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# Plumbagin attenuated oxygen-glucose deprivation/reoxygenation-induced injury in human SH-SY5Y cells by inhibiting NOX4-derived ROS-activated NLRP3 inflammasome

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**ABSTRACT**

Plumbagin (PLB), an alkaloid obtained from the roots of the plants of Plumbago genus, is an inhibitor of NADPH oxidase 4 (NOX4). This study aimed to investigate the beneficial effect of PLB against oxygen-glucose deprivation/reoxygenation (OGDR)-induced neuroinjury in human SH-SY5Y neuronal cultures. Our results showed that OGD/R stimulated NOX4 protein expression and reactive oxygen species (ROS) production in SH-SY5Y cells, whereas increased 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) production, resulting in the activation of the NLRP3 inflammasome. And PLB pretreatment reduced the ROS production by regulating the expression of NOX4 and downregulated NF-κB signaling which was induced by OGDR. Furthermore, PLB inhibited OGDR induced NLRP3 inflammasome activation but not PARP1. Overall, PLB improved OGDR induced neuroinjury by inhibiting NOX4-derived ROS-activated NLRP3 inflammasome.

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**KEYWORDS**
Plumbagin; SH-SY5Y; reactive oxygen species (ROS); NADPH oxidase 4 (NOX4); NLRP3

Cerebral ischemia-reperfusion injury is a complex pathophysiological processes, including inflammation, apoptosis, and excitotoxicity in the brain [1]. It remains lack of effective drug treatments for cerebral ischemia-reperfusion injury [2]. Ischemia-reperfusion injury can be mimicked in cultured neurons by using oxygen-glucose deprivation and reoxygenation (OGDR) [3]. SH-SY5Y cells are derived from human neuroblastoma and usually used as cell models for neurodegenerative diseases and cerebral ischemic disease [4]. Thus, the SH-SY5Y cell lines were employed in this study to mimic cerebral ischemia-reperfusion injury *in vitro*. The mechanisms of OGDR-induced neuronal injury include inflammation, oxidative stress, mitochondrial dysfunction and apoptosis, which eventually lead to irreversible neuronal cell death and brain injury [5].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is one of the most important reactive oxygen species (ROS) producers. It is a multi-component enzyme complex which is composed of membrane subunits, cytochrome b558, and multiple cytosolic subunits. Recently, NOX4 has been regarded as a major ROS source for the brain ischemia injury [6]. On the other hand, the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are key mediators of innate immune responses via inflammasome activation [7]. Among them, NLR pyrin domain containing 3 (NLRP3) has been involved in cerebral injuries and some neurodegenerative diseases [8]. NLRP3 inflammasome is a protein complex for processing the maturation of IL-1β and IL-18 [9]. And NLRP3 inflammasome is also a downstream molecule of oxidative stress [10]. Previous studies indicated that both NOX-derived ROS and mitochondrial ROS give rise to NLRP3 inflammasome activation [11,12]. Thus, the lipid peroxides (4-HNE and MDA) following OGDR could evaluate the peroxidation. Also oxidative stress could be evaluated by the mitochondrial membrane potential (ΔΨm), reactive oxygen species (ROS) and apoptosis. And the expression levels of NOX4, NLRP3, ASC and pro-caspase1 directly reflect the activation of NOX4-NLRP3 axis.

Plumbagin (PLB, 5-hydroxy-2-methyl-1,4-naphthoquinone) is a natural naphthoquinone constituent found in many plants, especially *Plumbago zeylanica L* (Plumbaginaceae) which is traditionally used in traditional Chinese medicine [13]. Previous studies have reported its role in inhibiting NOX4 and regulating redox signaling [14,15]. Also plumbagin protected cerebral infarction-reperfusion induced neuroinjury in rats through repression of apoptosis and NF-κB activation [16]. In this study, we aimed to investigate the beneficial effect of PLB on oxygen-glucose deprivation/reoxygenation (OGDR)-induced neuroinjury and its mechanisms in human SH-SY5Y cells.

## Materials and methods

### Reagents and antibodies

Plumbagin (S4777) was provided by Selleck Chemicals. Anti-NLRP3 antibody was provided by Boster (Wuhan,

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China). Anti-pro-caspase1 antibody was obtained from Abcam (Cambridge, UK). Anti-NOX4, anti-ASC, anti-TLR4, anti-Myd88, anti-NF-κB p65, anti-PARP1 antibodies were purchased from Proteintech (Wuhan, China).

### Cell culture

Human undifferentiated SH-SY5Y cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum. SH-SY5Y cells were incubated at 37°C in a humidified incubator containing 5% $CO_2$. Cells in exponential phase of growth were used for the subsequent experiments. The cultured cells were randomly divided into five groups. The control group was treated with complete culture medium without OGDR (later referred to as control); the OGDR group was treated with OGDR only (later referred to as OGDR); the OGDR+PLB groups were treated with different concentrations (5, 10 and 20 μM) of PLB for 24 h prior to OGDR (later referred to as OGDR+PLB).

### Oxygen–glucose deprivation/reoxygenation (OGDR) injury

Cells were pretreated with PLB at different concentrations in a DMEM medium for 24 h. To establish an *in vitro* ischemic injury model, SH-SY5Y cells were first incubated in glucose- and serum-free DMEM in an anaerobic humidified chamber filled with 95% $N_2$ and 5% $CO_2$ for 4 h at 37°C (mimic oxygen glucose deprivation). Afterward, the cells were then cultured in a normal medium under normoxic conditions and re-oxygenated for 24 h. Normoxic control cells without OGDR were placed in norm-oxygenated complete DMEM medium.

### Cell viability assays

After re-oxygenation, cell viability was determined using a CCK-8 kit (Beyotime Biotechnology, China). The absorbance was evaluated at 450 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

### Mitochondrial membrane potential assay

JC-1 dye was conducted to determine the mitochondrial membrane potential (ΔΨm) changes [17]. To determine the ΔΨm, cells were stained with a JC-1 assay kit (Bioswamp) and subsequently evaluated by flow cytometry with the CXP software 2.0.

### Measurement of intracellular ROS

To determine the levels of intracellular ROS, a DCF-DA staining was performed in SH-SY5Y cells. Fluorescence intensity was determined by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data was analyzed using the CXP software 2.0 (Beckman Colter, Pasadena, CA, USA).

### Neuronal apoptosis

Cell apoptosis assay was performed using an Annexin V-FITC/PI assay with an Annexin V-FITC/PI apoptosis detection kit (Beyotime Inst, China). SH-SY5Y cells were cultured in 60 mm cell culture plates ($1 \times 10^6$ cells/well), subjected to OGDR, harvested by trypsinization and washed three times with ice-cold PBS. Then the cells were resuspended in 200 μL of binding buffer. After Annexin V-FITC (10 μL, 20 μg/mL) and PI (10 μL, 1 mg/mL) were added, the cells were incubated for 30 min at 4°C. After that, 300 μL of binding buffer was added. Then flow cytometry was conducted for the determination of apoptotic cells. Each test was repeated three times. Data was analyzed using CXP software 2.0.

### Western blot analysis

To assess protein expression, western blot analysis was performed [18]. Briefly, the whole cell lysate was prepared to detect the protein level of NOX4, NLRP3, ASC, pro-caspase 1, TLR4, Myd88 and PARP1. The nuclear extracts were obtained to detect the protein level of nucleus NF-κB p65 by using a nuclear/cytoplasmic isolation kit (Beyotime Inst, China). The total proteins and nuclear proteins were separated by SDS-PAGE, transferred to a PDVF membrane. Then, the membranes were incubated overnight at 4°C in solution containing 0.1% Tween 20, 5% nonfat milk and the following primary antibodies: NOX4 (1:1000), NLRP3 (1:200), ASC (1:1000), pro-caspase 1 (1:1000); PARP1 (1:1000); TLR4 (1:1000); Myd88 (1:1000); NF-κB p65 (1:2000); GAPDH (1:1000); Histone H3 (1:1000). Levels of proteins were evaluated using a chemiluminescence detection system (IS4000MM Pro, Kodak, USA). GAPDH or Histone H3 was used as an internal control.

### Statistical analysis

Statistical analysis was carried out using SPSS version 13.0. All data were expressed as the mean ± S.D. Data were analyzed by one-way ANOVA with LSD post hoc analysis. The $p$ value <0.05 was considered to be statistically significant. And the $p$ value <0.01 was considered to be statistically extremely significant.

## Results

### Plumbagin protected SH-SY5Y cells against OGDR-stimulated cytotoxicity

CCK-8 assay was conducted to evaluate the cell viability. As shown in Figure 1, a significant decrease in

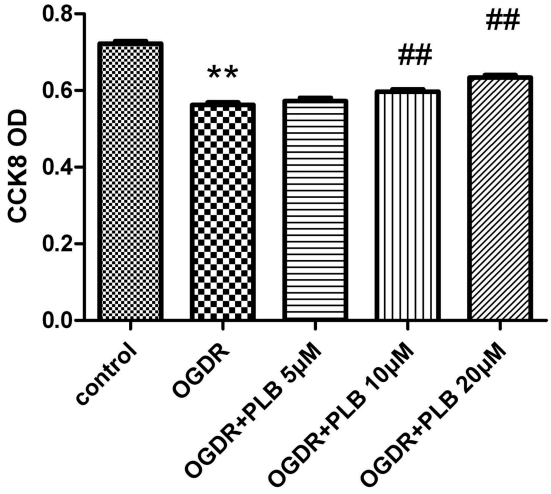


**Figure 1.** Effects of different concentration of PLB on the viability of OGDR-treated SH-SY5Ycells.

The data were obtained from at least three independent experiments. The results were represented as the means ± S.D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; $^{\#}$ $p < 0.05$ versus OGDR, $^{\#\#}$ $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

the cell viability of the OGDR group was observed as compared with the control group ($p < 0.01$). Pretreatment with PLB (10 and 20 μM) significantly increased the cell viability as compared with the OGDR group ($p < 0.01$).

### *Plumbagin attenuated OGDR-induced mitochondrial dysfunction and elevation of ROS production*

A JC-1 assay was conducted to determine the ΔΨm of SH-SY5Y cells. OGDR reduced the ΔΨm of SH-SY5Y cells ($p < 0.01$), pretreatment with PLB (20 μM) resulted in an obvious elevation in the ΔΨm ($p < 0.05$, Figure 2(a-f)).

In this study, the levels of ROS in SH-SY5Y cells were quantified using DCF-DA staining. OGDR significantly increased the intracellular levels of ROS in SH-SY5Y cells. And pretreatment with PLB (10 and 20 μM) reduced ROS production ($p < 0.01$, Figure 3(a-f)).

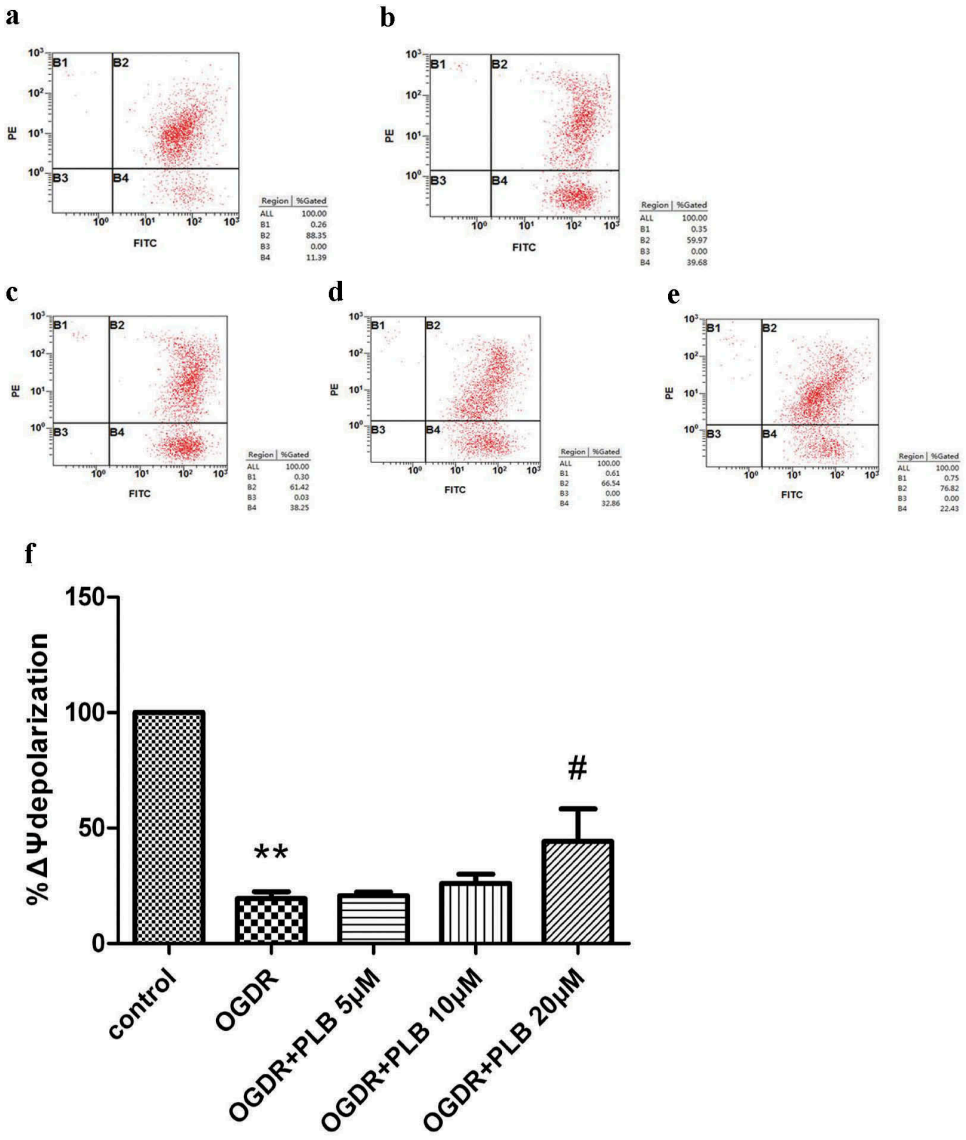


**Figure 2.** Effects of different concentration of PLB on mitochondrial membrane potential in OGDR-treated SH-SY5Ycells.

(a) Control group. (b) OGDR group. (c) OGDR+PLB (5 μM). (d) OGDR+PLB (10 μM). (e) OGDR+PLB (20 μM). (f) Bar graph represents ΔΨm changes of the different groups. The data were obtained from at least three independent experiments. The results were represented as the means ± S.D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; $^{\#}$ $p < 0.05$ versus OGDR, $^{\#\#}$ $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

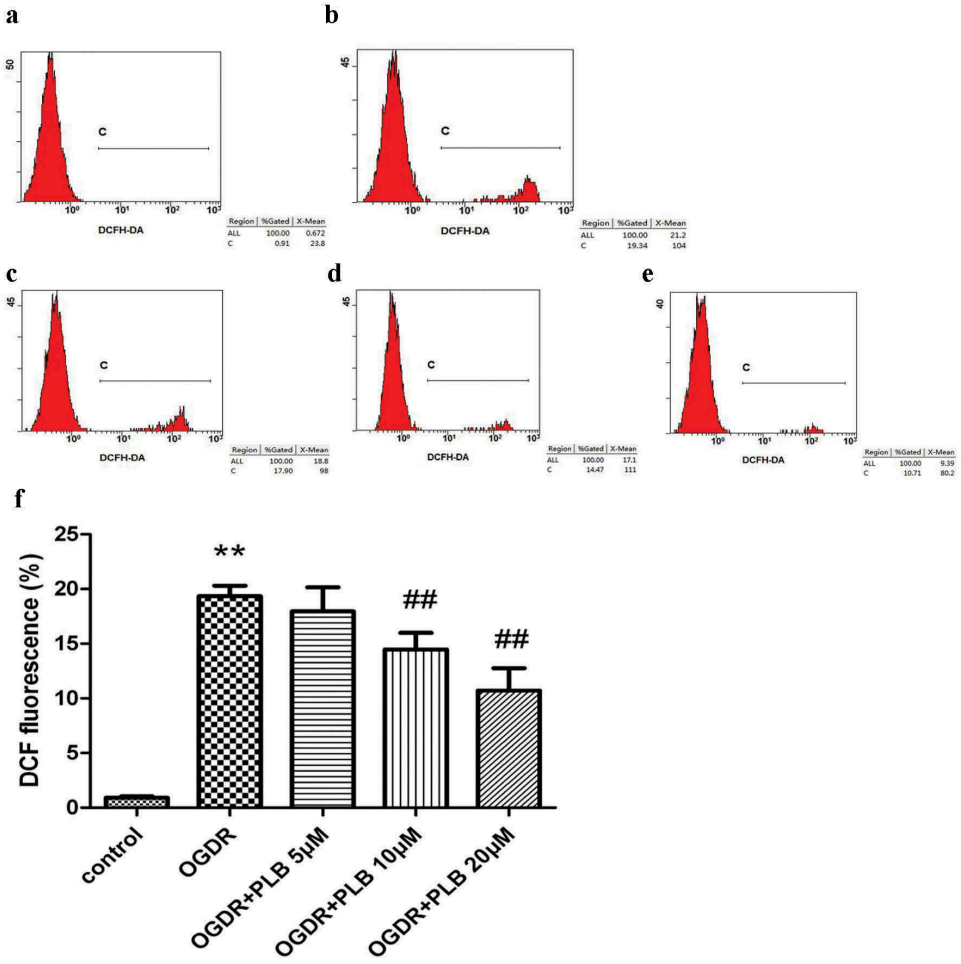


Figure 3. Effects of different concentration of PLB on the elevation of ROS in OGDR-treated SH-SY5Ycells.

(a) Control group. (b) OGDR group. (c) OGDR+PLB (5 μM). (d) OGDR+PLB (10 μM). (e) OGDR+PLB (20 μM). (f) Bar graph represents DCF fluorescence intensity of the different groups. The data were obtained from at least three independent experiments. The results were represented as the means ± S. D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus OGDR, ## $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

### *Plumbagin prevented OGDR-induced apoptosis of human neuronal cells*

As shown in Figure 4(a-f), the apoptotic rate was significantly higher in the OGDR group than in the control group ($p < 0.01$). Pretreatment with PLB (10 and 20 μM) significantly reduced the apoptotic rate as compared with OGDR alone ($p < 0.05$).

### *Plumbagin reduced the inflammatory cytokines and lipid peroxide in OGDR-treated SH-SY5Y cells*

The inflammatory cytokines (IL-1, IL-6 and TNF-α) and lipid peroxide (4-HNE and MDA) content were significantly increased in the OGDR group than in the control group ($p < 0.01$). Pretreatment with PLB (5, 10 and 20 μM) significantly reduced the inflammatory cytokines and lipid peroxide content as compared with OGDR alone ($p < 0.01$, Figure 5(a-e)).

### *Plumbagin inhibited the NF-κB signaling pathway*

As shown in Figure 6, the expression of TLR4, Myd88 and NF-κB p65 protein in the OGDR group was notably increased as compared with the control group ($p < 0.01$). In comparison, pretreatment with PLB (10 and 20 μM) significantly inhibited TLR4, Myd88 and NF-κB p65 protein expression as compared with the OGDR group ($p < 0.05$ and $p < 0.01$).

### *Plumbagin inhibited the NOX4/NLRP3 signaling pathway*

The expression of NOX4, NLRP3, ASC, pro-caspase 1 and PARP1 protein in the OGDR group was notably induced as compared with the control group ($p < 0.01$). In comparison, pretreatment with PLB (10 and 20 μM) significantly inhibited NOX4, NLRP3 and pro-caspase 1 protein expression as compared with the OGDR group ($p < 0.05$ and $p < 0.01$). Pretreatment with PLB (20 μM) significantly inhibited ASC protein expression as compared with the OGDR group ($p < 0.01$). And PLB failed to influence the PARP1 expression obviously (Figure 7).

## Discussion

In this study, PLB relieved apoptosis and reduced ROS production in OGDR-stimulated SH-SY5Y

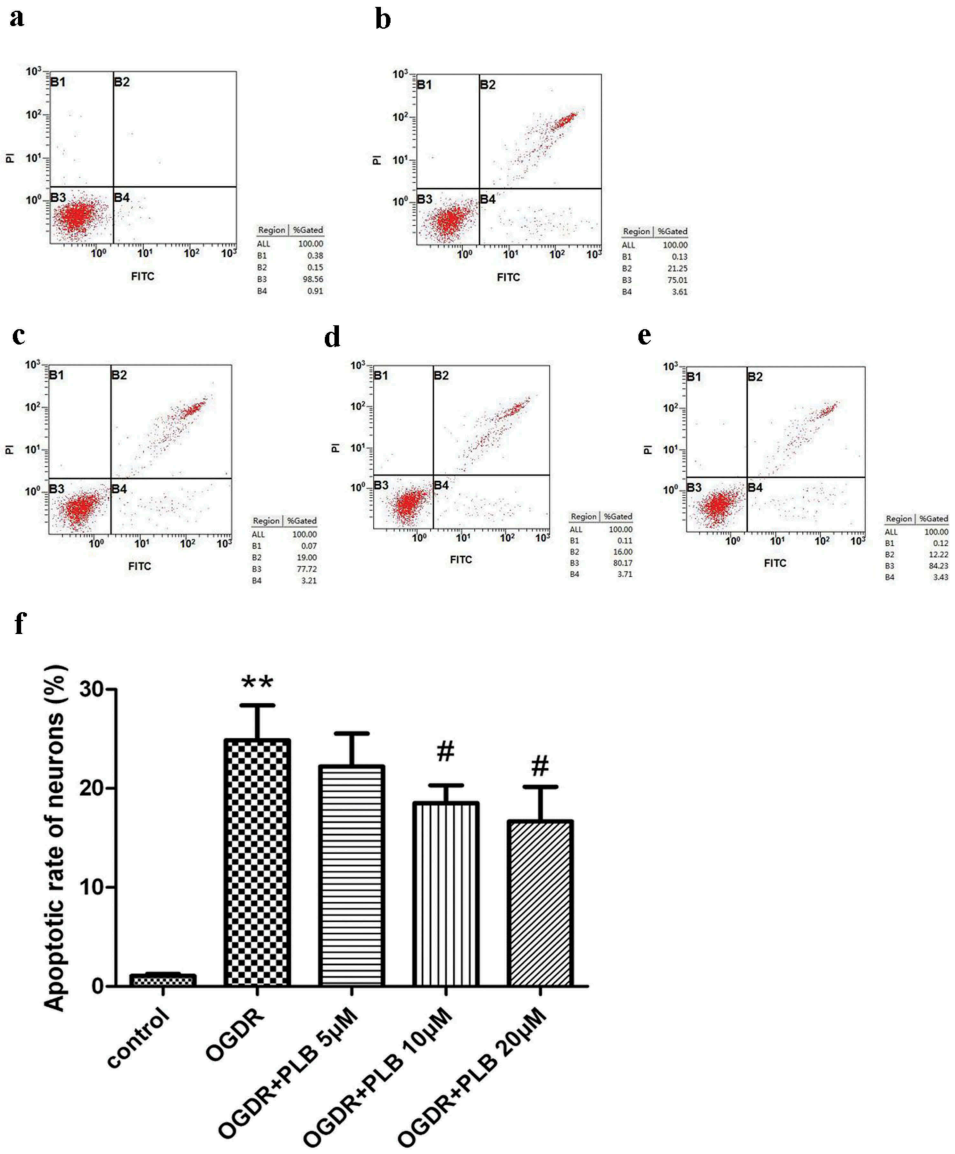


**Figure 4.** Effects of different concentration of PLB on cell apoptosis in OGDR-treated SH-SY5Ycells.

(a) Control group. (b) OGDR group. (c) OGDR+PLB (5 μM). (d) OGDR+PLB (10 μM). (e) OGDR+PLB (20 μM). (f) Bar graph represents apoptotic rates of the different groups. The data were obtained from at least three independent experiments. The results were represented as the means ± S.D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus OGDR, ## $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

cells by inhibiting NOX4-derived ROS-activated NLRP3 inflammasome.

OGDR-induced neuronal injury in SH-SY5Y cell lines is a classical cell model that mimics ischemia-reperfusion insult [19]. Ischemic injury causes massive releases of reactive oxygen species (ROS), which directly disrupt main cellular components. The restoration of oxygen levels in hypoxic tissues also stimulates ROS production [20]. And ROS further induces neuronal cell death in a time- and dose-dependent manner [21]. In this study, PLB pretreatment obviously reduced the OGDR-induced ROS production, which indicated that PLB relieved the OGDR-induced oxidative damage in SH-SY5Y cells.

Main sources of ROS in brain consist of the mitochondrial respiratory chain, xanthine oxidase and cyclooxygenase [22]. Previous studies revealed that NADPH oxidases (NOX) were important ROS producers. NOX consists of the membrane-bound cytochrome b558 (gp91phox and p22phox) and cytoplasmic proteins (p40phox, p47phox and p67phox) [23]. The NOX expression and activation are induced in brain tissues following ischemic stroke. And the NOX inhibitor (apocynin) obviously improves cerebral infarction, suggesting a crucial role of NOX in mechanism of cerebral ischemia/reperfusion injury [24]. Particularly, NOX4 is notably induced during ischemic stroke in mouse model [25]. $NOX4^{-/-}$ mice but neither $NOX1^{y/-}$ nor $NOX2^{y/-}$ mice are benefited in both transient and permanent ischemic stroke [26]. Protection from ischemic stroke in $NOX4^{-/-}$ mice is due to the repressed oxidative stress, inhibition of neurons apoptosis and improvement of blood-brain-barrier (BBB) leakage [27]. And our results indicated that PLB reduced the NOX4 protein level in OGDR-challenged SH-SY5Y cells. Thus, we

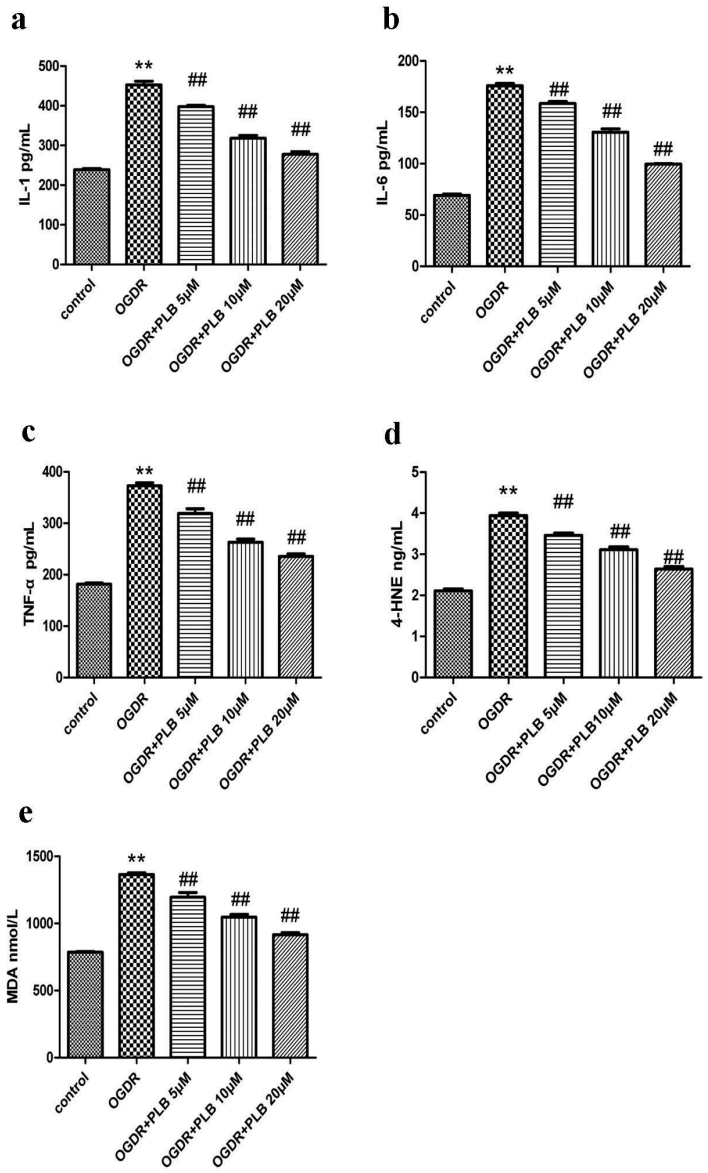


**Figure 5.** Effects of PLB on the levels of inflammatory cytokines and lipid peroxide in OGDR-treated SH-SY5Y cells.

(a) IL-1. (b) IL-6. (c) TNF-α. (d) 4-HNE. (e) MDA. The data were obtained from at least three independent experiments. The results were represented as the means ± S.D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus OGDR, ## $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

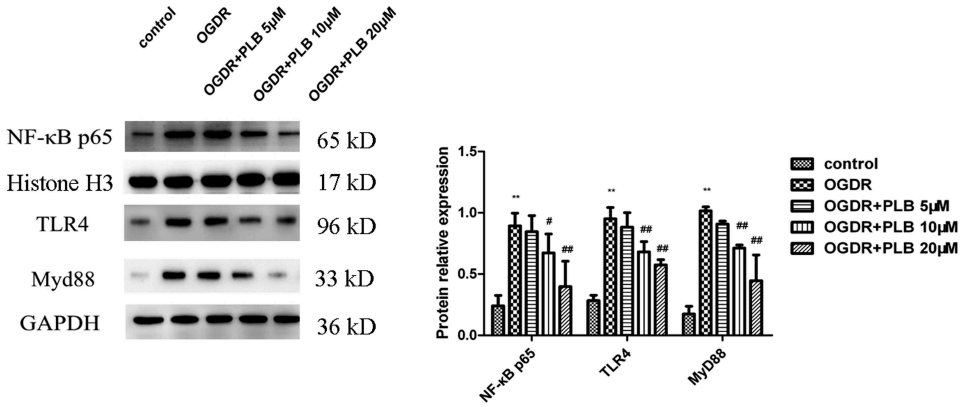


**Figure 6.** Effects of PLB on the NF-κB signaling pathway in OGDR-treated SH-SY5Y cells.

The data were obtained from at least three independent experiments. The results were represented as the means ± S.D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus OGDR, ## $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

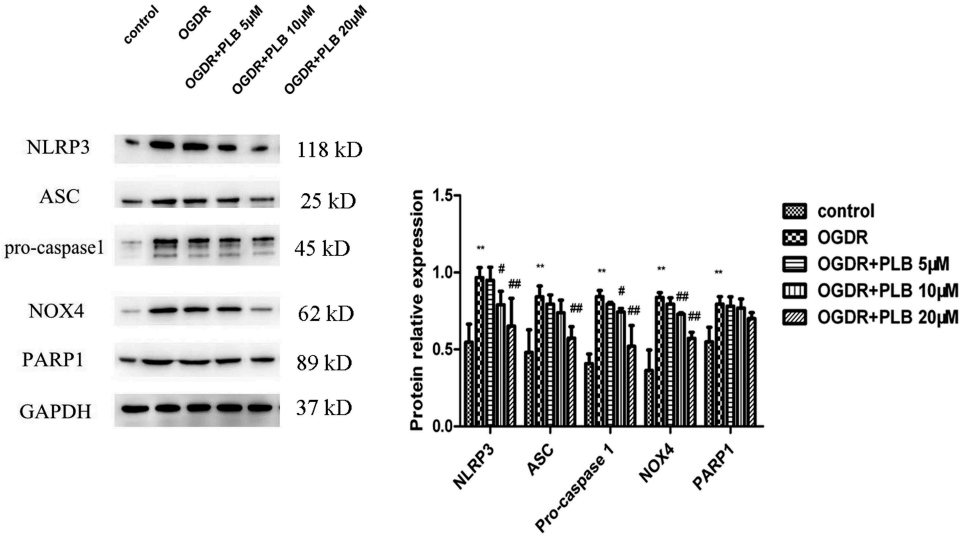


**Figure 7.** Effects of PLB on the NOX4/NLRP3 signaling pathway in OGDR-treated SH-SY5Y cells.

The data were obtained from at least three independent experiments. The results were represented as the means ± S.D. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus OGDR, ## $p < 0.01$ versus OGDR by one-way ANOVA and LSD post hoc test.

hypothesized that PLB might alleviate the OGDR-induced oxidative damage and protect against OGDR induced neuroinjury by down-regulating NOX4 *in vitro*.

Previous reports have revealed that nod-like receptor protein 3 (NLRP3) inflammasomes might be pivotal for mediating inflammatory responses and for inducing cellular damage following ischemic stroke [28,29]. Mitochondrial ROS is a major signal to trigger the NLRP3 inflammasome activation in ischemic stroke [30]. The activated NLRP3 inflammasome then form a molecular platform for caspase-1 activation leading to the production of IL-1β and IL-18, eventually magnifying the inflammatory responses [31]. Activation of the NLRP3 inflammasome requires two independent signals [32]. First, the precursor of IL-1β as well as the NLRP3 proteinis required to be transcriptionally activated. Second, subsequent activation of the NLRP3 inflammasome results in its oligomerization and inflammasome assembly. In macrophages, NF-κB activation is the first step to activate the NLRP3 inflammasome. The TLRs/Myd88/NF-κB pathway (signal 1) has been proved to induce NLRP3 as well as pro-IL-1β [33]. The signal 2 is provided by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that activate inflammasome assembly, IL-1β and IL-18 release. ROS (signal 2) is the most important molecule that activates NLRP3 inflammasome [34]. The presented results demonstrated that NLRP3 inflammasome components (NLRP3, ASC and pro-caspase 1) were significantly raised in SH-SY5Y cell 24 h post OGDR. And PLB pretreatment significantly repressed the NLRP3 inflammasome activation stimulated by OGDR. In addition, the elevated protein level of TLR4, Myd88 and nuclear NF-κB p65 was repressed significantly by PLB treatment. However, TLR signaling is not only TLR4 but also other TLR family. Thus we could not attribute the mechanism by which PLB inhibit NOX4-derived ROS-activated NLRP3 inflammasome to TLR4 only. .Further studies are still ongoing.

The mitochondrial membrane potential (ΔΨm) is generated by the special configuration of the outer and inner mitochondrial membranes. The ΔΨm decreases and membrane instability increases during mitochondrial dysfunction. Disruption of ΔΨm is an earliest event that occurs following the cellular apoptosis [35]. Loss of mitochondrial membrane is mostly due to activation of mitochondrial permeability transition pore which causes the release of Cytochrome C from mitochondria and then triggers apoptotic signals [36]. OGDR induces mitochondrial depolarization, which in turn influences apoptosis process in SH-SY5Y cells [37]. Our results showed that pretreatment with PLB at high concentration (20 μM) notably elevated the ΔΨm, thus improved the mitochondrial dysfunction and inhibited neuronal apoptosis following OGDR.

The excessive ROS and mitochondrial dysfunction not only trigger the apoptotic cascade of caspase-3, caspase-9, but also activate poly (ADP-ribose) polymerase (PARP) [38]. And PARP1 is a nuclear enzyme thatregulates various inflammatory genes [39]. After binding to damaged DNA, PARP1 completes its activation and auto-poly (ADP-ribosylation) which is critical for DNA repair. However, over activation of PARP1 results in intracellular depletion of β-nicotinamide adenine dinucleotide (NAD+) and adenosine triphosphate (ATP), driving cells into energy depletion and mitochondrial dysfunction [40]. In the present study, although OGDR notably induced

PARP1 expression, PLB pretreatment failed to significantly influence PARP1 level in OGDR challenged SH-SY5Y cells.

In summary, this study showed that PLB improved OGDR-induced neuronal injury in human SH-SY5Y cells by inhibiting NOX4-derived ROS-activated NLRP3 inflammasome.

## Authors' contributions

Qianrui Zhang and Tao Wu designed the study and wrote the manuscript. Sheng Zhao, Wenxia Zheng and Haitan Fu performed parts of the experiments. Fei Hu provided the technological guidance and was a major contributor in writing the article. All authors read and approved the manuscript.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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