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Modulation of endoplasmic reticulum chaperone GRP78 by high glucose in hippocampus of streptozotocin-induced diabetic mice and C6 astrocytic cells

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ABSTRACT

Diabetes mellitus is known to increase the risk of neurodegeneration, and both diseases are reported to be linked to dysfunction of endoplasmic reticulum (ER). Astrocytes are important in the defense mechanism of central nervous system (CNS), with great ability of tolerating accumulation of toxic substances and sensitivity in Ca^{2+} homeostasis which are two key functions of ER. Here, we investigated the modulation of the glucose-regulated protein 78 (GRP78) in streptozotocin (STZ)-induced diabetic mice and C6 cells cultured in high glucose condition. Our results showed that more reactive astrocytes were presented in the hippocampus of STZ-induced diabetic mice. Simultaneously, decrease of GRP78 expression was found in the astrocytes of diabetic mice hippocampus.

In *in vitro* study, C6 cells were treated with high glucose to investigate the role of high glucose in GRP78 modulation in astrocytic cells. GRP78 as well as other chaperones like GRP94, calreticulin and calnexin, transcription levels were down-regulated after high glucose treatment. Also C6 cells challenged with 48 h high glucose were activated, as indicated by increased level of glial fibrillary acidic protein (GFAP). Activated C6 cells simultaneously exhibited significant decrease of GRP78 level and was followed by reduced phosphorylation of Akt. Moreover, unfolded protein response was induced as an early event, which was marked by the induction of CHOP with high glucose treatment, followed by the reduction of GRP78 after 48 h. Finally, the upsurge of ROS production was found in high glucose treated C6 cells and chelation of ROS could partially restore the GRP78 expression. Taken together, these data provide evidences that high glucose induced astrocytic activation in both *in vivo* and *in vitro* diabetic models, in which modulation of GRP78 would be an important event in this activation.

1. Introduction

Diabetes mellitus (DM) is a systemic disease characterized by chronic hyperglycemia and with widespread malicious complications including blindness, renal failure, myocardial infarction and stroke (Brownlee, 2001). Research in the last few years has drawn more attention to diabetes effects on brain for both cerebrovascular disease and neurodegenerative diseases (Roriz-Filho et al., 2009). Multiple clinical studies have shown significant deterioration of cognitive functions including psychomotor efficiency, intelligence, attention and information processing in diabetic patients (Reijmer et al., 2011; Brands et al., 2005; Nooyens et al., 2010). The deficits were found in both type 1 and type 2 diabetic patients and were in part closely related to chronic hyperglycemia. These findings were obtained and evidenced by the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) study and several cross-sectional and longitudinal studies (Jacobson et al., 2007; Wessels et al., 2008; Cukierman-Yaffe et al., 2009). Apart from functional disorders, diabetes can also lead to slow progressive structural

Abbreviations: ER, Endoplasmic reticulum; DM, diabetes mellitus; GRP, glucose-regulated protein; CNS, central nervous system; UPR, unfolded protein response; PERK, PKR-like ER kinase; IRE1, inositol-requiring enzyme 1; ATF6, activating transcription factor 6; HO-1, Heme oxygenase 1; STZ, streptozotocin; H_2O_2, hydrogen peroxide; GFAP, glial fibrillary acidic protein; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; SOD, superoxide dismutases; GPx-1, glutathione peroxidase 1; CAT, catalase; NG, Normal glucose; HG, High glucose; OC, Osmotic control.

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abnormalities in the brain such as brain atrophy and blood brain barrier leakage which can further worsen the brain functioning and thus bring about diabetes related brain diseases (Bruehl et al., 2009; Perantie et al., 2011; Huber, 2008).

Astrocytes have an essential role in the brain and are responsible for maintenance of the extracellular environment, neuronal communication and metabolism, regulation of synaptic activity, regulation of the cerebral microcirculation and protection from various kinds of CNS insults such as oxidative stress and glutamate toxicity (Sofroniew and Vinters, 2010; Araque et al., 1999; Anderson and Nedergaard, 2003; Zonta et al., 2003). These properties of astrocytes made it a good candidate to study the mechanism of diabetes-induced cerebral changes. It was implicated in several reports that early astroglial responses were explicated in rodent diabetic model. Lebed et al. has shown a sequential event of astrocytes activation as early as 2 weeks after STZ-induced diabetes in rat (Lebed et al., 2008). In addition, other recent reports showed that glutamate uptake and gap junction communication of astrocytes were also found to be disturbed in the type 1 diabetic animal model (Ball et al., 2011; Coleman et al., 2004). It is worth noticing that dysfunction of astrocytes can also bring about detrimental consequences to neurons, and both neuronal damage and astrocytes malfunction in turn may contribute in neurodegenerative diseases (Langeveld et al., 1995; Dhandapani et al., 2003).

Despite the vast supporting data connecting diabetes and neurodegeneration, the detailed mechanism is still largely uncertain. Oxidative stress was suggested as the key pathway in the pathogenesis of diabetic complications (Ceriello, 2003). Increased production of free radicals was observed in astrocytes challenged by high glucose condition (Wang et al., 2012) and in brain tissue of the diabetic animals (Celik and Erdogan, 2008; Refaie et al., 2009). Nevertheless, with an exception of α-lipoic acid, traditional antioxidants such as vitamin C and E failed to demonstrate compelling clinical benefits in diabetic complications (Mayer-Davis et al., 2002; Lonn et al., 2002; Liu et al., 2006). This could be the reason that vitamin E and others antioxidants are not enzymatically regenerated and they mainly target on certain but not all the damaging ROS end-products produced in diabetic condition (Du et al., 2000; Cumbie and Hermayer, 2007). Another reason could be due to specificity in tissue response to oxidative stress. Other pathways or genes may also be involved in the pathogenesis of the complications (Scott and King, 2004; Kido et al., 2000).

Unfolded protein response (UPR) is one of the major responses that could be observed in diabetic models. Among various protein chaperones, GRP78 is one of the best known chaperone proteins commonly found in endoplasmic reticulum (ER). It functions as a regulator of ER homeostasis which involves in protein folding, calcium binding, and control of the ER stress signaling sensors. Upon ER stress activation, PERK, IRE1 and ATF6 lose their binding with GRP78 and starts the unfolded protein response (UPR) to combat the stress (Rasheva and Domingos, 2009). In neurons, cardiomyocytes and epithelial cells, GRP78 also acts as a major prosurvival modulator against various insults in cells (Liu et al., 1997; Shintani-Ishida et al., 2006; Qian et al., 2005; Yu et al., 1999). On the other hand, other ER chaperones such as GRP94, calreticulin and calnexin, found in ER lumen, also co-operate with GRP78 to form the protein degradation network (Ni and Lee, 2007). In particular GRP94 could assist GRP78 in controlling degradation of unfolded protein substrates and post-translational activities in affected cells. These two proteins therefore possess anti-apoptotic functions, in which ER stress response could be linked with apoptosis and result in cell death (Ni and Lee, 2007).

Accordingly, the diverse role of GRP78 and other chaperones implicated their involvement in several diseases, and was found with emerging role in cancer progression, neurodegeneration, and diabetes (Wang et al., 2009; Luo et al., 2006; Wang et al., 2009). However, the association of modulation of GRP78 with diabetic associated neurodegeneration has not been explored. Therefore we investigated the involvement of oxidative stress and GRP78, as well as other chaperones during high glucose-induced astrocyte activation in C6 cells and STZ type I diabetic mice hippocampus. And our results have demonstrated the involvement of GRP78 in astrocytic dysfunction and indicated that GRP78 may become a potential therapeutic target for treating diabetic induced neurodegeneration.

2. Methods

2.1. Animals

4 weeks male C57/BL6J black mice weighing 20–25 g were purchased from the Chinese University of Hong Kong. The handling of animal and all procedures were in accordance with National Institutes of Health guide for the care and use of Laboratory animals and Animals (Control of Experiments) Ordinance, Hong Kong, China. The use of animal was approved by Department of Health, Hong Kong (DH/HA&P/8/2/6) and the Committee of the Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University. All efforts were made to minimize animal number and suffering.

2.2. Materials

Anti-GRP78, anti-phospho-PERK, anti-CHOP, and anti-β-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin was purchased from Sigma (St. Louis, MO, USA). Anti-GFAP was purchased from Millipore (Billerica, MA, USA) and Dako (Copenhagen, Denmark). Anti-phospho-Akt (Ser473) and anti-total-Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma (St. Louis, MO, USA).

2.3. Cell culture and treatments

The C6 rat glioma cell line (ATCC cat. number CCL-107) (Rockville, Maryland, USA) was maintained in 5.5 mM D-glucose (normal glucose-NG) DMEM supplemented with 10% FBS, 100 U/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at a humidified atmosphere of 5% CO_2 at 37 °C. Cells were seeded to 96-well plate or 6 well plates for 24 h. Cells were then treated with 5.5 mM (normal glucose-NG), 12 mM, 25 mM, 35 mM, 45 mM D-glucose (high glucose-HG) or 5.5 mM D-glucose + 6.5 mM/19.5 mM/29.5 mM/39.5 mM D-mannitol (mannitol osmotic control-OC) containing DMEM under various time points and conditions as indicated in different individual experiment.

2.4. Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

In order to have a preliminary study of the chaperone expressions in astrocytic cells after high glucose treatment, RT-PCR was used to investigate the transcription activities of various chaperones and anti-oxidative stress gene prior to protein analysis. RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA from each sample was converted to cDNA and expressions of genes were investigated by real-time PCR reactions using SYBR green dye (Applied Biosystems, Foster City, CA, USA) as the reporting signals. Quantitative real-time PCR was performed using ABI Prism 7500 Sequence Detection System (ABI, Foster City, CA) with the