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ER stress-mediated regulation of immune function under glucose-deprived condition in glial cells: Up- and down-regulation of PGE_2 + IFNγ-induced IL-6 and iNOS expressions

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ABSTRACT

Glucose metabolism plays central role in maintaining brain function. Under ischemic condition, where glucose levels were reduced, glial cells induce pro-inflammatory cytokine production. In the present study, we found prostaglandin (PG) E_2 + interferon (IFN) γ-induced interleukin (IL)-6 production was enhanced under glucose-deprived condition in the primary cultured glial cells. On the other hand, to our surprise, we found that PGE_2 + IFNγ-induced iNOS expression was attenuated under glucose-deprived condition. These dual effects would be mediated through endoplasmic reticulum (ER) stress, because we observed (1) up-regulation of GRP78 and CHOP under glucose-deprived condition, which was inhibited by chemical chaperon TMAO, and (2) treatment with TMAO inhibited IL-6 production under glucose-deprived condition. By activating theses responses glial cells may protect neurons because we observed increased neuronal cell viability in the immune-activated glial cell conditioned medium. Overall, our results suggest a link between ER stress and immune reactions under glucose-deprived condition in the glial cells.

1. Introduction

Under normal conditions, brain uses glucose as a metabolic substrate, for both energetic and biosynthetic needs. However, under the ischemic condition, glucose level in the brain is reduced. Meanwhile, immune function was dramatically activated under the ischemic condition. Immune function was suggested to participate in the diseases, as the local activation of glial cells such as astrocytes and microglia resulted in induction of the production of pro-inflammatory cytokines. Several types of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and TNF-α, were produced under ischemic condition [1,2]. In addition, inducible NO synthases (iNOSs), which produces nitric oxide (NO), was also reported to be increased in the ischemic condition [3]. These observation raises possibility that low level of glucose may play a key role in activating immune reaction. However, the mechanisms regulating immune reaction under glucose-deprived condition has not yet been discovered.

Endoplasmic reticulum (ER) is an organelle, which plays an important role in folding aggregated protein. Under the stressed condition, unfolded protein will be accumulated, which results in ER stress. ER stress is involved in neurodegenerative disorders such as cerebral ischemia, Alzheimer's disease (AD) and Parkinson's disease (PD) [4–6]. Under the ER stressed conditions, cells activate the unfolded protein response (UPR) to cope. Several UPR related genes such as glucose-regulated protein 78 (GRP78) and CCAAT/enhancer-binding protein homologous protein (CHOP) were reported to be activated in cerebral ischemia [7,8]. Furthermore, glucose-deprivation was shown to activate UPR in several types of cells such as in cancer cell [9]. Therefore, glucose-deprivation may activate UPR.

As noted above, both immune reaction and ER stress were activated under glucose-deprived condition. However, the linkage between ER stress and immune reaction under glucose-deprived condition has not yet discovered. In the present study, we found up- and down-regulation of PGE_2 + IFNγ-induced IL-6 and iNOS expressions under glucose-deprived condition. Furthermore, we found that ER stress may play a key role in regulating immune reaction under glucose-deprived condition.

2. Materials and methods

2.1. Materials

Tunicamycin (Tm) was obtained from Wako Pure Chemical Ltd. (Japan). Trimethylamine N-oxide (TMAO) was purchased from Alfa

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Aesar (MA). Glucose-free or glucose-containing (4500 mg/L) DMEM medium was purchased from Life Technologies.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Preparation of primary cultured glial cells

Glial cells were prepared from the whole brains of neonatal C57BL/6 mice as described previously [10]. The cells were allowed to grow to confluency (10 days) in DMEM with 10% FCS, 100 units/ml penicillin G, and 100 mg/ml streptomycin. All cultured cells were kept at 37 °C in 5% CO_2/95% air. Subsequently, mixed glial cells were shaken at 120 rpm for 18 h, and cultured again for 4–6 days in 35-mm dishes. At this point, astrocyte cultures were routinely >95% positive for glial fibrillary acidic protein.

2.4. Measurement of IL-6 levels

The cultured medium was centrifuged at 800*g* at for 5 min 4 °C and the supernatant was used as the sample. IL-6 levels were measured using a ELISA (DuoSet® ELISA Development kit (R&D Systems, USA) according to the manufacturer's directions.

2.5. Western blotting

Western blotting was performed as described previously [11]. Cells were washed with ice-cold PBS and lysed in a buffer containing 10 mM HEPES–NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1% NP-40 for 20 min. The lysate was centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatant was collected. The samples were boiled with laemmli buffer for 3 min, fractionated by SDS–PAGE, and transferred at 4 °C to nitrocellulose membranes. The membranes were incubated with anti-KDEL (StressGen; diluted to 1:1000), anti-CHOP (Santa Cruz; diluted to 1:500) antibodies followed by anti-horseradish peroxidase-linked antibody. Peroxidase was detected by chemiluminescence using an ECL system.

2.6. Measurement of cell viability

Cell viability was measured using WST-1 reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's directions. Forty-eight hours after the stimulation, the cells were incubated with WST-1 reagent for 4 h and then analyzed for optical density.

2.7. Statistics

Results are expressed as the mean ± SE. Statistical analyses were performed using the Student's *t*-test or paired *t*-test.

3. Results

3.1. Glucose-starvation synergistically enhanced PGE_2 + IFNγ-induced IL-6 production

In the present study, we used mouse primary cultured glial cells as the glial cells plays central role in regulating the immune reaction in the brain. We tested the possible link between glucose starvation and inflammation by measuring IL-6 levels in the glial cells. The cells were exposed to glucose-free medium and measured IL-6 levels. We observed IL-6 induction on treating the cells with glucose-free medium (Fig. 1A). Stimulating glial cells with PGE_2 + IFNγ caused a increase in IL-6 production (Fig. 1). Thus, we next investigated the effect of glucose-starvation on PGE_2 + IFNγ-induced IL-6 production. We observed a synergistic effect on PGE_2 + IFNγ-induced IL-6 production under glucose-starved condition (Fig. 1A).

3.2. Glucose-starvation inhibited PGE_2 + IFNγ-induced iNOS induction

We next tested the possible link between glucose starvation and inflammation by measuring iNOS levels in primary cultured glial cells. We did not detect iNOS expression under normal condition in the glial cells. However, stimulating glial cells with PGE_2 + IFNγ caused a dramatic increase in iNOS production (Fig. 1B). Interestingly, in sharp contrast with IL-6, we did not observe induction of iNOS under glucose-starved condition. Furthermore, when the cells were treated with PGE_2 + IFNγ under glucose-deprived condition, iNOS level was dramatically attenuated (Fig. 1B).

Therefore, these results suggest that stress evoked by glucose deprivation causes an enhancement of IL-6 but attenuation of iNOS in immune activated cells.

3.3. Glucose starvation activated UPR

To determine whether glucose starvation could cause ER stress in the present conditions, the glial cells were cultured in glucose free medium and analyzed the expression levels of ER stress-specific UPR-regulated genes. We observed induction of GRP78 and CHOP in glucose-deprived cells (Fig. 2), indicating ER stress.

3.4. Involvement of ER stress on IL-6 production under glucose-deprived condition

To test the possibility that ER stress is involved in IL-6 production under glucose-deprived condition, we treated cells with chemical chaperon, which can reduce ER stress. Trimethylamine-N-oxide (TMAO) is a chemical chaperon which can reduce protein aggregation [12]. We treated cells with TMAO and analyzed the expression level of GRP78 under glucose-deprived condition. As shown in Fig. 3, we observed significant attenuation of GRP78 induction in TMAO treated cells. Therefore, TMAO may be acting

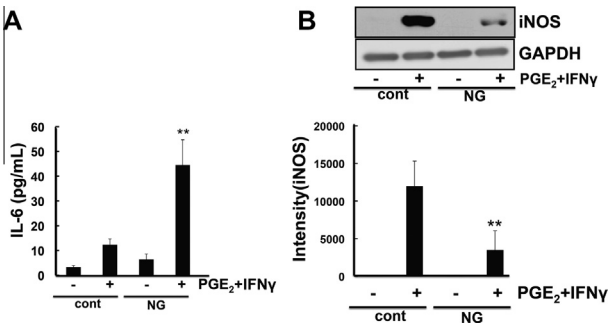

Fig. 1. Up- and down-regulation of PGE_2 + IFNγ-induced IL-6 and iNOS expressions under the glucose-deprivation. (A) Glial cells were cultured with non-glucose (NG) medium, treated with PGE_2 + IFNγ for 48 h, and analyzed for the production of IL-6 by ELISA. Glucose-deprivation enhanced PGE_2 + IFNγ-induced IL-6 production. $^{**}P < 0.01$ (B) Glial cells were cultured with non-glucose (NG) medium, treated with PGE_2 + IFNγ for 48 h, and analyzed for iNOS by Western blotting. Glucose-deprivation inhibited PGE_2 + IFNγ-induced iNOS expression. $^{**}P < 0.01$.

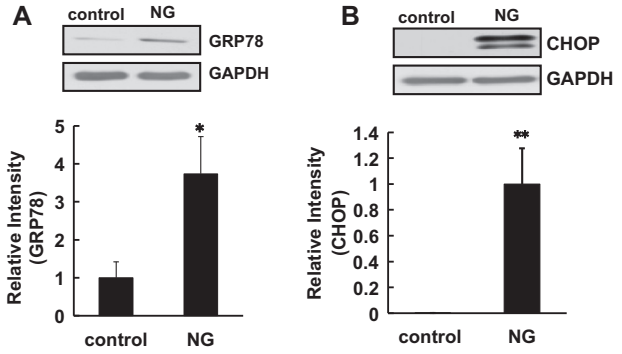

Fig. 2. Glucose-deprivation activated UPR in glial cells. Glial cells were treated with non-glucose (NG) medium for 48 h and GRP78 and CHOP levels were analyzed by Western blotting. Glucose-deprivation activated ER stress in the primary cultured mouse glial cells as evidenced by up-regulation of UPR. $^{*}P < 0.05$, $^{**}P < 0.01$.

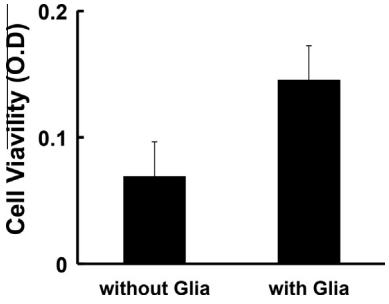

Fig. 4. Neuro-protective effect of the immune activated glial cells under glucose-deprivation. Glial cells were cultured with non-glucose (NG) medium which contained PGE_2 + IFNγ for 48 h. The medium was then transferred to SH-SY5Y human neuroblastoma cells and cellular viability was analyzed 48 h later (with glia). Control experiments were done in a similar fashion except that the medium was cultured without glial cells for 48 h (without glia).

as chemical chaperon, thereby reduced ER stress in the present condition. Under these conditions, we next measured IL-6 levels. IL-6 was induced in the glucose-deprived condition and the effect was significantly attenuated by treating cells with TMAO (Fig. 3). These results suggest that ER stress is involved in glucose starvation induced IL-6 production.

3.5. Glial cells protect against glucose deprivation-induced neuronal cell death

Increasing evidence suggests that neuron–glial communication to play an important role in maintaining normal brain functions [13]. As glial cells affect immune function under glucose-deprived condition, we next investigated the outcome of the observed effect. We examined whether immune-activated glial cells affect neuronal viability under glucose-deprived conditions. To this end, we treated glial cells with PGE_2 + IFNγ under glucose-deprived conditions for 48 h and transfered the medium (glial-conditioned medium) to neuronal cells (SH-SY5Y cell line). The neuronal cells were then incubated for 48 h and cell viability was analyzed using the WST1 assay. As shown in Fig. 4, we observed a recovery of cell viability, when we treated neuronal cells with the glial-conditioned medium (Fig. 4). Thus, it was suggested that immune-activated glial cells can transmit survival signals to neuronal cells under stressed conditions.

4. Discussion

Glucose starvation is a trigger of cellular stress and inflammation in the ischemic condition. However, the underlying mechanisms are unclear. We found that PGE_2 + IFNγ-induced IL-6 expression was enhanced under glucose-deprived condition. Meanwhile, PGE_2 + IFNγ-induced iNOS expression was attenuated. Moreover, we show a previously unknown link; i.e. involvement of ER stress on mediating inflammatory responses in glucose-deprived condition. In the previous study, we reported that ER stress is involved in regulating immune reaction [14]. We found that ER stress inducer, tunicamycin, up- and down-regulated PGE_2 + IFNγ-induced IL-6 and iNOS expressions in the glial cells [14]. Therefore, our present results suggest that ER stress may play a key role in regulating IL-6 and iNOS levels under glucose-deprived condition. These unique findings may provide information important for understanding the mechanisms of pathophysiological mechanism such as cerebral ischemia.

We next addressed the question about the physiological outcome of glial cell response under stressed environment. We observed that neuronal cell viability was increased when the cells were treated with the immune activated glial cells under glucose-deprived condition. In the physiological concentration, NO has been shown to function in neurotransmission and vasodilation [15]. However, if excess amounts of NO were produced, it will

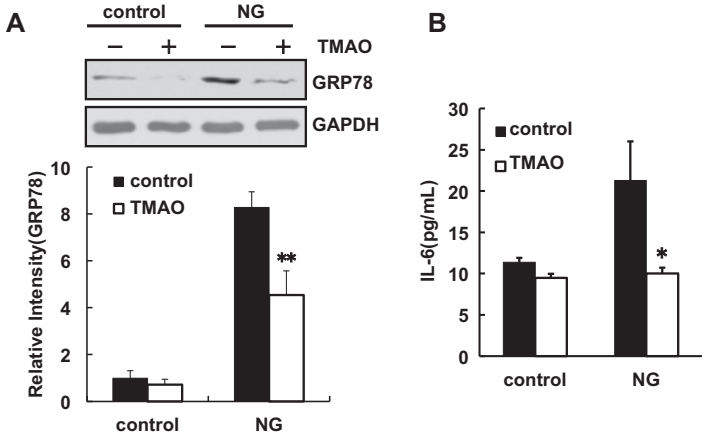

Fig. 3. Involvement of ER stress on non-glucose-induced IL-6 secretion. Mouse primary cultured glial cells were treated with TMAO (150 mM), which was simultaneously treated non-glucose (NG) medium for 48 h. (A) Densitometric analysis of GRP78, which were detected by Western blotting. TMAO attenuated ER stress. $^{**}P < 0.01$ (B) IL-6 levels were detected by ELISA. TMAO inhibited NG-induced IL-6 release. $^{*}P < 0.05$.

cause neuronal cell death [16]. On the hand, IL-6 has neuro-protective property [17–19]. Therefore, reduced amount of NO and increased amount of IL-6 under glucose-deprived condition in the glial cells may contribute in the neuro-protection. Therefore, these observations suggest an important function of glial cells in attenuating neuronal cell death to cope with stress.

The brain utilizes high levels of glucose. However, under cerebral ischemia, brain glucose levels will be lowered. On the other hand, positron emission tomography (PET) has suggested that glucose utilization is dramatically lowered in the AD brain compared with age-matched controls [20,21]. These observations suggest that glucose plays an important role in normal brain homeostasis and that a failure of this homeostasis results in the disease. In the present study, we found the mechanisms of the immune function activated under glucose-deprived condition, which may shed light on the potential pharmacological treatment against the disease.

5. Author contribution statement

T.H. and K.O. designed research; T.H., and T.O. performed research; T.H., T.O. and K.O. analyzed data; and T.H. wrote the paper.

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