High glucose-induced imbalance of mitochondria-associated ER membranes function promotes RSC96 cell damage

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ABSTRACT

To investigate the effect of high glucose on mitochondrial-related ER membranes (MAMs) in rat Schwann cells (SCs) and the mechanism of cell injury. SCs (RSC96) cells were used as the control group, and RSC96 cells cultured in a high glucose environment for 48 h were set as the experimental group. The level of intracellular calcium was observed by flow cytometry, and ROS levels were detected by DCFH-DA fluorescent probe. The subcellular structure was observed by transmission electron microscopy, focusing on the morphology of mitochondria and endoplasmic reticulum as well as the formation of MAMs. The expression levels of MAMs-related proteins Mfn2, PERK, VDAC1, and IP3R were detected by Western blot. Compared with the control group, after high glucose-induced cells, the level of calcium ion was significantly increased (p<0.01), the level of ROS was significantly increased (p<0.01), mitochondria and endoplasmic reticulum were damaged, and the number of MAMs was increased (p<0.05). Western blot analysis showed that the expression level of Mfn2 was significantly decreased (p<0.01), and the expression levels of PERK, VDAC1, and IP3R were significantly increased (p<0.01). By inducing the imbalance of MAMs function in SCs, high glucose promotes intracellular calcium overload and leads to cell damage.

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Introduction

Neurogenic bladder (NB), a lower urinary tract dysfuncion caused by damage to the central nervous system or peripheral nervous system that controls voiding function, usually occurs in patients with neurological lesions or trauma (1). Diabetes mellitus is one of the important factors predisposing to NB, and diabetic neurogenic bladder (DNB), the most significant complication of the urinary system in diabetic patients, can be as high as 25%-87% (2). The pathogenesis of DNB involves oxidative stress damage, abnormal expression of cell signaling molecules, and impaired neurotransmission pathways in a high-glucose environment (3). High glucose metabolism leads to excessive release of superoxide from intracellular mitochondria, resulting in neurogenic and myogenic alterations of the detrusor muscle, structural and functional dysfunction of the urinary epithelium and urethra, further leading to deterioration of bladder function (4).

With the intensive study of cellular ultrastructure, the structural functions and related roles of mitochondria and endoplasmic reticulum have shown to be important in many diseases (5, 6). Mitochondrial-related ER membranes (MAMs), a newly discovered subcellular structure, abnormalities in the function and structure of MAMs are closely associated with the development of neurological diseases (7, 8). MAMs are composed of multiple functional protein molecules and mediate functional effects, including mitofusin-2 (Mfn2), protein kinase R (PKR) like ER kinase (PERK), voltage-dependent anion channel 1 (VDAC1), inositol 1, 4,5-triphosphate receptor (IP3R), etc (8). These functional proteins are involved in regulating biological processes such as functional homeostasis of MAMs, endoplasmic reticulum stress (ERS), and calcium signaling. However, how high glucose in diabetes mediates peripheral neuropathy is still not clearly elucidated. In this study, we intend to observe the structure of MAMs and the detection of related proteins after high glucose induction in rat Schwann cells (SCs) and provide research ideas and theoretical basis for clinical prevention and treatment of DNB.

Materials and Methods

Cell culture and grouping

RSC96 cells were purchased from American Tissue Culture Collection (ATCC, Rockville, MD, USA). RSC96 was cultured in DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. When the cells were fused to 90%, the old medium was discarded, and the cells were washed twice with 2 mL PBS. After discarding PBS, cells were treated with 0.25% trypsin-0.02% EDTA mixed digestive solution at 2mL. When the cells were rounded, a 2mL full medium was quickly added to terminate digestion and the cells were gently blown up for collection. After centrifugation at 800 g for 5 min at 4°C, the supernatant was discarded and the cells were resuspended in a complete medium.

RSC96 cells were incubated normally for 48 h as the Control group, and RSC96 cells were incubated with a medium containing 150 mmol/L glucose for 48 h as the...
HG group.

**Percoll centrifugation for separation of MAMs**

Approximately $2 \times 10^9$ RSC96 cells were homogenized on ice. 600 g for 5 min and centrifuged to separate the nuclei and unbroken cells. The supernatant was collected and the crude mitochondria (Mc) were separated from the microsomal and endoplasmic reticulum fractions by centrifugation at 7000 g for 20 min. The Mc fractions were suspended in 2 mL of mitochondrial resuspension buffer (MRB, pH 7.4) and covered with a layer of 30% percoll, then centrifuged at 95,000 g for 30 min. MAMs were extracted from the percoll in a gradient. all fractions were stored at -70°C for use.

**Detection of intra-mitochondrial calcium ion concentration by flow cytometry**

RSC96 cells were collected, washed twice with PBS, and transfected with Fluo-3 AM (5 μM) for 30 min at 37°C under light-proof conditions. Cells were then washed three times with buffer without probe to fully remove residual Fluo-3 AM working solution. The green fluorescence signal was observed by flow cytometry at an excitation wavelength of about 488 nm and an emission wavelength of 525-530 nm.

**Intracellular ROS assay**

Experiments were performed using the ROS assay kit. DCFH-DA was diluted with serum-free culture medium at 1:1000 to a final concentration of 10 μM. RSC96 cells were collected and suspended in diluted DCFH-DA at a concentration of $1 \times 10^6$ cells/mL and incubated for 20 min at 37°C in a cell incubator protected from light. The cells were washed three times with a serum-free cell culture medium to fully remove the DCFH-DA that had entered the cells. Then the fluorescence intensity was analyzed at excitation 488 nm and emission 525 nm using a multifunctional enzyme marker.

**Transmission electron microscopy experiments**

RSC96 cells were fixed with 1 mL of 2.5% glutaraldehyde for 1 h, followed by 0.5 mL of 1% starvation acid for 1 h, followed by rinsing with PBS solution. The samples were dehydrated, embedded, sectioned to 70 nm thickness, stained with lead citrate for 10 min and then uranyl acetate for 30 min, and images were obtained using transmission electron microscopy. Set to a high resolution of 40,000-80,000×, the number of MAMs was recorded, and the mitochondrial and endoplasmic reticulum morphology was observed.

**Western blot analysis**

Samples of MAMs obtained by lysing the extracts with a protease inhibitor mixture in radioimmunoprecipitation assay (RIPA) buffer were subsequently quantified by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). The protein expression was also analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After incubation with primary and secondary antibodies (Table 1), the membranes were observed using an enhanced chemiluminescence system. ImageJ software was used to quantify the strength of the immunoblot bands.

**Statistical analysis**

The experimental data were analyzed and plotted using Graphpad Prism 9 (Version 9.4.0, La Jolla, CA, USA), and Adobe Illustrator 2022 (Version 2022) was used for collation. All data were expressed as means ± SD, and statistical differences between groups were determined by one-way ANOVA and Tukey’s test, and P values less than 0.05 were considered significant.

**Results**

**Effect of high glucose on Ca$^{2+}$ level in RSC96 cells.**

RSC96 cells were incubated with 150 mmol/L glucose for 48 h. As shown in Figure 1, flow cytometry analysis showed that high glucose-induced a significant increase in calcium concentration in RSC96 cells ($P<0.01$), which was 3.04 times that of the control group.

**Effect of high glucose on ROS level in RSC96 cells.**

As displayed in Figure 2, under the induction of a high glucose environment, the ROS level of RSC96 cells increased significantly ($P<0.01$), which was 1.30 times

![Figure 1](image1.png)  
**Figure 1.** The effect of high glucose on Ca$^{2+}$ level in RSC96 cells.  
The level of Ca$^{2+}$ (A, B) was detected by flow cytometry. Results were mean ± SD for three individual experiments. Compared with the control, **$P<0.01$.**

**Table 1.** Antibodies and dilution.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution (application)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mfn2</td>
<td>1:2000 (WB)</td>
</tr>
<tr>
<td>PERK</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>VDAC1</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>IP3R</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>CNX</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>Rabbit Anti-Mouse IgG H&amp;L (HRP)</td>
<td>1:10000 (WB)</td>
</tr>
<tr>
<td>Goat Anti-Rabbit IgG H&amp;L (HRP)</td>
<td>1:10000 (WB)</td>
</tr>
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shown that cell damage and apoptosis of peripheral nerve tissue are involved in the pathogenesis of diabetic peripheral neuropathy. Schwann cells are myelin-forming cells of the peripheral nervous system and play an important role in maintaining the structure and function of peripheral nerve tissue.

Effect of high glucose on the structure of MAMs in RSC96 cells.

The TEM images of the microscopic morphology of RSC96 cells are presented in Figure 3, compared with the control group, the structure of MAMs in RSC96 cells induced by high glucose was more compact, and the number of MAMs increased significantly (Figure 4). In addition, obvious mitochondria and endoplasmic reticulum damage can be observed, manifested as mitochondrial swelling and destruction, mitochondrial cristae vacuolization, and disappearance, while the endoplasmic reticulum cavity was significantly enlarged and swollen.

High glucose can induce the imbalance of MAMs function in RSC cells.

As shown in Figure 5, we found that the expression levels of Mfn2, PERK, VDAC1, and IP3R proteins were significantly affected after high-glucose-induced RSC96 cells. Compared with the control group, the expression level of Mfn2 in RSC96 cells in the HG group was significantly decreased ($P<0.01$), suggesting that the function of MAMs was unbalanced. The protein expression levels of PERK, VDAC1, and IP3R were increased ($P<0.01$). The up-regulated expression of the above MAMs junction proteins suggested the increase of MAMs formation and mediated cell damage through different pathways.

Discussion

Diabetic neurogenic bladder (DNB), also known as diabetic bladder dysfunction or diabetic cystopathy, is one of the common complications of diabetes (9, 10). The pathogenesis of DNB is complex and may be closely related to high-glucose-induced oxidative stress, inflammatory response, microcirculatory dysfunction, and metabolic abnormalities. High glucose-induced increased oxygen radical formation, activation of polyol pathways and accumulation of late glycosylation products lead to nerve axon degradation, which in turn leads to peripheral neuropathy, and bladder afferent nerve damage leads to decreased bladder filling (11, 12). Numerous studies have shown that cell damage and apoptosis of peripheral nerve tissue are involved in the pathogenesis of diabetic peripheral neuropathy. Schwann cells are myelin-forming cells of the peripheral nervous system and play an important role in maintaining the structure and function of peripheral nerve tissue.

**Figure 2.** The effect of high glucose on ROS level in RSC96 cells. DCFH-DA ROS fluorescent probe was used to detect the level of ROS. Results were mean ± SD for three individual experiments. Compared with the control, **$P<0.01$.**

**Figure 3.** The effect of high glucose on MAMs in RSC96 cells. The morphology of MAMs in RSC96 cells was observed by transmission electron microscope, Bar=2 μm.

**Figure 4.** The effect of high glucose on the number of MAMs in RSC96 cells. Results were mean ± SD for three individual experiments. Compared with the control, *$P<0.05$.**

**Figure 5.** The effect of high glucose on RSC96 cells. RSC96 cells were incubated with 150 mmol/L glucose for 48 h. The expression of Mfn2 (A, B), PERK (A, C), VDAC1 (A, D) and IP3R (A, E) from the indicated group were detected by Western Blot assay. Results were mean ± SD for three individual experiments. Compared with the control, **$P<0.01$.**
nerves (13, 14). The mitochondrial pathway, endoplasmic reticulum pathway and death receptor pathway are important pathways of hyperglycemic oxidative stress-induced apoptosis in Schwann cells. Mitochondria and some endoplasmic reticulum regions are highly co-localized intracellularly, and both form close physical and functional connections through MAMs, which play important roles in oxidative stress, changes in mitochondrial dynamics, ERS, apoptosis, and other life processes (15, 16). Disruption of MAMs integrity leads directly or indirectly to an imbalance in Ca\(^{2+}\) homeostasis and induces ERS and oxidative stress. The presence of MAMs facilitates the transfer of Ca\(^{2+}\) from the endoplasmic reticulum to the mitochondria, while excess Ca\(^{2+}\) interferes with the mitochondrial oxidative phosphorylation process, thereby increasing the production of ROS (14, 17).

MAMs can regulate endoplasmic reticulum stress and activate apoptotic pathways in peripheral neural tissues through pathways such as PERK (18). Mfn2 plays a key role in maintaining the morphological and functional integrity of MAMs (19, 20). It can affect Ca\(^{2+}\) transmission signals between mitochondria and the endoplasmic reticulum, and Mfn2 deficiency can promote endoplasmic reticulum stress responses (18, 21). Loss of function of Mfn2 leads to sustained activation of the PERK pathway, and increased ROS levels, causing mitochondrial calcium overload and functional impairment (22, 23). Ca\(^{2+}\) is released from the endoplasmic reticulum via IP3R and absorbed into the mitochondria via VDAC1 in the outer mitochondrial module, resulting in calcium overload (24, 25). The results of this study showed that high glucose-induced a significant increase in calcium ion levels in RSC96 cells. It is suggested that high glucose induces calcium overload in glial cells, exerts cytotoxic effects, and may lead to nerve damage. High glucose-induced ROS increase in RSC96 cells. Excessive ROS will cause oxidative damage to nucleic acids, proteins, and biofilms of cells, leading to changes in intracellular redox homeostasis, oxidative stress, and cell damage and apoptosis. In addition, high glucose regulates the function of MAMs by affecting the expression of Mfn2 in RSC96 cells, and the occurrence of mitochondrial and endoplasmic reticulum damage was observed, which may be closely related to the regulation of PERK, VDAC1, IP3R, and other proteins.

In this paper, high glucose may activate endoplasmic reticulum stress by regulating the function of MAMs in SCs cells, prompting mitochondrial calcium overload, inducing an increase in ROS levels and triggering cell injury. However, there are limitations in this study, such as the need to verify that high glucose modulates the function of MAMs mediating peripheral nerve injury in vivo through animal models. In conclusion, this study may provide an important reference for uncovering the pathogenesis of DNB and provide theoretical support for exploring the subcellular structure and function mediating peripheral neuropathy.

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Authors’ contributions
Housheng Fu and Jianbing Xu contributed equally to this work.

Conflict of interests
The authors declared no conflict of interest.

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