLBD1 were predicted using the DeepKla database, with western blotting and co-immunoprecipitation (Co-IP) confirming the lactylation site. **Results**: PLBD1 was significantly upregulated in the brain tissue of MCAO animal models and OGD-R-treated cells. PLBD1 knockdown markedly mitigated OGD-R-induced cellular injury, suppressed glycolysis *in vitro*, and reversed MCAO-induced brain damage *in vivo*. Furthermore, lactylation at the K155 site of PLBD1 enhanced its expression in response to elevated lactate levels following OGD-R treatment. These results indicated that the upregulation of PLBD1 *via* K155 site lactylation plays a pivotal role in exacerbating ischemic stroke-induced brain damage. **Conclusion**: Targeting the lactate/PLBD1 axis presents a promising therapeutic strategy for ischemic stroke management.

Keywords: ischemic stroke; brain injury; PLBD1; lactylation

1. Introduction

Acute stroke, characterized by high morbidity and mortality, poses a significant global threat to human health. Hemorrhagic and ischemic strokes are the two main forms of stroke [1]. While ischemic stroke is brought on by an obstruction in the blood flow to the brain, which results in limited oxygen and nutrient delivery and ultimately irreparable brain damage, hemorrhagic stroke is brought on by the bursting of cerebral blood vessels [2]. Notably, the majority of stroke cases (around 80%) are ischemic strokes, which are more prevalent than hemorrhagic stroke [3]. The current therapeutic approach for ischemic stroke focuses on restoring cerebral blood flow through pharmacological or mechanical interventions [4]. However, increasing evidence indicates that reperfusion (I/R) often results in severe secondary brain injury, manifesting as inflammation, cerebral edema, expanded infarct volume, and intracranial hemorrhage [5]. Consequently, further investigation into the underlying mechanisms driving ischemic brain injury is crucial to developing effective therapeutic strategies that can enhance patient outcomes and improve prognosis.

With advances in science and technology, gene expression analysis has expanded beyond tumor classification, prognosis, and treatment response prediction. The development of ischemic stroke animal models has circumvented the need for brain tissue biopsies and surgical samples from patients with stroke, enabling direct analysis of gene expression in affected brain tissues. Research indicates that ischemic stroke leads to a wide range of differential gene expressions in both brain tissue and peripheral blood mononuclear cells, offering potential therapeutic and prognostic targets for this condition [6,7]. Phospholipase B domain-containing protein 1 (PLBD1) is a key regulator involved in phospholipid metabolism. Recent findings have demonstrated that PLBD1 is highly expressed in various cardiovascular and cerebrovascular conditions, as well as immune-mediated skin disorders, including acute myocardial infarction, ischemic stroke, aneurysmal subarachnoid hemorrhage, and psoriasis [8]. These studies suggest that PLBD1 holds significant value for the diagnosis and prognosis of such diseases. For instance, in acute myocardial infarction, elevated PLBD1 levels in peripheral blood are closely associated with cardiac injury and inflammation, serving as an independent predictor of left ventricular dysfunction [9]. Similarly, in psoriasis, high PLBD1 expression in affected skin correlates with the development and exacerbation of the inflammatory response [8]. Existing literature also highlights a marked upregulation of

PLBD1 in glioma tumor cells and macrophages, where it is significantly associated with advanced pathological stages and decreased survival rates. Knocking down PLBD1 significantly weakened the proliferation and invasion abilities of glioma cells, indicating that it could serve as a targeted marker for prognosis and treatment in glioma. Furthermore, biogenic analysis indicates that PLBD1 modulates various immune checkpoints and immune cell infiltration, thus promoting glioma progression and influencing the tumor immune microenvironment, which contributes to poorer immunotherapy outcomes [10]. Moreover, PLBD1 levels are confirmed to be heightened in circulating white blood cells in patients with ischemic stroke, suggesting its potential as a diagnostic marker for this condition [11]. However, there has been no research conducted on the specific regulatory role of abnormally high expression of PLBD1 in ischemic stroke-induced brain injury. Therefore, this study aims to investigate the effects of knocking down PLBD1 both *in vitro* and *in vivo* on brain damage caused by ischemic stroke, with the goal of revealing its potential applications in the treatment of ischemic stroke.

The regulation of essential cellular processes, including gene expression, protein activity, stability, and molecular recognition, is largely dependent on protein post-translational modifications (PTMs). PTMs involve chemical modifications that occur after protein synthesis, altering the stability, activity, and subcellular localization of proteins, thus playing a pivotal role in maintaining protein structure and function under both physiological and pathological conditions. They are fundamental mechanisms for modulating protein function [12].

Recent research has identified lysine lactylation (Kla) as a novel PTM. This modification involves lactic acid-mediated lactylation of histones or proteins, which regulates gene transcription and participates in diverse pathophysiological processes [13]. For example, in anaplastic thyroid carcinoma (ATC), accumulated lactic acid promotes malignant progression by enhancing histone H4 Lysine 12 (H4K12) lactylation, thereby activating genes linked to ATC proliferation [14]. Similarly, hypoxia-induced lactate production in the retina drives lactylation of Yin Yang 1 (YY1) in microglia, upregulating fibroblast growth factor 2 (FGF2) expression and leading to blindness [15]. In ischemic stroke, the hypoxic-ischemic environment triggers anaerobic glycolysis, resulting in lactate accumulation and increased Kla. Nevertheless, little is known about how lactylation contributes to ischemic stroke-induced brain damage, and it remains unclear whether lactate accumulation directly influences PLBD1 expression *via* lactylation.

The objectives of this work were to clarify the regulatory role of PLBD1 on brain injury following ischemic stroke and to explore the mechanisms behind its abnormal expression. Findings demonstrated that PLBD1 was significantly upregulated in ischemic stroke animal and cell models. Moreover, PLBD1 was identified as a lactylation substrate, with lactylation occurring at the K155 site in response to elevated lactate levels in ischemic stroke cell models. Evidence further indicated that lactylation of PLBD1 at this site exacerbated ischemic stroke-induced brain injury by increasing PLBD1 protein expression.

2. Materials and Methods

2.1 Cell

Obtained through the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (SCSP-5208, Shanghai, China), the murine microglial BV2 cells were validated by Short Tandem Repeat (STR) Profiling analysis and confirmed free of mycoplasma contamination. High-glucose Dulbecco's Modified Eagle Medium (DMEM) (11965092, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, A5670701, Gibco, Grand Island, NY, USA) and 1% streptomycin (ST487-50g, Beyotime, Shanghai, China) used as the cell culture medium. Cells were maintained at 37 °C in a 5% CO_2 incubator. The oxygen-glucose deprivation and reperfusion (OGD-R) model was established by replacing the complete medium with glucose-free DMEM, followed by incubation in a 95% N_2 and 5% CO_2 atmosphere for 4 hours. Cells were subsequently grown for 4 more hours under standard conditions after the medium was changed back to the complete culture medium. According to the manufacturer's instructions, BV2 cells were treated with glycolysis inhibitors 2-Deoxy-D-glucose (2-DG) (25 mM, HY-13966, MedChemExpress, Shanghai, China) and lactate (15 mM, HY-B2227, MedChemExpress, Shanghai, China) for 24 hours.

2.2 Cell Transfection

Plasmids for PLBD1 wild type (WT), K155R and K338R mutants, as well as the PLBD1 knockdown plasmid (shPLBD1) and its negative control (shNC), were offered by GENE Biotechnology (Shanghai, China). The shPLBD1 and shNC plasmids were cloned into lentiviral vectors, which were then used to infect BV2 cells. Stable transfectants were selected using 5 µg/mL puromycin (540411, Sigma-Aldrich, St. Louis, MO, USA) over 2 weeks. Additionally, PLBD1 WT, K155R, and K338R plasmids were transfected using Lipofectamine 3000 (L3000150, Invitrogen, Carlsbad, CA, USA), and cells were harvested 24 hours post-transfection for subsequent analyses.

2.3 Western Blotting

The bicinchoninic acid (BCA) method was adopted to quantify the total protein from BV2 cells. 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate equal amounts of protein, which were then electrophoresed onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hour at 37 °C in non-fat milk, and then they underwent incubation with primary antibodies for the entire

night at 4 °C. The next day, the membranes were then treated with horseradish peroxidase-conjugated IgG secondary antibody (ab7621, Abcam, Eugene, OR, USA) for 2 hours at 37 °C. Protein bands were visualized using chemiluminescence. Primary antibodies included anti-PLBD1 (PA5-68857, Invitrogen, Carlsbad, CA, USA), anti-pan-Kla (PTM-1401RM, PTM Biolabs, Hangzhou, Zhejiang, China), anti-NLRP3 (MA5-32255, Invitrogen, Carlsbad, CA, USA), anti-ASC (ab283684, Abcam, Eugene, OR, USA), anti-β-actin (ab8226, Abcam, Eugene, OR, USA), anti-GSDMD-N (ab255983, Abcam, Eugene, OR, USA), and anti-IL-1β (ab315084, Abcam, Eugene, OR, USA), anti-cleaved caspase-1 (PA5-99390, Invitrogen, Carlsbad, CA, USA).

2.4 Lactate Dehydrogenase (LDH) Release

To measure Lactate Dehydrogenase (LDH) release, a sign of cell injury, the LDH Cytotoxicity Colorimetric Assay Kit (ab65393, Abcam, Eugene, OR, USA) was utilized. In brief, 10 µL of the supernatant from BV2 cell culture, obtained by centrifugation, was added to a 96-well plate. Each well was then filled with 100 µL of LDH Reaction Mix. After 30 minutes of incubation at 37 °C, absorbance at 490 nm was measured with a microplate reader.

2.5 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from BV2 cells and brain tissue using Trizol reagent (15596026CN, Invitrogen, Carlsbad, CA, USA), followed by quantification and quality control. Subsequently, 1–2 µg of total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (RR036A, TaKaRa, Kyoto, Japan), and polymerase chain reaction (PCR) amplification was performed. The relative mRNA expression levels of *PLBD1* were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the internal control using the $2^{-\Delta\Delta CT}$ method.

2.6 Co-Immunoprecipitation (Co-IP)

Cells were lysed using immunoprecipitation (IP) lysis buffer (P2179M, Beyotime, Shanghai, China). Protein samples were obtained by centrifuging the lysates for 10 minutes (12,000 rpm, 4 °C). Following that, these samples were gently rotated while incubated with the anti-PLBD1 primary antibody (PA5-68857, Invitrogen, Carlsbad, CA, USA) for an entire night at 4 °C. After that, 40 µL of Protein A/G beads were added, and the mixture was incubated at 4 °C for the entire night. After five rounds of washing with cooled IP lysis solution, the eluted proteins were gathered for further western blotting examination.

2.7 Glucose Metabolism

Following the manufacturer's instructions, BV2 cells' glucose uptake and lactate production were measured using a glucose uptake colorimetric assay kit (ab136955, Abcam, Eugene, OR, USA) and a lactate colorimetric assay kit (MAK570, Sigma-Aldrich, St. Louis, MO, USA), respectively.

2.8 Extracellular Acidification Rate (ECAR) Assay

For Extracellular Acidification Rate (ECAR) measurement, 1×10^4 BV2 cells were seeded into each well of a Seahorse XF cell culture microplate. Glucose (49163, Sigma-Aldrich, St. Louis, MO, USA), oligomycin A (495455, Sigma-Aldrich, St. Louis, MO, USA), and 2-DG (HY-13966, MedChemExpress, Shanghai, China) were sequentially added at 20, 50, and 90 minutes, respectively. ECAR was then measured using a Seahorse XFe 96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA).

2.9 Cell Immunofluorescence

For immunofluorescence analysis, BV2 cells were grown in confocal culture dishes until 80% confluence. Cells were incubated with primary antibodies against PLBD1 (PA5-68857, Invitrogen, Carlsbad, CA, USA) and ionized calcium-binding adapter molecule 1 (Iba-1, a microglia marker) (019-19741, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 1 hour. The cells were left to incubate with IgG fluorescent secondary antibodies (ab150077, Abcam, Eugene, OR, USA) for two hours after being rinsed three times with PBS. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (ab104139, Abcam, Eugene, OR, USA) for 5 minutes, and fluorescence microscopy was performed using a Leica microscope (DMi8, Leica Microsystems, Wetzlar, Hesse, Germany).

2.10 Animal

All animal experiments adhered to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and received approval from the Ethics Committee of Renmin Hospital, Hubei University of Medicine (NO. syrmyy2022-065). The study used 24 male adult Sprague Dawley (SD) rats (2–3 months old, 250–300 g), which were randomly assigned to four groups (n = 6 per group): (1) sham group; (2) middle cerebral artery occlusion (MCAO) group; (3) MCAO + shNC group; and (4) MCAO + shPLBD1 group. Rats in the MCAO + shNC and MCAO + shPLBD1 groups received injections of shNC and shPLBD1, respectively, into the cortical and hippocampal regions. Two weeks post-injection, rats underwent MCAO to induce the ischemic stroke model. The surgical procedure involved anesthetizing the rats intraperitoneally with sodium pentobarbital (40 mg/kg, 11715, Sigma-Aldrich, St. Louis, MO, USA), followed by exposing the carotid arteries. The wound was then closed after a surgical suture was placed into the lumen of the right common carotid artery and advanced until resistance was encountered. Reperfusion was achieved by removing the sutures one hour later. The identical process was performed on the sham group,

but no sutures were inserted. One week after surgery, the rats were euthanized by an intraperitoneal overdose of sodium pentobarbital (150 mg/kg, 11715, Sigma-Aldrich, St. Louis, MO, USA), and brain tissues were taken for additional examination.

2.11 Neurological Assessment

Neurological function was evaluated using Bederson's scoring method [16], based on forelimb behavior when rats were suspended by their tails. Higher scores indicated more severe neurological deficits. The scoring criteria were: 0 points for free movement of the forelimbs, 1 point for difficulty moving the left forelimb, 2 points for reduced lateral thrust resistance, 3 points for unilateral rotation during free walking, and 4 points for the inability to walk independently.

2.12 2,3,5-Triphenyl Tetrazolium Chloride (TTC) Staining

Cerebral infarct areas were assessed using 2,3,5-Triphenyl Tetrazolium Chloride (TTC) staining. Brain tissues (2 µm) were stained with 2% TTC (G3005, Solarbio, Beijing, China) for 30 minutes in the dark, followed by overnight fixation in 10% formalin solution (G2161, Solarbio, Beijing, China). Infarcted areas appeared milky white. ImageJ software (1.54, National Institutes of Health, Bethesda, MD, USA) was used to measure the areas of the left hemisphere and the infarcted right hemisphere. The following formula was used to get the infarction rate (%): (left hemisphere area – infarct area) / left hemisphere area $\times$ 100%.

2.13 Determination of Brain Tissue Water Content

Cerebral edema in the rats was evaluated by measuring brain tissue water content. After TTC staining, brain tissue sections were immediately weighed to determine the wet weight. After being dried for 24 hours at 110 °C in an oven, the sections were weighed once more to determine their dry weight. The following formula was used to get the water content (%): (1 – dry weight / wet weight) $\times$ 100%.

2.14 Biochemical Analysis

On the seventh day post-surgery, apical blood sampling was conducted to collect blood samples. To extract the serum, these samples were centrifuged at 1000 g for 15 minutes at 4 °C. A chemical analyzer (Roche Cobas c 501, Roche Diagnostics, Rotkreuz, Zug, Switzerland) was used to detect the levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in the serum.

2.15 Statistical Analysis

GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) was implemented for statistical analysis. The mean $\pm$ SD was employed to express the data. Analysis of variance (ANOVA) or Student's *t*-test was utilized for group comparisons, with a significance level of $p < 0.05$.

3. Results

3.1 PLBD1 is Up-Regulated in Ischemic Stroke

Following ischemic stroke, white blood cells rapidly infiltrate the ischemic site, exacerbating brain injury through the release of neurotoxic factors [17]. Previous study has reported a significant elevation of PLBD1 in circulating leukocytes of patients with ischemic stroke [11]. To further investigate PLBD1 expression patterns in ischemic stroke, this study established an OGD-R cell model and an MCAO animal model to mimic the onset and progression of the condition. The findings demonstrated that, in comparison to the sham group, PLBD1 expression was markedly elevated in the brain tissues of rats exposed to MCAO (*T*-test: $p < 0.0001$, the data met the assumptions of normality and equal variance) (Fig. 1A,B). For original western blotting figures in Fig. 1B see **Supplementary Material**. Likewise, under OGD-R circumstances, BV2 microglial cells showed elevated PLBD1 levels (*T*-test: $p = 0.0004$, the data met the assumptions of normality and equal variance.) (Fig. 1C–E). For original western blotting figures in Fig. 1E see **Supplementary Material**. These results indicated that PLBD1 was markedly upregulated in ischemic stroke models.

3.2 PLBD1 Knockdown Inhibits OGD-R-Induced Cell Injury, Pyroptosis, and Inflammatory Responses

To elucidate the function of PLBD1, sh-PLBD1 transfection into BV2 cells was performed to down-regulate PLBD1 expression (*T*-test: $p < 0.0001$, the data met the assumptions of normality and equal variance.) (Fig. 2A). OGD-R exposure substantially elevated LDH release, indicating cellular damage, while PLBD1 knockdown markedly attenuated this increase (One-way ANOVA: Control vs. OGD-R, $p < 0.0001$; OGD-R + shNC vs. OGD-R + shPLBD1, $p < 0.0001$, the data met the assumptions of normality and equal variance.) (Fig. 2B). Molecular assays revealed that OGD-R consistently up-regulated the inflammatory cytokines interleukin (IL)-1β (One-way ANOVA: Control vs. OGD-R, $p < 0.0001$, the data met the assumptions of normality and equal variance.) and IL-18 (One-way ANOVA: Control vs. OGD-R, $p < 0.0001$, the data met the assumptions of normality and equal variance.), along with pyroptosis-related proteins GSDMD-N and ASC. However, PLBD1 knockdown significantly reversed the increased levels of IL-1β (One-way ANOVA: OGD-R + shNC vs. OGD-R + shPLBD1, $p < 0.0001$, the data met the assumptions of normality and equal variance.), IL-18 (One-way ANOVA: OGD-R + shNC vs. OGD-R + shPLBD1, $p = 0.0002$, the data met the assumptions of normality and equal variance.), as well as the pyroptosis-associated proteins Gasdermin D N-terminal domain (GSDMD-N) and Apoptosis-associated speck-like protein containing a CARD (ASC) (Fig. 2C–E). For original western blotting figures in Fig. 2E see **Supplementary Material**.

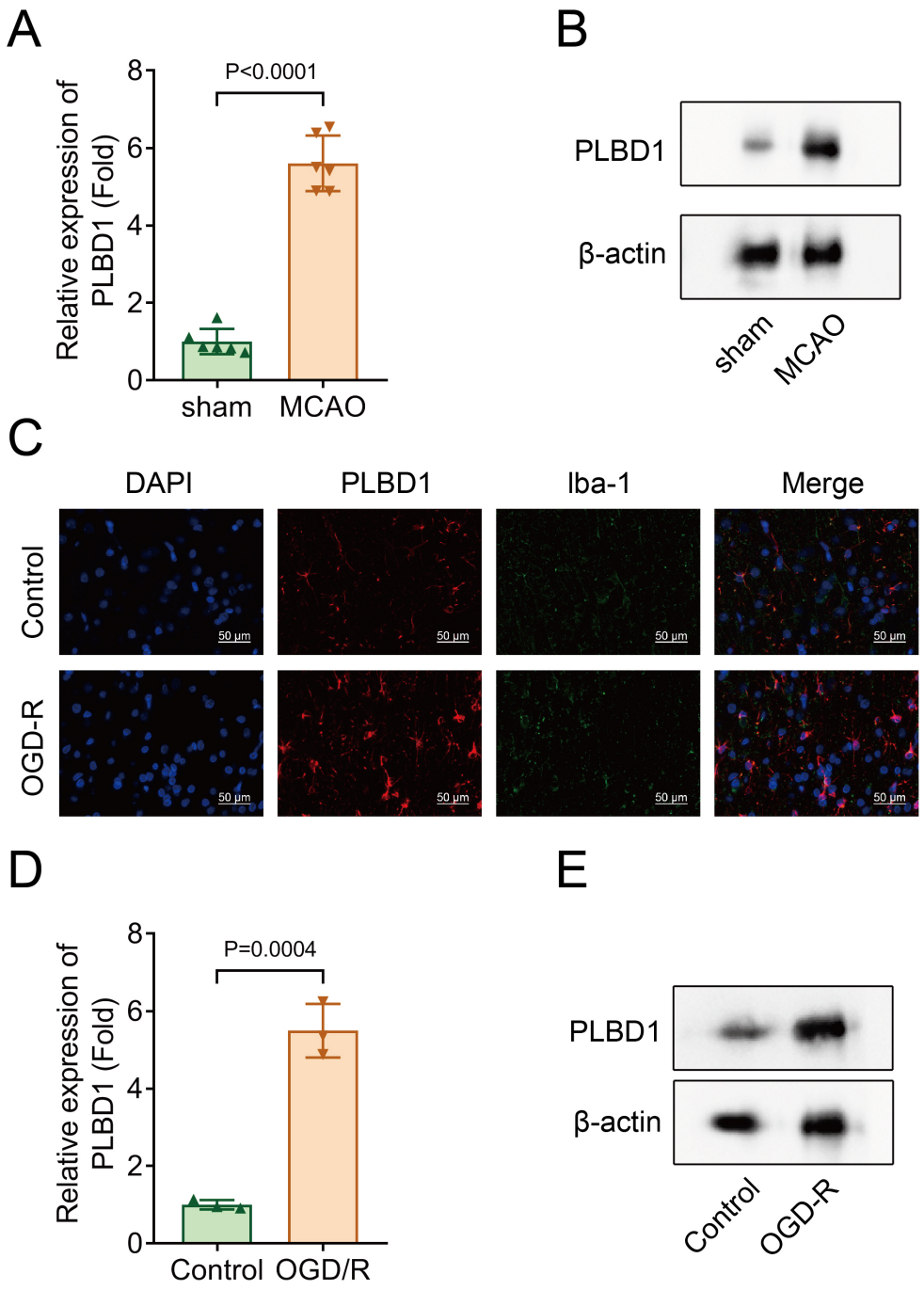

Fig. 1. Phospholipase B domain-containing protein 1 (PLBD1) is up-regulated in ischemic stroke. (A,B) Analysis of RT-qPCR (A) (n = 6) and western blotting (B) (n = 6) showed a significant upregulation in the mRNA and protein expression levels of PLBD1 in rats following middle cerebral artery occlusion (MCAO) treatment. (C) Double immunofluorescence staining for Iba-1 (green) and PLBD1 (red) revealed a obvious upregulation in the protein expression levels of PLBD1 in BV2 cells 24 hours post-OGD-R stimulation (n = 3). The scale bar=50μm. (D,E) Analysis of RT-qPCR (D) (n = 3) and western blotting (E) (n = 3) showed a significant upregulation in the mRNA and protein expression levels of PLBD1 in BV2 cells after OGD-R treatment. OGD-R, oxygen-glucose deprivation and reperfusion. Iba-1, ionized calcium-binding adapter molecule 1; RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction; PLBD1, Phospholipase B domain-containing protein 1; DAPI, 4′,6-diamidino-2-phenylindole.

Considering the pivotal role of nucleotide-binding domain (NBD), leucine-rich repeat (LRR), and pyrin domain (PYD)-containing protein 3 (NLRP3) inflammasome activation in microglial pyroptosis and the release of inflammatory mediators [18,19], the effect of PLBD1 knockdown on OGD-R-induced NLRP3 activation was examined. The

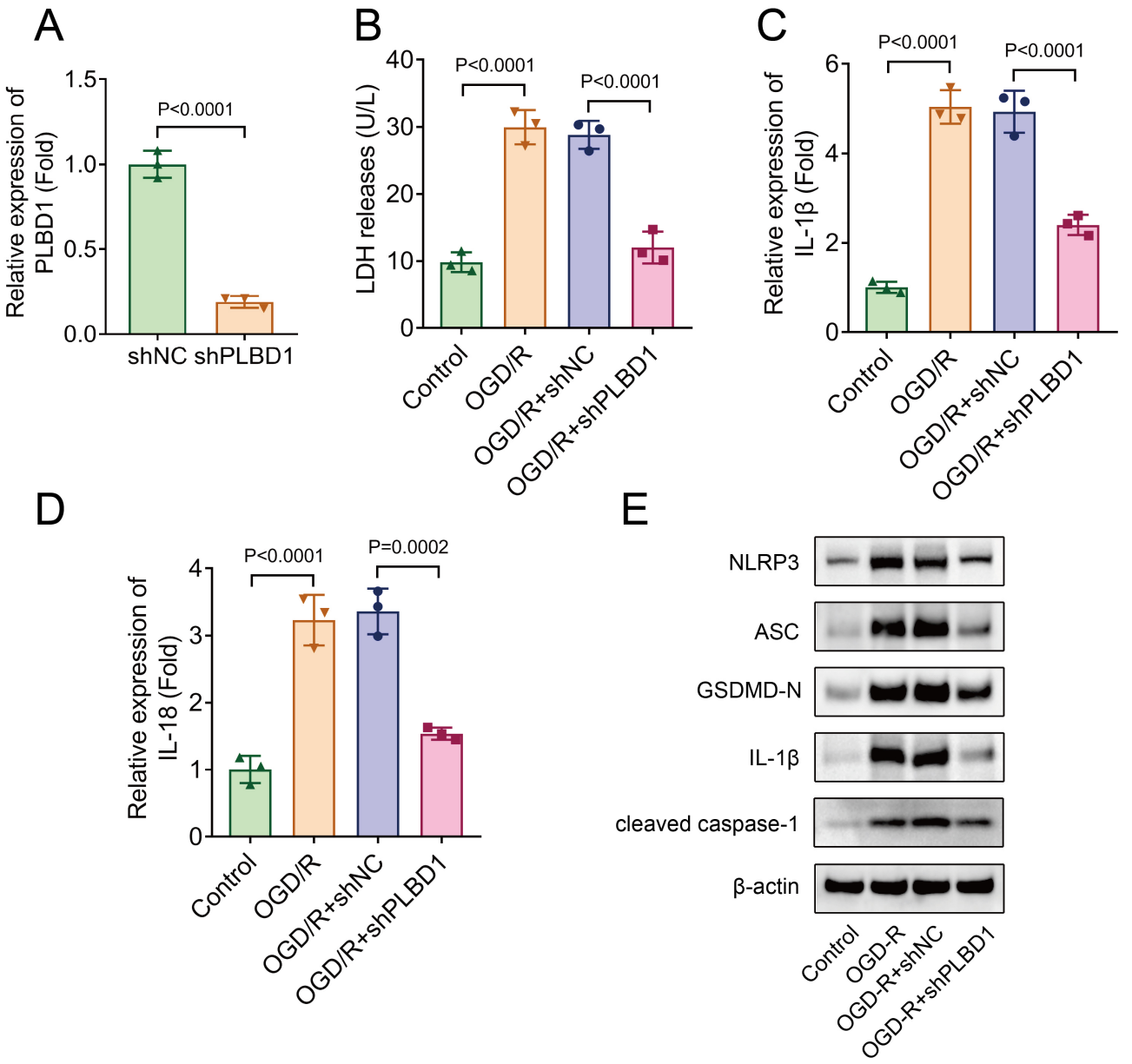

Fig. 2. PLBD1 knockdown inhibits oxygen-glucose deprivation and reperfusion (OGD-R)-induced cell injury, pyroptosis, and inflammatory responses. (A) Analysis of RT-qPCR showed a reduced *PLBD1* mRNA expression in BV2 cells transfected with shPLBD1 (n = 3). (B) PLBD1 knockdown markedly attenuated elevated lactate dehydrogenase (LDH) release caused by OGD-R treatment in the supernatants of BV2 cells (n = 3). (C,D) Analysis of RT-qPCR revealed that PLBD1 knockdown significantly reversed the increased *IL-1β* (C) (n = 3) and *IL-18* (D) (n = 3) mRNA expression in BV2 cells treated with OGD-R. (E) Analysis of western blotting indicated that PLBD1 silencing dramatically reduced the overexpression of NLRP3-associated proteins (NLRP3, cleaved caspase-1, and IL-1β) and pyroptosis-related proteins (GSDMD-N and ASC) expression in BV2 cells treated with OGD-R (n = 3). NLRP3, NOD-like receptor thermal protein domain associated protein 3; GSDMD-N, Gasdermin D N-terminal domain; shNC, PLBD1 negative control; shPLBD1, PLBD1 knockdown plasmid; ASC, apoptosis-associated speck-like protein containing a CARD; IL, interleukin.

findings demonstrated that under OGD-R circumstances, PLBD1 silencing dramatically reduced the overexpression of NLRP3-associated proteins, which includes NLRP3, cleaved caspase-1, and IL-1β (Fig. 2E), suggesting a suppression of inflammasome-driven inflammatory responses.

3.3 PLBD1 Knockdown Alleviates OGD-R-Induced Glycolysis

Analysis of glycolysis indicated that PLBD1 knockdown significantly reduced OGD-R-induced lactate production (One-way ANOVA: Control vs. OGD-R, $p < 0.0001$; OGD-R + shNC vs. OGD-R + shPLBD1, $p =$

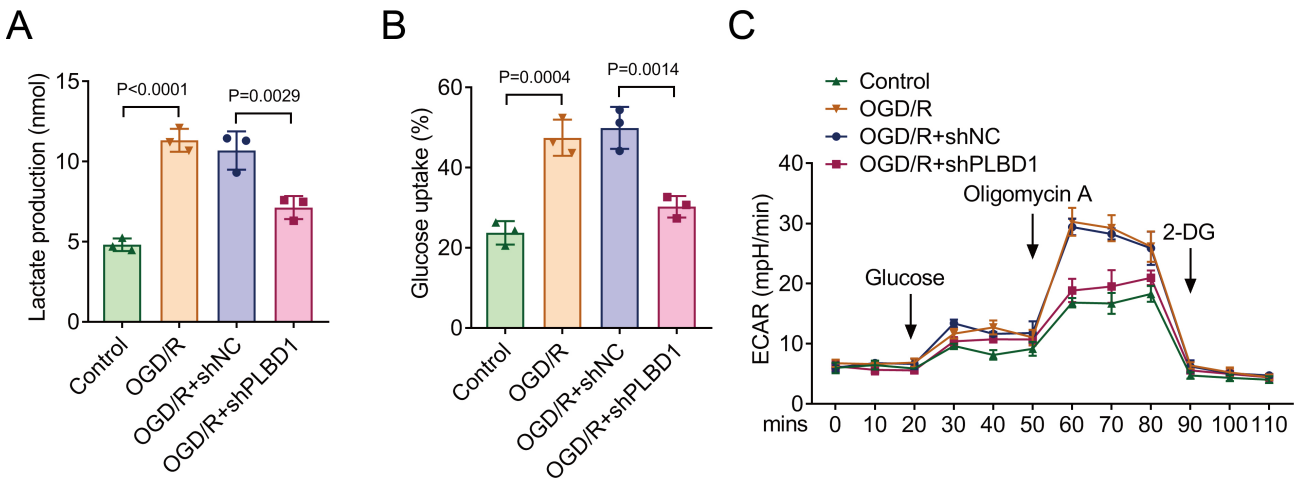

Fig. 3. PLBD1 knockdown alleviates OGD-R-induced glycolysis. (A–C) Glycolysis analysis indicated that silencing of the PLBD1 gene significantly suppressed the increase in lactate production (A) (n = 3), glucose uptake (B) (n = 3), and extracellular acidification rate (ECAR) (C) (n = 3) in BV2 cells induced by OGD-R. The arrows indicates that specific reagents are added at specific time points. 2-DG, 2-deoxy-d-glucose.

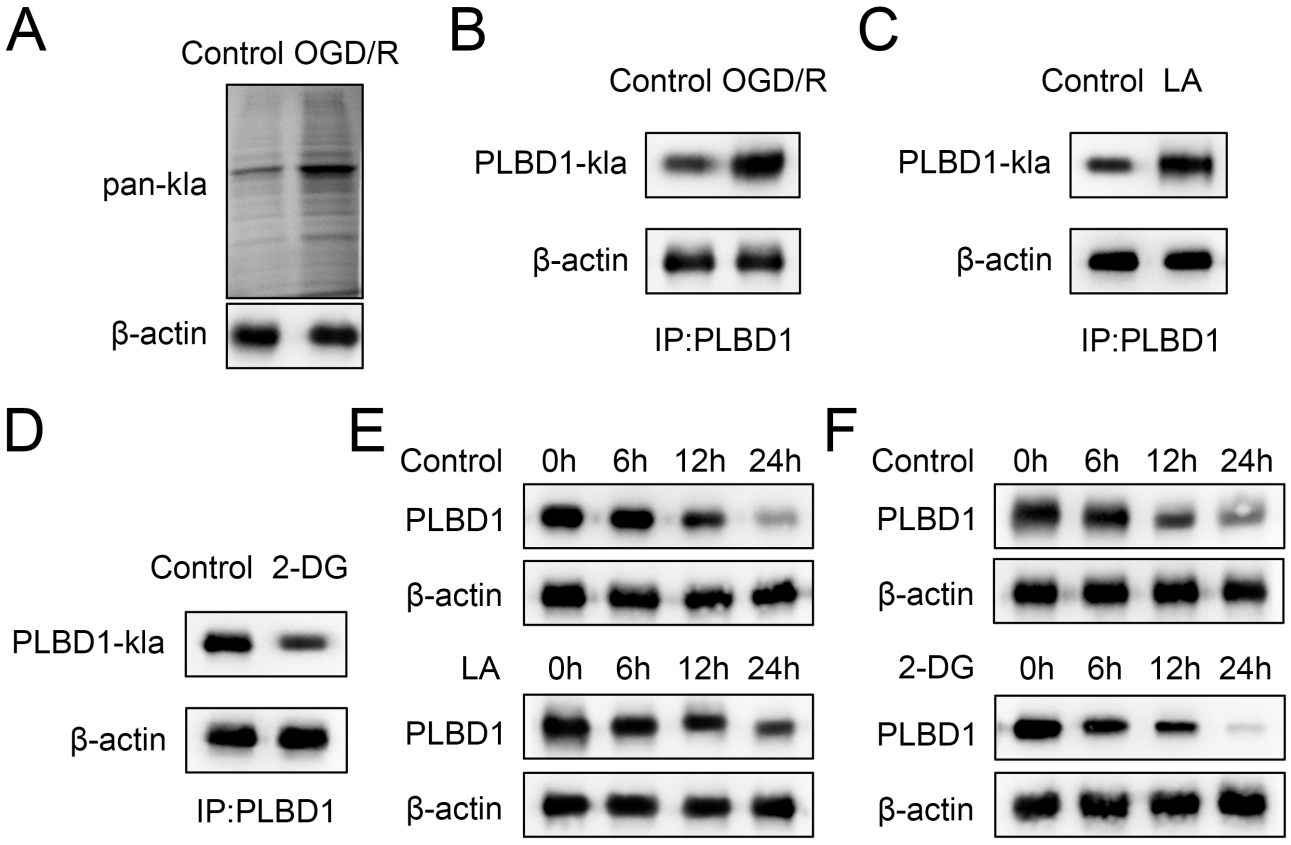

Fig. 4. OGD-R promotes PLBD1 lactylation by inducing lactate production. (A,B) Analysis of western blotting revealed increased the overall lactylation level (A) (n = 3) and the expression level of lactylated PLBD1 (B) (n = 3) in BV2 cells with OGD-R treatment. (C,D) Analysis of Co-IP assays revealed increased expression level of lactylated PLBD1 in BV2 cells treated with LA (C) (n = 3) and decreased expression level of lactylated PLBD1 in BV2 cells treated with 2-DG (D) (n = 3). (E,F) Analysis of western blotting revealed strengthened PLBD1 stability in BV2 cells after LA treatment (E) (n = 3) and weakened PLBD1 stability in BV2 cells after 2-DG treatment (F) (n = 3). IP, immunoprecipitation; LA, lactic acid.

A

ID	Sequence	Position	Residue	PTM scores
PLBD1	IKAQKDDPF	155	K	98.72%
PLBD1	AEGGKEWAQ	338	K	84.68%

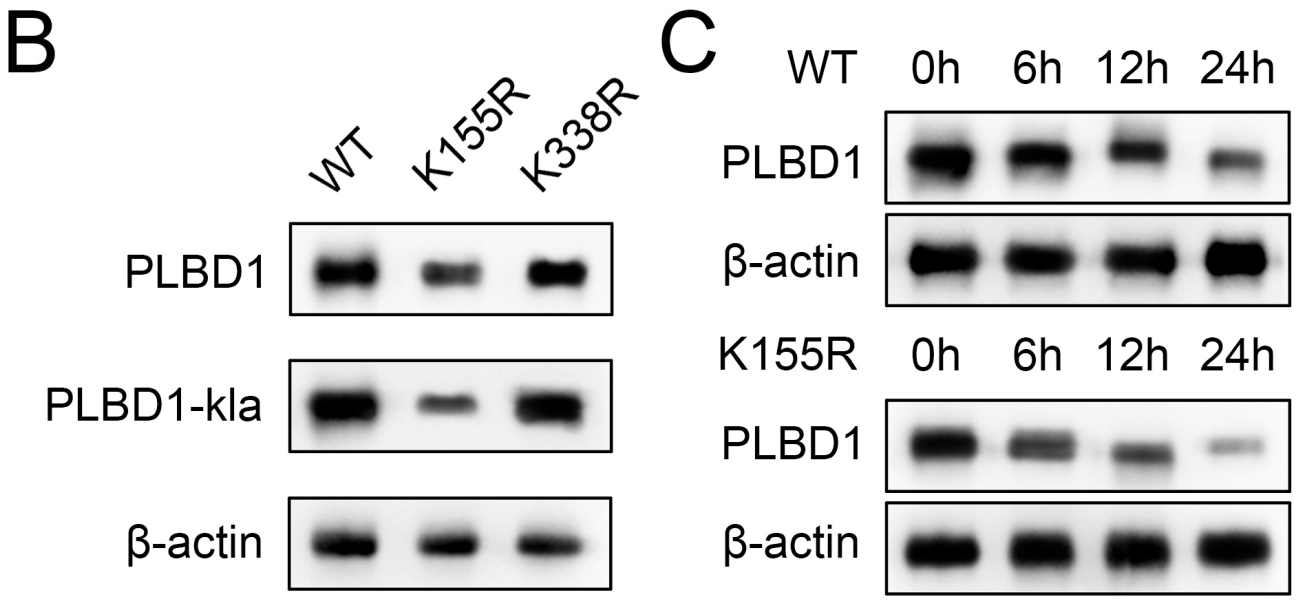

Fig. 5. K155 is the lactylation modification site of PLBD1. (A) The DeepKla database revealed two potential lactylation modification site of PLBD1 (K155 and K338). (B) Analysis of western blotting showed mutation at K155 markedly decreased both the protein level and lactylation of PLBD1 in BV2 cells (n = 3). (C) Analysis of western blotting showed a weakened PLBD1 stability in BV2 cells at 6 h, 12 h, and 24 h post-transfection with PLBD1 K155R mutant plasmids (n = 3). PTM, post-translational modification; WT, wild type.

0.0029, the data met the assumptions of normality and equal variance.), glucose uptake (One-way ANOVA: Control vs. OGD-R, $p = 0.0004$; OGD-R + shNC vs. OGD-R + sh-PLBD1, $p = 0.0014$, the data met the assumptions of normality and equal variance), and ECAR (Fig. 3A–C), thereby alleviating OGD-R-induced glycolytic activity.

3.4 OGD-R Promotes PLBD1 Lactylation by Inducing Lactate Production

Research indicates that ischemic stroke-induced oxygen deprivation favors anaerobic glycolysis, resulting in the conversion of pyruvate into protons and lactic acid, leading to substantial intracellular lactic acid accumulation [20]. Lactic acid mediates various pathophysiological processes by facilitating protein lactylation [21]. In this study, changes in lactylation levels were assessed in OGD-R-treated BV2 cells, revealing a marked increase in overall lactylation following OGD-R exposure (Fig. 4A). These results suggested that OGD-R-induced lactate production contributes to stroke-related cellular damage *via* lactylation modification. To test this hypothesis, the lactylation status of PLBD1 was evaluated in BV2 cells post-OGD-R treatment, showing a significant elevation in PLBD1 lactylation (Fig. 4B). Additionally, exogenous lactic acid (LA) administration further promoted PLBD1 lactylation, whereas treatment with the glycolysis inhibitor 2-DG produced the opposite effect, reducing lactylation (Fig. 4C,D). Subsequently, we further explored the effects of LA and 2-DG on the stability of PLBD1. As shown in Fig. 4E,F, compared with the control group, the was also investigated; LA treatment enhanced PLBD1 stability of PLBD1 in the LA-treated group was strengthened, while the, while 2-DG treatment accelerated its degradation (Fig. 4E,F). For original western blotting figures in Fig. 4 see **Supplementary Material**. These results indicated that OGD-R-induced lactate enhanced PLBD1 stability by increasing its lactylation.

3.5 K155 is the Lactylation Modification Site of PLBD1

The findings indicated that lactate induces PLBD1 lactylation, prompting further investigation into the underlying mechanisms. The DeepKla database was utilized to predict potential lactylation sites on PLBD1, revealing two

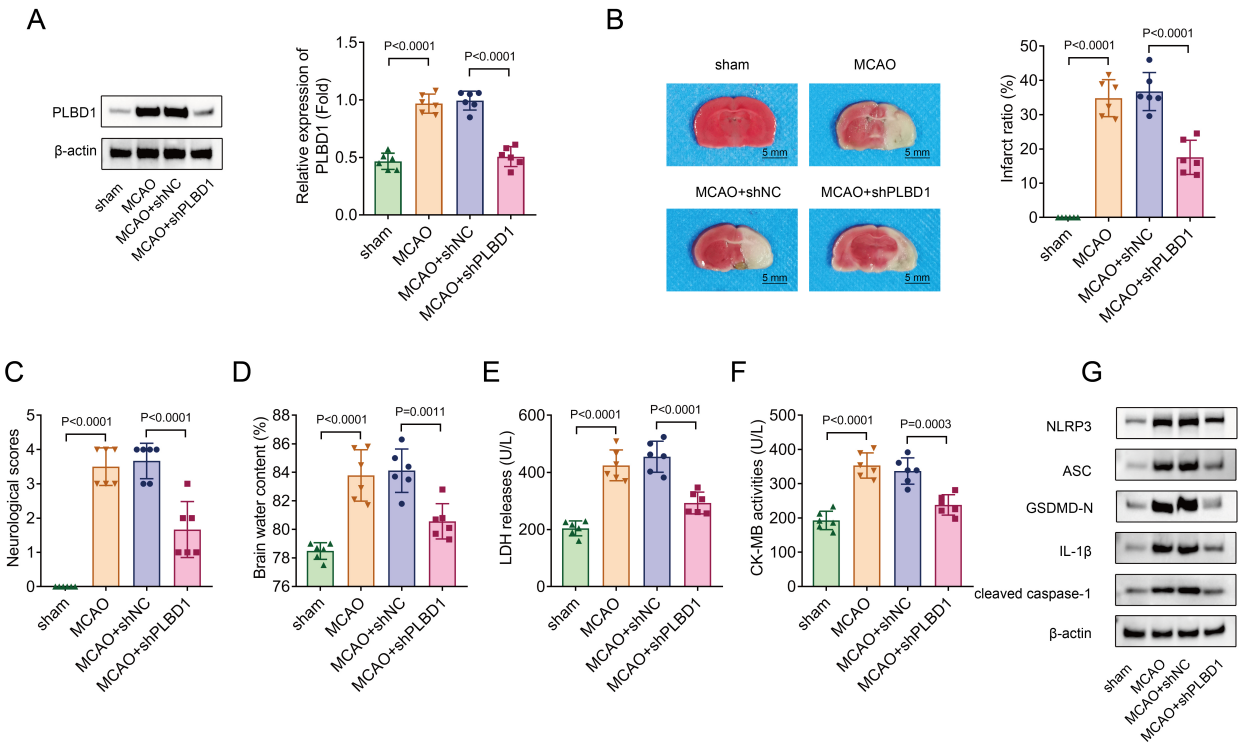

Fig. 6. PLBD1 knockdown alleviates brain damage after ischemic stroke. (A) Analysis of western blotting indicated that shPLBD1 lentivirus significantly reduced PLBD1 expression in the brain tissues of MCAO rats (n = 6). (B) Representative TTC-stained brain slices and analysis of the infarction ratio showed a reduced cerebral infarction rate in MCAO mice after shPLBD1 lentivirus treatment (n = 6). The scale bar = 5 mm. (C) PLBD1 knockdwon reduced the neurological deficit scores in MCAO rats (n = 6). (D–F) PLBD1 knockdwon reduced the brain water content (D) (n = 6) and serum levels of LDH (E) (n = 6) and CK-MB (F) (n = 6) in MCAO rats. (G) Analysis of western blotting indicated that PLBD1 silencing dramatically reduced the overexpression of NLRP3-associated proteins (NLRP3, cleaved caspase-1, and IL-1β) and pyroptosis-related proteins (GSDMD-N and ASC) expression in the brain tissues of MCAO rats (n = 6). TTC, 2,3,5-Triphenyl Tetrazolium Chloride; CK-MB, creatine kinase-MB. For the full TTC images see **Supplementary Material**.

candidate sites, K155 and K338, with K155 displaying the highest PTM score, suggesting it as the primary site for lactylation modification (Fig. 5A). To confirm the lactylation site, lysine residues at positions 155 and 338 were mutated to arginine (R) in PLBD1. The results showed that mutation at K155 markedly decreased both the protein level and lactylation of PLBD1, whereas mutation at K338 did not produce a significant effect (Fig. 5B). Further analysis demonstrated that the K155 mutation led to a significant reduction in PLBD1 stability compared to the control, resulting in accelerated protein degradation (Fig. 5C). For original western blotting figures in Fig. 5B,C see **Supplementary Material**. These results corroborated the prediction that K155 served as the primary lactylation modification site on PLBD1.

3.6 PLBD1 Knockdown Alleviates Brain Damage after Ischemic Stroke

To assess the therapeutic potential of PLBD1 in mitigating stroke-induced brain injury, an MCAO rat model was established, followed by the administration of shNC or shPLBD1 lentivirus to evaluate the effects of PLBD1 knockdown on brain damage. Western blotting analysis confirmed that shPLBD1 lentivirus significantly reduced PLBD1 expression in the brain tissue of MCAO rats (One-way ANOVA: sham vs. MCAO, $p < 0.0001$; MCAO vs. MCAO + shPLBD1, $p < 0.0001$, the data met the assumptions of normality and equal variance.) (Fig. 6A). TTC staining revealed substantial infarct areas in the right hemisphere post-surgery, indicating pronounced brain injury, while the cerebral infarct volume was markedly reduced in the shPLBD1 group compared to the shNC group (One-way ANOVA: sham vs. MCAO, $p < 0.0001$; MCAO vs. MCAO + shPLBD1, $p < 0.0001$, the data met the assumptions of normality and equal variance.) (Fig. 6B). Neurological assessment showed that PLBD1 knockdown significantly alleviated surgery-induced neurological deficits (One-way ANOVA: sham vs. MCAO, $p < 0.0001$; MCAO vs. MCAO + shPLBD1, $p < 0.0001$, the data met the assumptions of normality and equal variance.) (Fig. 6C). Additionally, brain tissue from MCAO and MCAO + shNC groups exhibited marked edema (wa-

ter content), whereas the MCAO + shPLBD1 group showed a significant reduction in brain edema (One-way ANOVA: sham vs. MCAO, $p < 0.0001$; MCAO vs. MCAO + shPLBD1, $p = 0.0011$, the data met the assumptions of normality and equal variance.) (Fig. 6D). Biochemical analysis indicated that MCAO significantly elevated serum levels of LDH (One-way ANOVA: sham vs. MCAO, $p < 0.0001$, the data met the assumptions of normality and equal variance.) and CK-MB (One-way ANOVA: sham vs. MCAO, $p < 0.0001$, the data met the assumptions of normality and equal variance), along with increased expression of pyroptosis-related proteins (GSDMD-N and ASC) and NLRP3 inflammasome-associated proteins (NLRP3, cleaved caspase-1, and IL-1β) in brain tissue. However, PLBD1 knockdown significantly suppressed these elevations (LDH: One-way ANOVA, MCAO vs. MCAO + shPLBD1, $p < 0.0001$; CK-MB: One-way ANOVA, MCAO vs. MCAO + shPLBD1, $p = 0.0003$, the data met the assumptions of normality and equal variance.) (Fig. 6E–G). For original western blotting figures in Fig. 6A,G see **Supplementary Material**. Overall, these results demonstrated that PLBD1 knockdown effectively mitigated brain injury in the MCAO rat model.

4. Discussion

Ischemic stroke is a severe condition that ranks second only to heart ischemia as the primary reason of death globally. Following cerebral ischemia, the immune system plays a key role in mediating brain damage. White blood cells contribute to the inflammatory response by releasing pro-inflammatory substances, triggering an immune-inflammatory cascade that exacerbates brain tissue damage and significantly impacts patient survival and prognosis [22]. Thus, a deeper understanding of the interplay between the immune system and ischemic brain injury is essential for improving clinical outcomes. Prior research has found that PLBD1 is significantly upregulated in circulating leukocytes of patients with ischemic stroke, suggesting its diagnostic potential for this condition [11]. Furthermore, PLBD1 expression is also elevated following acute myocardial infarction, where it acts as an inflammatory driver in myocardial injury [9]. Based on these insights, the purpose of this study was to investigate the regulatory role of PLBD1 in ischemic stroke-induced brain injury.

Our findings revealed that PLBD1 exhibited abnormally high expression in both the brain tissues of ischemic stroke rats and in the OGD-R cell model. Research has established that modulating protein stability and expression levels can influence various cellular functions, including cell proliferation, cell cycle regulation, apoptosis, tumor growth, metastasis, and invasion. In this study, PLBD1 knockdown, achieved through viral vector delivery, significantly attenuated ischemic stroke-induced brain damage both *in vivo* and *in vitro*. This included reductions in cell damage, pyroptosis, inflammatory responses, abnormal glucose metabolism, cerebral infarct volume, and neurological deficits. According to these findings, PLBD1 downregulation may be the target of a viable ischemic stroke treatment approach.

Abnormalities in protein stability and expression are closely associated with disease development, making the investigation of PLBD1 expression mechanisms essential for elucidating the molecular basis of ischemic stroke. Recent studies have identified lactylation as a significant PTM that directly alters protein structure and function, thereby regulating the expression of target genes [23,24]. Subsequent research has established that lactate mediates protein lactylation in various diseases, including embryogenesis, inflammatory metabolism, neurological disorders, and pulmonary fibrosis, indicating its broad biological significance [25]. In conditions of hypoxia or ischemia, such as ischemic stroke, restricted oxygen availability triggers anaerobic glycolysis, leading to the accumulation of lactic acid in affected tissues. Elevated lactic acid levels play a role in disease progression by inducing lactylation modifications on proteins, thereby influencing cellular processes [26]. For instance, Yao *et al*. [27] identified 49 upregulated and 99 downregulated lactylated proteins in cerebral endothelial cells from ischemic stroke rats, shedding light on the role of protein lactylation in the underlying mechanisms of disease progression. Additionally, lactate dehydrogenase A-driven lactylation of high mobility group protein B1 (HMGB1) facilitates its degradation, thereby exacerbating cerebral ischemia-reperfusion injury [28]. Research by Zhang's team demonstrated that inhibiting glycolysis in ischemic stroke leads to a significant decrease in the lactylation and stability of lymphocyte cytosolic protein 1 (LCP1), ultimately reducing brain injury [29]. Similarly, Zhou *et al*. [30] reported that low phosphate root 1 (LPR1) mitigates disease progression by inhibiting glycolysis and lactate production, resulting in lowered lactylation levels of ADP-ribosylation factor 1 (ARF1). Consistent with previous findings, our study confirmed an upward trend in lactate levels in the OGD-R cell model. However, the role of lactate as a substrate in regulating PLBD1 transcription *via* lactylation requires further investigation. This study verified the hypothesis, demonstrating that both OGD-R and exogenous lactate treatment significantly increased PLBD1 lactylation in BV2 cells, enhancing PLBD1 stability, while the glycolysis inhibitor 2-DG had the opposite effect. Although previous studies have identified the regulatory role of protein lactylation in ischemic stroke progression, specific lactylation sites have not been thoroughly explored [31,32]. This study predicted and confirmed K155 as a critical lactylation site on PLBD1, thus elucidating the mechanism by which lactate-mediated lactylation regulates PLBD1 during ischemic stroke. Collectively, the findings indicate that elevated PLBD1 expression and stability in ischemic stroke are driven by lactate-mediated lactylation at the K155 site.

In conclusion, the effect of elevated PLBD1 expression on ischemic stroke-induced brain damage is being investigated for the first time in this work. It was demonstrated that lactate-mediated lactylation at the K155 site enhances PLBD1 stability and expression, thereby exacerbating ischemic stroke-related brain damage. These results contribute to a deeper understanding of the role of lactylation modification in ischemic stroke progression and offer new perspectives for potential therapeutic approaches. These findings advance our knowledge of the part lactylation alteration plays in the development of ischemic stroke and provide fresh insights into possible treatment strategies. However, this research has limitations. The K155 mutation's effect on PLBD1 down regulation was only assessed in a single cell line (BV2 cells). Moreover, the potential of this mutation to mitigate brain injury in ischemic stroke animal models has not been explored. Additional studies should investigate the function of PLBD1 in other ischemic stroke cell lines and animal models and evaluate the therapeutic potential of targeting PLBD1 lactylation in this context, which would further enhance the significance of these findings.

5. Conclusion

In conclusion, this study demonstrated elevated protein and mRNA expression levels of PLBD1 in both the brain tissue of ischemic stroke animal models and OGD-R cell models. Additionally, lactylation at the K155 site enhanced the stability and expression of PLBD1. Viral vector-mediated PLBD1 knockdown significantly alleviated ischemic damage. These insights not only advance the understanding of epigenetic regulation in ischemic stroke but also suggest that targeting PLBD1 knockdown could be a promising gene therapy approach for treating ischemic stroke.

Abbreviations

MCAO, middle cerebral artery occlusion surgery; OGD-R, oxygen glucose deprivation; PTMs, post-translational modifications; ATC, acid in thyroid hyperplastic carcinoma; RT-qPCR, Quantitative (real-time) polymerase chain reaction; Co-IP, Co-immunoprecipitation; ECAR, Extracellular acidification rate; SD, Sprague Dawley; TTC, triphenyl tetrazolium chloride; LDH, lactate dehydrogenase; CK-MB, creatine kinase MB.

Availability of Data and Materials

The data in this study are available from the corresponding author upon request.

Author Contributions

FZ designed the study, performed the experiments, and drafted the manuscript. XL collected the clinical data and processed statistical data. XY analyzed and interpreted the data. YY partly contributed to the experiments and data analysis. GC designed, supervised the study, and revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Renmin Hospital, Hubei University of Medicine (SYSRMYY-074). All animal experiments were conducted in accordance with Chinese animal welfare regulations on the care and use of animals Animal studies and were performed in compliance with the ARRIVE guidelines.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN25949.

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