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PERK Signaling Pathway Involved in Lactic Acid Induced Astrocyte Damage

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Abstract: Diabetes is an important modifiable risk factor for cerebral ischemic stroke. It increases infraction area and restrains astrocyte activation. Meanwhile, hyperglycemia increase lactic acid and decreases pH. Lactic acid may partially be accountable for the detrimental effects of diabetes on ischemic stroke. The objective of this study was to investigate the effect of lactic acid on astrocyte viability and to explore the potential mechanisms that lactic acid enhances astrocyte cell death. Astrocytes were challenged by various concentrations of lactic acid (0, 2.5, 5， 10 and 15 mM). Cell viability and PERK pathway were examined. The results showed that lactic acid resulted in astrocyte death (p<0.05) and activation of GRP78 and PERK (p<0.05). It is concluded that lactate acidosis causes stress to the endoplasmic reticulum.

Keywords: Acidosis; Astrocyte; Lactic acid; GRP78; PERK

Introduction

Cerebral ischemia causes damage to both neurons and astrocytes. During experimental cerebral ischemia, cerebral blood is reduced to less than 5% of control value and oxygen delivery is dramatically decreased. The anaerobic metabolism of glucose produces H^+ and lactate, causing tissue acidosis [1,2]. Preexisting diabetes or hyperglycemia further enhances acidosis due to excessive amount of glucose going through anaerobic glycolysis [1,2].

Diabetes mellitus increases neuronal damage after global cerebral ischemia and enlarges infarct size after focal ischemia [3-6]. The mechanisms of diabetes or hyperglycemia enhanced ischemic brain damage are not fully understood. Lactic acidosis, increased ROS production, early damage to the mitochondria, and damage to astrocytes may be accountable for the diabetes-exacerbated ischemic brain damage [5,7-9].

Astrocytes, the most abundant cell type in the central nervous system (CNS) play a pivotal role in the construction of the blood-brain barrier (BBB), regulation of metabolisms of the neurons, promotion of synaptic plasticity, and communication between vascular compartment and neurons [10,11]. Astrocytes have been shown to be specifically sensitive to lactic acidosis [12]. In animal models, diabetes inhibits astrocyte activation following cerebral ischemia [8,13]. Although lactic acidosis is partially responsible for the deteriorative effect of

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diabetes on cerebral ischemia and reperfusion injury, the action mechanism of lactic acidosis on ischemic stroke remains unclear. A previous study has shown that endoplasmic reticulum (ER) stress plays an important role in mediating hyperglycemia-enhanced ischemic brain damage [14,15]. ER stress activates the protein kinase R-like ER kinase (PERK) and glucose regulated protein 78 (GRP78), also known as the immunoglobulin heavy chain binding protein (BIP) [16]. Therefore, elevations of GRP78 and PERK have been used as markers for ER stress. The objective of this study was to examine whether lactate acidosis causes stress to the ER.

Materials and Methods

Cell culture

Mouse hippocampal astrocyte cell line (MA-h) was obtained from Ohio State University (Columbus, OH, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 2% penicillin/streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO2. The cells were seeded at the density of 10^5 and 10^6 cells per well in 12 or 96 well plates and incubated with vehicle or different concentrations of lactic acid (0.5, 2.5, 5 and 15 mM) for 24 h. Cells were trypsinized and collected after 24 h of lactate incubation and their viabilities were measured using the MTT reduction assay. All experiments were performed in triplicate and repeated in at least three separate experiments.

Western blotting analyses

At 24 h following Lactic acid treatment, the cells were detached using trypsin, lysed using cell lysis buffer (Life Technologies) and then centrifuged at 3,000 *g* for 15 min at 37°C. The pellet was collected, sonicated, and then centrifuged at 2,000 *g* for 15 min at 4°C and the supernatant was collected to detect GRP78 and PERK by Western blotting. The protein contents were measured using Microplate BCA Protein Assay Kit (Thermo Scientific). Equal amount of protein (20 µg) was loaded into each lane of 10% NUPAGE BT gel (Invitrogen), electrophoresed, transferred to a PVDF membrane (Millipore) and incubated with antibodies against GRP78 (1:1000, BiP antibody #3183, Cell Signaling Technology), PERK (1:1000, Rabbit mAb #5683, Cell Signaling Technology), and phosphor-PERK (1:1000, Rabbit mAb #3179, Cell Signaling Technology).

Immunocytoochemistry

Immunocytochemistry was performed using antibody against phosphor-PERK (1:500, Rabbit mAb #3179, Cell Signaling Technology) and secondary donkey anti-rabbit antibody conjugated with Alexa Fluor 488 (1:2000, Invitrogen, Carlsbad, CA, USA). The specimens were mounted with Vectashield Mounting Media (H-1200) containing DAPI and examined under a confocal microscope (Nikon Eclipse C1). Three microscopic fields at 400X were captured and number of positively stained cells was counted.

Statistics

Data are presented as means ± s.d. One-way ANOVA followed by Tukey's multiple comparison test by using GraphPad Prism 5.0 was used for statistical analysis. A p value of < 0.05 was considered statistical significant.

Results

Cell viability

The cell viability in control was treated as 100%. Cell viability was significantly decreased in the presence of lactic acid in a concentration-dependent manner (**Fig. 1**). Compared with PBS control, 2.5 and 5 mM of lactic acid caused a reduction of cell viability by 27.7% and 30.1%, respectively. When lactic acid concentration increased to 10 and 15 mM, the cell viability decreased by 39.1% and 48.1% of the control, respectively.

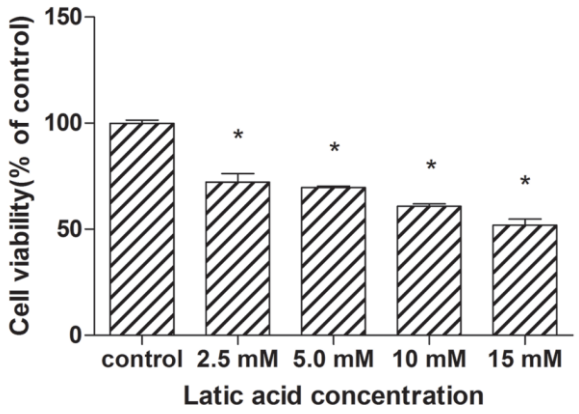

Fig. 1. Cell viability after incubation with various concentrations of lactic acid. Data were collected from 3 independent experiments and presented as means ± s.d. *p<0.05 *vs.* control. Bar =50 μM.

Lactic acid increases the protein levels of GRP78 and phosphor-PERK

Western blotting revealed that the protein content of GRP78 significantly increased when astrocytes were incubated with 2.5 mM lactic acid and the protein level further elevated when lactic acid increased to 5 mM (Fig. 2). The changes of phospho-PERK was the same as GRP78. Thus, phospho-PERK moderately increased in 2.5 mM lactate medium and markedly elevated in 5.0 mM lactate medium (**Fig. 2**).

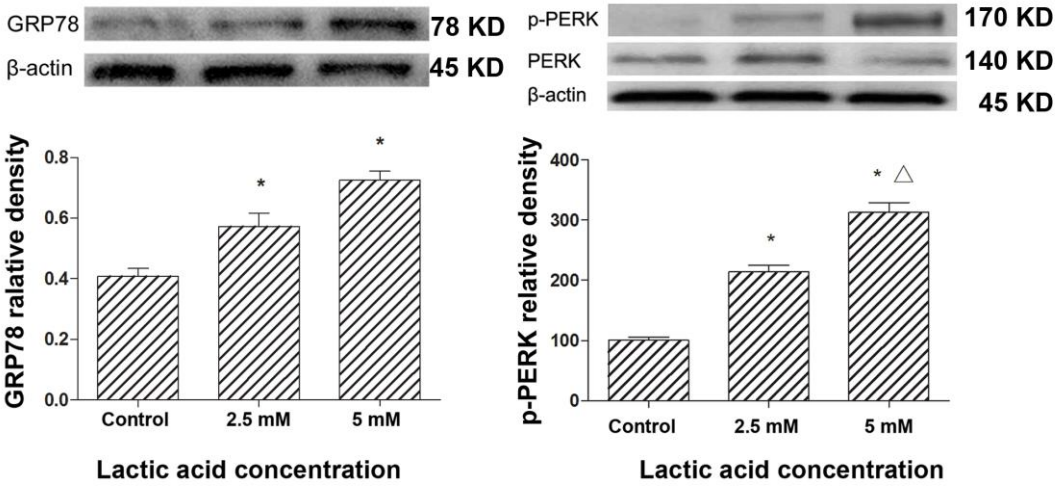

Fig. 2. GRP78, PERK and P-PERK protein levels detected by Western blot in cultured astrocytes. *Upper panels*, representative protein blots of GRP78, p-PERK and PERK; *lower panels*, summarized bar graph showing the target protein bands ratio to actin. Data were collected from 3 independent experiments and presented as means ± s.d. *p ＜0.05 *vs.* control.

Lactic acid enhances phospho-PERK immunoreactivity

Double immune labeling of phospho-PERK (pPERK) and nuclear marker DAPI showed a colocalization (**Fig. 3A**). Furthermore, the number of phospho-PERK positive astrocytes increased significantly in a dose dependent manner when lactic acid was present (**Fig. 3B**).

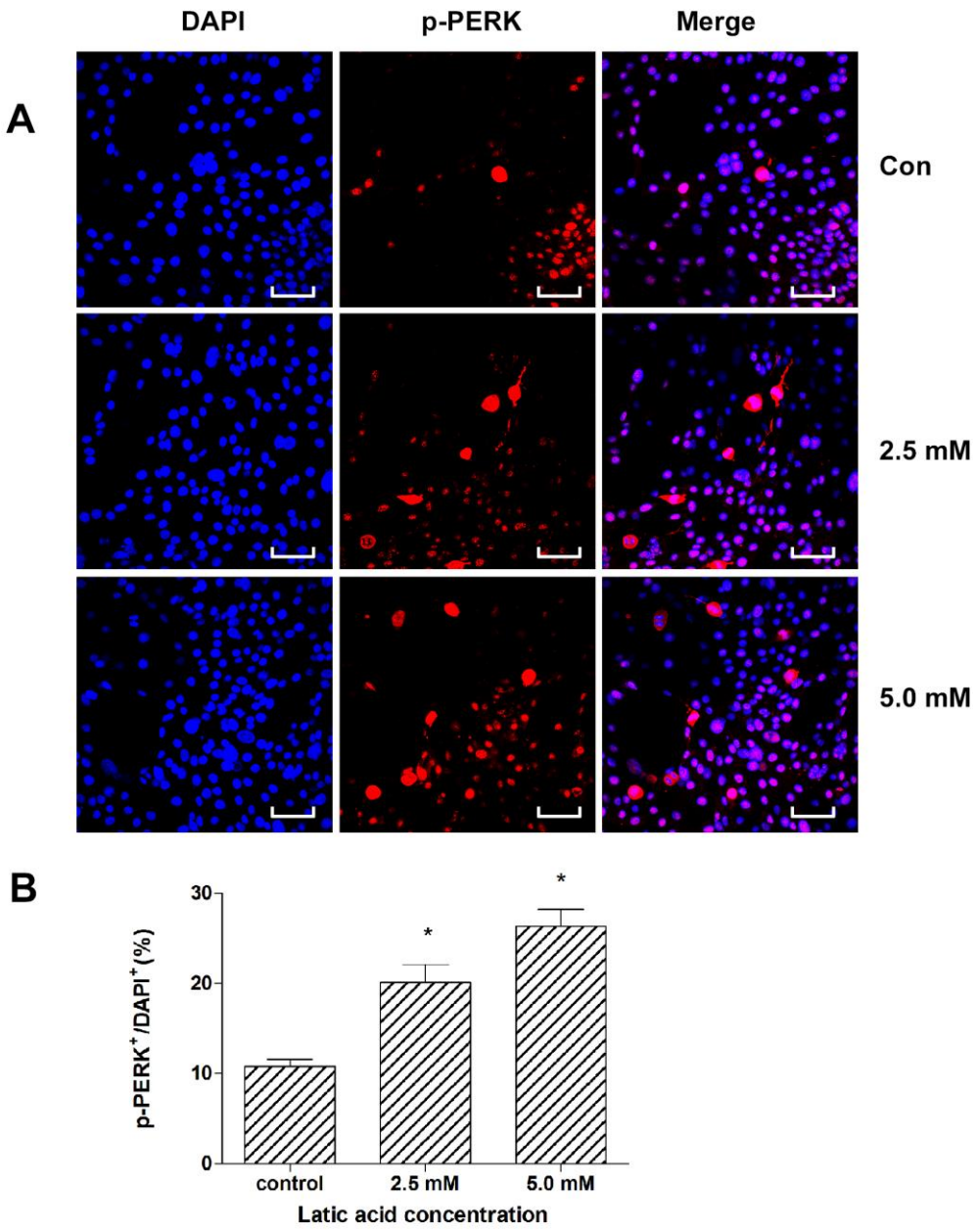

Fig. 3. **A**: Representative immuno-fluorescent microscopy images of p-PERK; **B**: Graph showing quantification of immune fluorescent-positive signal in panel A. Astrocyte was challenged with lactic acid (2.5 mM and 5.0 mM). Data were collected from 3 independent experiments and presented as means ± s.d.*$p<0.05$, compared with non-lactate treated cells and $^{\triangle}p<0.05$, compared with lactate 2.5 mM. Bar =50 μM

Discussion

Astrocytes are main site of glucose consumption and lactate production in a living brain [10,17]. Diabetic hyperglycemia *per se* has been shown to activate astroglial cells as early as 2 weeks following streptozotocin injection [18,19]. However, long lasting and/or fluctuating glucose level diminish astroglial function through high rates of glycolytic activity in astroglia, resulting in dysfunction of astroglia [3]. Under ischemic condition, brain lactate soon accumulated to high level due to anaerobic glycolysis, resulting in tissue acidosis [1,3].

Astrocytes have been shown to be vulnerable to lactate acidosis [12]. Pericytes and endothelial cells and astrocytes are important factors that constitute the BBB. In diabetes, all these tree types of cells are severely impacted [20-22]. Our previous studies have revealed that hyperglycemia suppressed ischemia-induced astrocyte activation, induced withdrawal of the astrocyte end-foot from the cerebral blood vessel walls, resulted in severe demyelination and hindered the remyelination processes [8,13,23]. In the present study, we observed that astrocyte cell viability significantly decreased in the presence of lactic acid, and this detrimental effect exhibited a concentration-dependent manner as assayed by the MTT assay.

The ER is an intracellular organelle that is involved in folding proteins, transporting proteins, regulating intracellular calcium levels [24,25]. Under non-stress conditions, rough ERs efficiently fold newly synthesized proteins. This function is carried out by the ER-resident molecular chaperone GRP78 located in the ER lumen and 3 effectors of the unfoled protein response, PERK, IRE-1 and AFT6. Under ER stress conditions, misfoled and dysfunctional proteins accumulate in the ER lumen, which causes GRP78 to translocate from the 3 effector domains to the misfolded proteins in the ER to assist in folding. Under these conditions, PERK, AFT6 and IRE-1 are activated. In the present study, GRP78 and PERK protein levels significantly increased, suggesting lactic acid elicited ER stress in astrocytes. Phosphorylation of PERK sets off a phosphorylation cascade that culminates in the phosphorylation and inactivation of the translation eukaryotic initiation factor eIF-2α, resulting in protein synthesis being switched off. This process may lead to the deleterious effects on astrocytes viability. The fact that inhibition of ER stress by pharmacological means reduced ischemic damage in diabetic animals [26,27] further support our notion that activation of PERK pathway my mediate the detrimental effects of hyperglycemia on ischemic brain.

Conclusion

Our results suggest that the PERK signaling pathway may be involved in astrocyte damage resulting from lactic acid. This pathway could be a target for therapeutic strategies to reduce brain injury associated with hyperglycemia or diabetes.

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Authors Contributions

The work presented here was carried out in collaboration among all authors. Conceived and designed the experiments: LJ, JZZ, QPH, PAL. Performed the experiments: RY, XMC. Wrote the manuscript: LJ, RY, JZZ, PAL.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Competing Interests

The authors have declared that no competing interest exists.

Li Jing, Ru Yan, Xiang-Mei Cao, Qing-ping He, Jian-Zhong Zhang and P. Andy Li declare that they have no conflict of interest.

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