Investigation and comparison of the protective activities of three functional proteins—lactoferrin, α -lactalbumin, and β -lactoglobulin—in cerebral ischemia reperfusion injury

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ABSTRACT

The objective of this study was to evaluate the protection conferred by lactoferrin, α -lactalbumin, and β -lactoglobulin in cerebral ischemia reperfusion (I/R) injury. Rat pheochromocytoma (PC12) cells were used to construct an oxygen and glucose deprivation model in vitro, and ICR mice underwent carotid artery “ligation-relaxation” to construct a cerebral I/R injury model in vivo. The levels of toll-like receptor 4 (TLR4) and downstream factors including nuclear factor- κ B, tumor necrosis factor- α , and IL-1 β were measured. Metabonomics detection and data mining were conducted to identify the specific metabolic sponsor of the 3 proteins. The results showed that lactoferrin, α -lactalbumin, and β -lactoglobulin protected neurons from cerebral I/R injury by increasing the level of bopindolol and subsequently inhibiting the TLR4-related pathway to different degrees; β -lactoglobulin had the strongest activity of the 3 proteins. In summary, this study is the first to investigate and compare the protective effects of lactoferrin, α -lactalbumin, and β -lactoglobulin in a cerebral stroke model. The results implicate TLR4 as a novel target of the 3 bioactive proteins to prevent cerebral I/R injury.

Key words: lactoferrin, α -lactalbumin, β -lactoglobulin, cerebral ischemia reperfusion, toll-like receptor 4

INTRODUCTION

A stroke involves an ischemic or hemorrhagic injury to the brain tissue and results in high fatality and disability rates (Cao et al., 2007; Liu et al., 2017).

Clinically, strokes are divided into 2 types: hemorrhagic strokes, which are characterized by the presence of a subarachnoid hemorrhage, and ischemic reperfusion (I/R) strokes, which are characterized by thrombosis or cerebral infarction. The morbidity rate of I/R injuries exceeds 85% (Cao et al., 2007; Liu et al., 2017; Lu and Wang, 2017). The mortality rate of stroke increases with age, but no efficacious clinical forewarning of this condition exists. Therefore, risk factors and premonitory signs of stroke, especially those involving cerebral I/R, as well as the development of preventive drugs, require greater attention and research (Cao et al., 2007; Liu et al., 2017; Lu and Wang, 2017).

Increasing evidence has demonstrated that cerebral I/R is accompanied by marked inflammatory reactions, which are initiated by the activation of chemokines, proinflammasomes, and adhesion molecules (Nagahiro et al., 1998; Nilupul Perera et al., 2006; Li et al., 2012). Nuclear factor- κ B (NF- κ B) is a key sensor in several cell-signaling pathways related to inflammation (Schwaninger et al., 2006). Pathogen-associated molecular pattern receptors, such as toll-like receptor 4 (TLR4), recognize the molecular structure of microorganisms and combine with an adapter molecule called myeloid differentiation protein 88 (MyD88). This triggers a prompt innate immune response, leading to activation of downstream inflammatory factors such as tumor necrosis factor- α (TNF- α) and IL-1 β (Medzhitov, 2001; Campos and Gazzinelli, 2004; Lu and Wang, 2017). Research has confirmed that TLR4 in microglia is closely involved in the immune reactions that occur during cerebral I/R, alleviating inflammation in both microglia and neurons (Tang et al., 2008; Napoli and Neumann, 2009; Teeling and Perry, 2009; Ock et al., 2010; Lu and Wang, 2017).

Milk and dairy products are widely recommended as part of a healthy diet; they contain multiple functional proteins, such as lactoferrin (LF), α -LA, and β -LG (Chagas et al., 2012). These functional proteins are

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reported to exert antioxidant, anti-inflammatory, antiviral, and antitumor effects (Hattori et al., 1995; Jing et al., 2006; Chung et al., 2012; Yang et al., 2015; Donovan, 2016; Legrand, 2016; Okubo et al., 2016; Redwan et al., 2016; Krämer et al., 2017; Gao et al., 2018; Li et al., 2019), all of which are beneficial to health. In several studies, these active proteins, especially LF and α -LA, have been shown to play a protective role on neurons in stroke injury, inhibiting oxidation and inflammation and alleviating damage in cerebral I/R models (Yamaguchi and Uchida, 2007; Zhang et al., 2012; Ikeda et al., 2013; van de Looij et al., 2014). In a recently published article, regular milk intake was shown to lower the risk of stroke and other cardio-cerebrovascular diseases, and this may be related to its nutritional components (Dehghan et al., 2018). However, little research exists to compare the protective activities of the 3 aforementioned proteins. Additionally, whether these proteins protect neurons through regulation of a TLR4-related pathway in a cerebral I/R model has seldom been investigated. Therefore, in this study, we constructed in vitro and in vivo cerebral I/R models and studied the protective effects of the 3 bioactive proteins. We also investigated the mechanisms underlying the observed protective effects.

MATERIALS AND METHODS

Chemicals

The PC12 cell line was obtained from the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium, glucose-free medium, fetal bovine serum (FBS), penicillin-streptomycin solution, and methylthiazoltetrazolium (MTT) were all purchased from Solarbio (Beijing, China). Lactoferrin (iron-saturated), α -LA, and β -LG, all with a purity >95%, were purchased from Sigma (St. Louis, MO). The TLR4 short interfering (si)RNA fragment was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against TLR4, MyD88, NF- κ B, TNF- α , IL-1 β , and β -actin, as well as all related secondary antibodies, were purchased from Santa Cruz Biotechnology. Reagents for the Western blotting assay were purchased from Solarbio. An enhanced chemiluminescence (ECL) reagent was purchased from Thermo Fisher Scientific (Waltham, MA).

Animals

Forty male ICR mice (BW of 18–22 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China; license number SCXK 2012-0001).

Table 1. The ingredients of the diet fed to mice

Component	%
Moisture	6.6000
Total fat	7.0000
Total carbohydrate	63.6000
Total protein	18.7000
Ash	4.1000
Corn starch	39.7486
Casein	20.0000
Maltodextrin	13.2000
Sucrose	10.0000
Soybean oil	7.0000
Powdered cellulose	5.0000
AIN 93G mineral mix	3.5000
AIN 93 vitamin mix	1.0000
L-Cystine	0.3000
Choline bitartrate	0.2500
<i>t</i> -Butylhydroquinone	0.0014

Animals were kept in cages at a constant temperature of 25°C and relative humidity of 50%. The mice were acclimated for 5 d before the formal experiment. The dietary ingredients were prepared according to AIN-93G (Reeves et al., 1993, Table 1). This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health (Bethesda, MD). The protocol in the present study was approved by the Committee on the Ethics of Animal Experiments of Chinese Academy of Agriculture Sciences (Beijing, China; permission number: IAS20181020). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell Culture and Cell Viability Detection

For cell viability detection, protein samples (LF, α -LA, and β -LG; 0–5,000 mg/L) were added into PC12 cells (in 96-well plates) and incubated for 48 h. Then, MTT (500 mg/L) was added, the medium was aspirated 4 h later, dimethyl sulfoxide (DMSO, 200 μ L/well) was added, and absorbance values at 490 nm were measured. The protein dosages resulting in cell viability >90% were then selected.

Oxygen-Glucose Deprivation Model Construction

To construct the oxygen-glucose deprivation (OGD) model, the RPMI 1640 medium was replaced with a glucose-free RPMI 1640 medium, and plates were incubated in 95% N₂ and 5% CO₂ at 37°C for 120 min. Cells were then returned to normal RPMI 1640 medium for reperfusion. In the protein treatment groups, proteins were added to the wells before replacement of medium, and OGD model induction began 4 h later.

Cerebral I/R Model Construction

Forty healthy male ICR mice (18–22 g) were randomly divided into 8 groups: control (no treatment); I/R group (model), LF group, α -LA group, β -LG group, I/R+LF combination group, I/R+ α -LA combination group, and I/R+ β -LG combination group ($n = 5$). The I/R model was achieved by a 15-min period of carotid artery ligation, followed by a 5-min period of carotid artery relaxation; this “ligation-relaxation” procedure was repeated 3 times. Mice in the protein treatment groups were given protein orally 6 h before I/R model induction. The dosage of LF or α -LA used was 100 mg/kg of BW, and that for β -LG was 50 mg/kg of BW. The mice were killed 4 h later and tissue from the hippocampus was isolated.

Metabonomics Detection and Analysis

Samples of PC12 cells were divided into the following groups: control, OGD model (M), LF, α -LA, β -LG, M+LF, M+ α -LA, and M+ β -LG. Special metabolites in the cell samples were separated using a ultra-HPLC system (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA) equipped with a Waters column (Acquity BEH C18 1.7 μ m, 2.1 $\times$ 50 mm) at 40°C. The mobile phase consisted of water containing 1.8 mM ammonium formate and 0.1% formic acid (solvent A, vol/vol); acetonitrile containing 0.1% formic acid and 2 mM ammonium formate (solvent B, vol/vol); a flow rate of 200 μ L/min; and the following gradient elution program: 0–1.0 min, 5% B; 1.0–5.0 min, 5% to 60% B; 5.0–8.0 min, 60% to 100% B; 8.0–11.0 min, 100% B; 11.0–14.0 min, 100% to 60% B; 14.0–15.0 min, 60% to 5% B; 15.0–18.0 min, 5% B. A Q-Exactive instrument (Thermo Fisher Scientific) equipped with electrospray ionization in positive and negative switching modes was used to detect the above samples, and the system was calibrated and controlled with Xcalibur 3.1 and Q-Exactive Tune software (Thermo Fisher Scientific). The UHPLC Q-Orbitrap analysis produced large amounts of raw data using TraceFinder software (Thermo Fisher Scientific). The data were exported into Excel spreadsheets using Simca-P software (Umetrics, Umeå, Sweden) for the following analyses: principal components analysis, partial least squares discriminant analysis, *t*-test, volcano plot, and variable importance in projection (VIP) plot analysis. Metabolites with VIP value > 1 and *P*-value < 0.05 were considered to be significant in this study.

Western Blotting Analysis

The PC12 cells were plated in a 6-well plate (1 $\times$ 10⁶ cells per well) with a confluence of 75%; 24 h later,

protein samples (final concentration: 500 mg/L LF, α -LA, or β -LG) were added into the wells for 48 h. Based on a supplementary experiment of cell viability (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17725>), 50 mg/L bopindolol was added to the cells. All proteins in the cells were lysed using lysis buffer (Solarbio) containing protease inhibitors, and then centrifuged at 7,500 $\times g$ at 4°C for 5 min. Hippocampal tissue was frozen in liquid N₂, homogenized rapidly, treated with lysis buffer, and centrifuged at 10,000 $\times g$ (4°C, 10 min). Following heat treatment at 90°C, the protein samples were added to 12% SDS-polyacrylamide gels and the samples were transferred onto a nitrocellulose membrane by dry Trans-Blot machines (Tanon, Shanghai, China) after electrophoresis. The membrane was blocked with 2% BSA in Tris-buffered saline-Tween (TBST) buffer for 1.5 h at 25°C. The proteins (TLR4, MyD88, NF- κ B, TNF- α , IL-1 β , and β -actin) were then probed with primary antibodies for 2 h at 37°C. β -Actin was used as an internal reference to ensure equal loading. After 3 washes with TBST buffer (6 min $\times$ 3), the membrane was incubated with secondary antibodies at 25°C for 1.5 h and then washed (10 min $\times$ 4). The protein bands in the membranes were then captured with ECL reagent using an imaging workstation (Tanon, Shanghai, China) and analyzed using Image J software (National Institutes of Health).

Statistical Analysis

All data are presented as means $\pm$ standard deviations and analyzed using GraphPad Prism 6.0 software (GraphPad Inc., San Diego, CA). All statistical analyses were conducted using a Student's *t*-test or a one-way ANOVA. In the tests of cell viability and Western blotting, *P*-values < 0.05 were considered to indicate significant differences between the control and other groups.

RESULTS

LF, α -LA, and β -LG Inhibited the Viability of PC12 Cells to Different Degrees

To observe and compare the effects of the 3 functional proteins on PC12 cell survival, the MTT assay was performed. Cell viabilities for the 100 mg/L LF, α -LA, and β -LG groups were 97.1 $\pm$ 1.1%, 94.5 $\pm$ 2.1%, and 90.4 $\pm$ 1.9%, respectively (Figure 1); cell viability differed between α -LA or β -LG group and the control (*P* < 0.05), whereas LF did not differ from control. The cell viabilities in the 500 mg/L LF, α -LA, and β -LG groups were 93.5 $\pm$ 2.4%, 90.1 $\pm$ 1.9%, and 84.3 $\pm$ 3.3%, respectively (Figure 1); cell viabilities in all

3 groups were inhibited compared with the control ($P < 0.05$). The PC12 cells were more sensitive to β -LG than to LF and α -LA. Given that cell viabilities in the 500 mg/L LF, α -LA, and β -LG groups were $>80\%$ and significantly lower than the controls ($P < 0.05$), we selected 500 mg/L as the appropriate dose of the 3 proteins for use in subsequent experiments.

Expression of the TLR4/MyD88/NF- κ B/TNF- α /IL-1 β Pathway in a Cell OGD Model

Western blotting was carried out to evaluate the expression of TLR4, MyD88, NF- κ B, TNF- α , and IL-1 β proteins in a cell OGD model and to investigate and compare the effects of the 3 proteins in regulating this pathway. Results demonstrated that levels of protein in this pathway exhibited no apparent change following the single addition of LF, α -LA, or β -LG. However, in the OGD group, expression of TLR4, MyD88, NF- κ B, TNF- α , and IL-1 β was upregulated significantly compared with the control ($P < 0.05$; Figure 2). In the OGD+LF, OGD+ α -LA, and OGD+ β -LG combination groups, expression of TLR4 and related proteins was significantly inhibited compared with that of the OGD group ($P < 0.05$). This inhibition of the TLR4-related pathway was more significant in the β -LG group than in the LF and α -LA groups ($P < 0.05$; Figure 2).

Expression of the TLR4/MyD88/NF- κ B/TNF- α /IL-1 β Pathway in a Mouse I/R Model

To validate and compare the protective effect of the 3 functional proteins through regulation of the TLR4-related pathway, expression of TLR4, MyD88, NF- κ B, TNF- α , and IL-1 β was measured in hippocampal tis-

sue using Western blotting. Because viability of PC12 cells was more sensitive to β -LG than to LF and α -LA, we used 50 mg/kg for β -LG and 100 mg/kg for LF and α -LA as the candidate dose. The levels of TLR4, MyD88, NF- κ B, TNF- α , and IL-1 β were significantly upregulated in the cerebral I/R model compared with the control ($P < 0.05$). We observed no difference in the levels of TLR4, MyD88, NF- κ B, TNF- α , or IL-1 β between the control and the single protein groups (Figure 3). Compared with protein levels in the I/R model, levels in the I/R+protein combination groups were inhibited significantly ($P < 0.05$). This inhibition of protein expression was significantly greater in the LF and α -LA groups than in the β -LG group ($P < 0.05$; Figure 3).

Expression of the TLR4/MyD88/NF- κ B/TNF- α /IL-1 β Pathway in PC12 Cells Treated with TLR4 SiRNA

To further investigate the role of TLR4 in OGD model and to evaluate the regulation of the 3 proteins on TLR4, we measured the expression of TLR4, MyD88, NF- κ B, TNF- α , and IL-1 β in PC12 cells treated with TLR4 SiRNA (5 μ M). Proteins were downregulated following treatment with TLR4 SiRNA (compared with control, $P < 0.05$), and no apparent change was observed in the TLR4 SiRNA group compared with that in the OGD model + TLR4 SiRNA group or OGD model + TLR4 SiRNA + LF/ α -LA/ β -LG groups, indicating that the 3 bioactive proteins exerted a protective effect by targeting TLR4 in the OGD model (Figure 4).

Effects of Bopindolol on Expression of the TLR4/MyD88/NF- κ B/TNF- α /IL-1 β Pathway in PC12 Cells

To investigate the mechanism of the 3 functional proteins in protecting against stroke injury, we first performed metabolomics detection and data analysis. Results showed that the level of bopindolol was significantly reduced in the OGD model compared with the control ($P < 0.05$). The 3 functional proteins were shown to significantly upregulate the level of bopindolol, both in single protein groups and in model+protein combination groups, compared with the OGD group ($P < 0.05$; Figure 5A). We chose bopindolol as a metabolite of interest in the present study because of previous studies (Arakawa et al., 1989; Hattori et al., 1999). To further investigate the role of this special metabolite in regulating the TLR4-related pathway, we used bopindolol to treat PC12 cells under OGD conditions and then detected protein expression. We found that bopindolol significantly inhibited the expression of the TLR4-related pathway compared with the control ($P < 0.05$; Figure 5B, 5C).

Figure 1. The effects of no treatment (control, C) or different concentrations of lactoferrin (LF), α -LA, and β -LG on survival of PC12 cells. * $P < 0.05$ compared with control (means $\pm$ SD; $n = 8$).

Figure 2. Protein expression in toll-like receptor-4 (TLR4)-related pathway in PC12 cells. (A) Expression of toll-like receptor (TLR)4, myeloid differentiation protein 88 (MyD88), nuclear factor (NF)- κ B, tumor necrosis factor (TNF)- α , and IL-1 β ; and (B) densitometric quantitation of proteins normalized to expression of β -actin (%) in PC12 cells. C = control group; LF = lactoferrin, M = model group. All values were calculated from 3 independent experiments. * $P < 0.05$ compared with control; # $P < 0.05$, compared with the oxygen-glucose deprivation (OGD) model group (means $\pm$ SD; $n = 3$).

DISCUSSION

The increasing morbidity and high mortality of cerebral I/R injury have drawn much attention in the area

of clinical medicine and drug development. Researchers are focused on finding sensitive markers for early diagnosis and on developing new drugs or health-enhancing products to prevent I/R. In the field of food science,

scientists are trying to establish potential causal connections between dietary components and disease prevention. Milk is a commonly consumed and inexpensive food and it contains many useful and bioactive ingredients that help in immune system maturation and recovery

(Palmer et al., 2007; Sisk et al., 2007; Nanthakumar et al., 2011) and in preventing cardiovascular disease (Dehghan et al., 2018). The published literature shows that LF, α -LA, and β -LG are partly resistant to enzymatic digestion during the transit in the stomach and

Figure 3. Protein expression in toll-like receptor-4 (TLR4)-related pathway in hippocampus tissue. (A) toll-like receptor (TLR)4, myeloid differentiation protein 88 (MyD88), nuclear factor (NF)- κ B, tumor necrosis factor (TNF)- α , and IL-1 β ; and (B) densitometric quantitation of proteins normalized to expression of β -actin (%). C = control group; LF = lactoferrin, M = model group. All values were calculated from 3 independent experiments. * $P < 0.05$ compared with control; # $P < 0.05$, compared with the oxygen-glucose deprivation (OGD) model group (means $\pm$ SD; $n = 3$).

Figure 4. Protein expression in toll-like receptor-4 (TLR4)-related pathway in PC12 cells treated with TLR4 short interfering (Si)RNA and 3 proteins. (A) Expression of toll-like receptor (TLR)4, myeloid differentiation protein 88 (MyD88), nuclear factor (NF)- κ B, tumor necrosis factor (TNF)- α , and IL-1 β ; and (B) densitometric quantitation of proteins normalized to expression of β -actin (%) in PC12 cells. C = control group; LF = lactoferrin, M = oxygen-glucose deprivation (OGD) model group. All values were calculated from 3 independent experiments. * $P < 0.05$ compared with control; # $P < 0.05$, compared with (model + TLR4 SiRNA) group (means $\pm$ SD; $n = 3$).

Figure 5. The content of bopindolol in PC12 cells, and protein expression in toll-like receptor-4 (TLR4)-related pathway in PC12 cells treated with bopindolol (50 mg/L). (A) Relative content of bopindolol in PC12 cells; (B) expression of toll-like receptor (TLR)4, myeloid differentiation protein 88 (MyD88), nuclear factor (NF)-κB, tumor necrosis factor (TNF)-α, and IL-1β; and (C) densitometric quantitation of proteins normalized to expression of β-actin (%). C = control group; LF = lactoferrin, M = oxygen-glucose deprivation (OGD) model group. All values were calculated from 3 independent experiments. * $P < 0.05$, compared with control; # $P < 0.05$, compared with model group (means $\pm$ SD; $n = 3$).

intestine (Hirai et al., 1992; Caillard and Tomé, 1995; Rytönen et al., 2006; Riihimäki et al., 2008; Jiang et al., 2011; Jiang and Lönnnerdal, 2014). According to the literature (Troost et al., 2001), 60 to 80% of orally administered bovine LF survives gastric digestion in the adult human. Thus, the 3 proteins could reach the surface of enterocytes and be further absorbed into cells through different transport routes. In intestinal epithelial cells, part of these proteins are degraded by lysosomes, and the remainder are transported intact to the basal side of epithelium. Then, the peptides or proteins can enter the blood by exchange and participate in body fluid circulation.

In this study, we chose 3 the lactoproteins that are most studied in immunomodulation and antioxidation and that might have latent effects against I/R. Because I/R is always accompanied by an inflammatory response, and TLR-4 related factors are reported to participate this process (Napoli and Neumann, 2009; Teeling and Perry, 2009; Ock et al., 2010), it might be possible to ameliorate or prevent I/R injury by suppressing the TLR-4 related pathway. Thus, we tested whether LF, and α-LA and β-LG, could inhibit the induction of TLR-4 and its downstream proteins, respectively, in the OGD cellular model and in the I/R mouse model. Lactoferrin (80 kDa) is a cationic glycoprotein that belongs to the nonheme iron-binding protein family. Its positively charged surface facilitates the binding of LF with DNA, heparin, and LPS (He and Furmanski, 1995; van Berkel et al., 1997; Lizzi et al., 2016). The unique structural characteristics of LF provide a variety of nutritional and medicinal benefits, including antimicrobial (Lee et al., 2005; Ostan et al., 2017), antiviral, and anti-inflammatory activities (Beljaars et al., 2004; Sagel et al., 2009; Pfefferkorn et al., 2010) and anticarcinogenic effects (Amiri et al., 2015; Gibbons et al., 2018). This indicates that LF may be a novel candidate in the field of biomaterial development. In studies of antitumor mechanisms, LF was proven to trigger the apoptosis pathway and inhibit angiogenesis and to elevate the expression of tumor suppressor proteins (Jiang and Lönnnerdal, 2017). Growing evidence also suggests that LF may be a valuable therapeutic compound in the clinical treatment of Alzheimer's disease, inhibiting cognitive decline (Hardy and Selkoe, 2002; Guo et al., 2017). In addition, LF can improve innate immunity and prevent the over-release of proinflammatory cytokines to balance T helper (Th)1/Th2 cells (Donovan, 2016; Legrand, 2016). α-Lactalbumin (14.2 kDa) is an abundant protein in milk; it is synthesized in the rough endoplasmic reticulum of mammary epithelial cells. It helps alleviate inflammation by directing neutrophils, monocytes, and lymphocytes to inflammatory sites (Rusu et al., 2010). Yamaguchi et

al. (2009) investigated the effect of α -LA in various animal models, demonstrating inhibition of acetic acid-induced writhing in mice and suppression of nociception and inflammation in rat footpads. Russo et al. (2012) reported that in *N*-methyl-D-aspartate (NMDA)- and pentylenetetrazol (PTZ)-induced seizure models, α -LA demonstrated a protective reduction of spontaneous seizure development, indicating this protein may have potential applications in the treatment of epilepsy. β -Lactoglobulin (18.4 kDa) is a bovine-derived milk whey protein that is not present in human milk and to which a minority of infants are allergic. β -Lactoglobulin and its hydrolytic peptides have high nutritional value and important functional properties, including antioxidant, immunomodulatory, antihypertensive, and antimicrobial functions (Mullally et al., 1997; Peña-Ramos et al., 2004; Huang et al., 2010; Théolier et al., 2013). In a study by Bamdad et al. (2017), the peptides of β -LG were found to alleviate inflammation by decreasing production of nitric oxide (NO) and proinflammatory cytokines.

The protective activity of LF in ischemic injury has been investigated and validated in several studies. Ikeda et al. (2013) found that bovine milk-derived LF (bLF) promoted vascular endothelial cell function and thereby contributed to the revascularization response to ischemic injury. Zhang et al. (2012) found that LF may serve as a potent supplement in protecting the gut from intestinal I/R-induced injury via its antioxidant, anti-inflammatory and antiapoptotic activities. van de Looij et al. (2014) showed that LF had neuroprotective effects on brain metabolism of rat pups with cerebral hypoxia-ischemia injury, and the cerebral gray and white matter of pups' brains recovered after LF ingestion (van de Looij et al., 2014). In the present study, the underlying mechanism of neuronal protection from cerebral ischemia reperfusion injury by LF was validated. Yamaguchi and Uchida (2007) reported that α -LA had a suppressive effect against cerebral I/R injury by inhibiting IL-6 release in a mouse model, showing a positive correlation between serum α -LA level and efficacy for I/R. An inflammatory response to the injury is the major cause of hypoxia-ischemia brain damage, and upregulated inflammatory factors, notably TNF- α and IL-6, contribute to early damage to neurons and the blood-brain barrier in ischemic tissues (Vasiljevic et al., 2011). The suppressive effect of α -LA on IL-6 expression suggests that α -LA may have potential as a safe, natural drug for I/R. To verify this, the protective effects of α -LA were investigated here in *in vitro* and *in vivo* cerebral I/R models to provide new insight into the prevention and therapy of cerebral hypoxia-ischemia injury. Research into the role of β -LG in brain damage is scarce. Accumulating evidence has shown,

however, that β -LG and its derived peptides are able to inhibit the activity of angiotensin-converting enzyme (Vermeirssen et al., 2004; Ortiz-Chao et al., 2009). This is notable given that angiotensin-converting enzyme is a crucial molecule participating in the pathology of various diseases, including hypertension and cerebral I/R injury (Eriksson et al., 2002; Balyasnikova et al., 2005; Oyamada et al., 2010). Thus, we can speculate that β -LG may play a protective role in cardio-cerebrovascular diseases.

The multiple functions of these 3 bioactive proteins have been widely reported and validated. However, a comparison of their protective effects against stroke, and the molecular mechanisms underlying this protection, has not been conducted. Therefore, our study aimed to investigate the protective effects of LF, α -LA, and β -LG against stroke using *in vitro* and *in vivo* cerebral I/R models, and to further explore the molecular mechanisms through which these proteins confer this protection. Results demonstrated that the 3 proteins inhibited the expression of the TLR4-related pathway to different degrees, with LF having the greatest effect.

Moreover, a metabolite of interest in PC12 cells, bopindolol, was identified and quantified through metabolomics analysis. As a nonselective β -adrenergic blocker, bopindolol ($C_{23}H_{28}N_2O_3$, molecular weight of 380.48) has possesses multiple unique properties and pharmacological actions, such as vasodilatory action, intrinsic sympathomimetic action (ISA), membrane-stabilizing action (MSA), and anti-serotonin action (Berthold et al., 1981; Nagatomo et al., 2001). Bopindolol can be metabolized to an active metabolite and benzoic acid by esterase, and the indole, amino and *tert*-butyl groups are thought to be the main functional groups responsible for binding to the β -adrenergic receptor (Hattori et al., 1999). The hypotensive effect of bopindolol has been well described. Fedelesová et al. (1989) reported that bopindolol could significantly reduce the blood pressure of patients with hypertension. Similar results were confirmed by Arakawa et al. (1989), who revealed that bopindolol (1 mg once daily) markedly reduced both diastolic and systolic pressure in patients with essential hypertension after 2 d of therapy. These findings suggest that bopindolol may be beneficial in the treatment of cardiovascular diseases such as hypertension and stroke. In the present study, the level of bopindolol in the cerebral I/R model group was sharply reduced. In addition, levels of bopindolol in the I/R+active protein combination groups were significantly increased, suggesting that the 3 bioactive proteins could upregulate expression of bopindolol in PC12 cells. Moreover, the regulatory effect of bopindolol on the TLR4-related pathway was investigated using Western blotting, which further indicated that

the 3 functional proteins protect against cerebral I/R injury by upregulating the level of bopindolol and subsequently inhibiting the TLR4-related pathway.

This work is the first to explore and compare the protective effects of 3 proteins against stroke and to identify their underlying mechanistic pathways. This could ultimately provide novel strategies for the prevention and therapy of several neurogenic diseases.

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