Effect of High Glucose Concentration on Mitochondrial Metabolism in Neuronal Cells

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Abstract: Mitochondrial dysfunction caused by elevated extracellular glucose may play an important role in the pathogenesis of diabetic neuropathy. In this study, we investigated the effect of high glucose both on the mitochondrial respiratory chain and on the energy production in SH-SY5Y neuroblastoma cells, a model of peripheral neurons. We first measured total oxygen consumption rate of the cells cultured in various glucose concentrations using a polarographic method. We then measured the activity of each respiratory chain complex of the mitochondrial fractions of the cells cultured in either normal or high glucose. Finally, we measured the accumulation of the reactive oxygen species (ROS) in the cells. The total oxygen consumption of cells in high glucose concentration decreased in a dose- and time-dependent manner. Inhibitors of mitochondrial electron transport system reduced the oxygen consumption, while antioxidants improved it. We observed a significant decrease in the activity of respiratory chain complex I and complex III+IV of the cells in high glucose. We also observed an increase of ROS in the cells cultured in high glucose for more than 2 days. In conclusion, we propose that the impairment of the mitochondrial respiratory chain and the decrease in the energy production caused by oxidative stress in high glucose may be closely related to the pathogenesis of diabetic neuropathy.

Key words: diabetic neuropathy, mitochondrial dysfunction, oxidative stress, reactive oxygen species, apoptosis

INTRODUCTION

Metabolic factors such as activation of polyol pathway or protein kinase C cascade, and vascular factors such as endoneuronal hypopfusion or hypoxia have been implicated for the pathogenesis of diabetic neuropathy1-3). Recently, mitochondrial dysfunction has become more postulated as a new candidate for diabetic neuropathy, since Low et al. reported a reduction of oxygen consumption in rat sciatic nerves using a chronic model of diabetic endoneuronal hypoxia4) and others reported an increase of lactate level in sciatic nerves of diabetic rats5,6). Moreover, Suzuki et al. demonstrated an increase in mitochondrial DNA deletions in proportion to the severity of diabetic complications7,8), and Kaur et al. observed a decrease in mitochondrial electron transport in the brains of diabetic rats9). Despite these potential implications of mitochondrial dysfunction in the pathogenesis of diabetic neuropathy, there has been no study that directly demonstrated the correlation between the dysfunction of the energy production in peripheral nerves and the exposure to the high glucose levels. Therefore,
we investigated the direct effect of a high glucose milieu on the mitochondrial respiratory chain complexes and the energy production system using SH-SY5Y neuroblastoma cells, a model of peripheral neurons.

**MATERIALS AND METHODS**

**Cell culture**

SH-SY5Y neuroblastoma cells were cultured for 5 to 7 days in various glucose concentrations with Dulbecco’s modified Eagle medium (DMEM) containing 10% calf serum (Gibco BRL, NY, U.S.A.). The media was changed every other day until day 5. Thereafter, it was changed every day. Various agents were added from day 5 to 24 hours prior to the collection of cells.

**Determination of total oxygen consumption**

After trypsinization, cells were centrifuged and suspended in phosphate buffer saline (PBS). For the determination of total oxygen consumption, 3 ml of the cell suspension was used. Oxygen consumption rates were measured using Granger’s method with YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Inc., OH, U.S.A.). The determined oxygen consumption rates were normalized by the atmospheric pressure and the temperature of the incubator, and by the cell numbers calculated with automatic cell counter (Erma Automatic Cell Counter Model PC-602A, Erma Inc., Tokyo, Japan).

**Isolation of mitochondrial fractions and determination of activities of mitochondrial respiratory chain complexes**

All the steps for the isolation were carried out at 0–4°C. The cells cultured for 7 days were trypsinized, suspended in DMEM, and centrifuged. The cell pellet of 1 ml was resuspended in 11 ml of hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5), and transferred to a 15 ml Dounce homogenizer kept on ice. After 10 min, the cell suspension was homogenized by 15 strokes of the pestle. An 8 ml aliquot of hypertonic buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5) was added to make the suspension isotonic, then 10 ml of isotonic buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5) was added to bring the volume to 30 ml. The homogenate was centrifuged at 850 x g for 5 min to remove nuclei and unbroken cells. This procedure was repeated twice. The last supernatant was transferred to a clean centrifuge tube and the mitochondrial fraction was sedimented by centrifugation at 19,000 x g for 15 min. The precipitated pellet was resuspended in isotonic buffer and 19,000 x g sedimentation was repeated. The pellet was suspended in 2 ml of respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, pH 7.2, 5 mM KH₂PO₄, pH 7.2) for the following determination.

**Determination of the activity of each mitochondrial respiratory chain complex**

For determining the activity of each mitochondrial complex, 2 ml of mitochondrial suspension was maintained at 37°C throughout the experiment. The substrate and inhibitor of each complex were added into the chamber in the following order: 1) 5 mM pyruvate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and malate (Sigma Chemical Co., MO, U.S.A.) (complex I substrate); 2) 250 nM Rotenone (ICN Biomedicals Inc., OH, U.S.A.) (complex I inhibitor); 3) 5 mM succinate (ICN Biomedicals Inc., OH, U.S.A.) (complex II substrate); 4) 0.4 mM TTFA (thenoyltrifluoro-acetone, ICN
Biochemicals Inc., OH, U.S.A.) (complex II inhibitor); 5) 5 mM L-α-glycerophosphate (Sigma Chemical Co., MO, U.S.A.) (complex III substrate); 6) 50 nM Antimycin A (Sigma Chemical Co., MO, U.S.A.) (complex III inhibitor); 7) 0.4 mM TMPD (N, N, N’, N’-tetramethyl-p-phenylene-diamine, ICN Biomedicals Inc., OH, USA) (complex IV substrate). The changes in oxygen consumption rate in the presence or absence of inhibitor of each respiratory complex were calculated and regarded as the activity of each complex. The measured consumption rates were normalized by the atmospheric pressure, by the temperature of the chamber, and by the protein concentrations of the mitochondrial suspension.

**Determination of intracellular ATP**

For the determination of intracellular ATP content, 200 μl of suspension was saved from the cell suspension used for oxygen consumption analyses. The sample was centrifuged at 12,000 x g for 60 sec and the pellet was dissolved in 100 μl of 0.1 N NaOH and heated for 5 min at 94°C, followed by centrifugation at 12,000 x g for 5 min. The supernatant was stored at 80°C. ATP concentrations were measured by an enzymatic method, and the results were normalized to the protein concentrations.

**Determination of intracellular lactate and pyruvate**

Cells were collected and suspended in PBS. The suspensions were stored at -20°C. Prior to use, the samples were thawed and sonicated. The concentrations of lactate and pyruvate in the samples were measured by an enzymatic method, and the results were normalized to the protein concentrations.

**Determination of total oxygen consumption of cells exposed to antioxidants**

Cells were cultured in the presence of either 5.5 mM (100 mg/dl) or 30 mM (540 mg/dl) glucose for 5 days, then, one of the following antioxidants was added to one half of the culture dishes; 50 μg/ml α-tocopherol acetate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 μM α-lipoic acid (Sigma Chemical Co., MO, U.S.A.) or 35 μg/ml L-ascorbic acid (ICN Biomedicals, OH, U.S.A.). The cells were exposed to the antioxidants for 3 days. Total oxygen consumption of cells exposed to antioxidant was determined.

**Determination of intracellular reactive oxygen species (ROS)**

The cells were cultured in the presence of 5.5, 30, or 50 mM (900 mg/dl) glucose for 1, 2 or 3 days. The cells were collected and suspended in D-PBS (PBS lacking Ca²⁺ and Mg²⁺, and with 0.1% gelatin). The suspension was filtered through a layer of nylon mesh and incubated for 15 minutes at 37°C with 50 μM DCFH-DA (2’,7’-dichlorofluorescin, Molecular Probes Inc., OR, U.S.A.), followed by 5 mM EDTA. The intensities of fluorescence in the cells were measured using a flow cytometer (FACS Caliber, Becton Dickinson and Co., NJ, U.S.A.).

**Protein assay**

The protein concentrations of the mitochondrial fractions and the cell suspensions were determined with a biocinchoninic acid protein assay kit (Sigma Chemical Co., MO, U.S.A.).

**Statistical Analyses**

Statistical analyses were performed on replicate (n = 5 or 6) samples, determined from individual wells, in one experiment in Figs. 1–4, and Tables 1 and 2. The difference among the
groups was evaluated for significance by one-way analysis of variance (ANOVA) in experiments of Figs. 1, 3, 4, and Table 1 and 2, whereas that was tested by two-way ANOVA in experiment of Fig. 2 followed by post hoc comparison test (Schaffe’s F test) using Stat View software (Stat View J 4.5, Abacus Concepts, Inc. CA, U.S.A.).

RESULTS

Concentration-dependent effect of glucose on total oxygen consumption

The oxygen consumption rate of the cells cultured for 7 days in the presence of 5.5 mM glucose was $12.5 \pm 1.5 \times 10^{-5}$ mol O2/10^6 cells/10 min, while those of cells cultured in 10, 20 or 30 mM (180, 360, 540 mg/dl) glucose were $15.7 \pm 1.8, 9.0 \pm 1.0, \text{ and } 7.2 \pm 1.5 \times 10^{-5}$ mol O2/10^6 cells/10 min, respectively (mean \pm SD). The oxygen consumption rate decreased significantly in a dose-dependent manner above 20 mM as shown in Fig. 1.

Time-dependent effect of glucose on total oxygen consumptions

Total oxygen consumption rates were measured for various periods at various concentrations of glucose. The results are shown as percentage of total oxygen consumption rates at 5.5 mM glucose (Fig. 2). When exposed to 20 mM glucose, the total oxygen consumption rate fell significantly at day 7. As the concentration of glucose increased, the time period to obtain a significant reduction in total oxygen consumption was shortened. For example, when exposed to 50 mM glucose, total oxygen consumption rate fell significantly at day 3.

Effects of mitochondrial respiratory chain inhibitors on the total cellular oxygen consumption

We compared the total oxygen consumption rates of cells cultured for 7 days in either normal (5.5 mM = 100 mg/dl) or high (30 mM = 540 mg/dl) glucose. On day 5, we added either 50 nM Rotenone, 35 μg/ml TTFA, or 40 nM Antimycin A. The inhibitor concentrations were maintained until day 7. Total consumption rates

![Fig. 1](image-url)
were expressed as the percentage of the rate in cells cultured in 5.5 mM glucose. Rotenone and Antimycin A significantly inhibited the oxygen consumptions of the cells, which was independent of the concentrations of the glucose level (Fig. 3). However, the oxygen consumption rates of cells exposed to TTFA did not decrease significantly (Data not shown).

Inhibitory effect of high glucose on mitochondrial respiratory complexes

To identify the site whereby high glucose impairs mitochondrial function, we determined the activity of each mitochondrial respiratory chain complex in cells cultured for 5-7 days in either 5.5 or 30 mM glucose. We found that the activities of complex I and complex III + IV of the cells cultured in 30 mM glucose were significantly lower than those in 5.5 mM glucose. We did not observe significant difference in the activity of complex II (Table 1).

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Effect of high glucose on ATP production and anaerobic metabolism

We compared the intracellular ATP concentration of cells cultured for 7 days in either 5.5 or 30 mM glucose. The ATP content of cells in 30 mM glucose was significantly lower than in cells in 5.5 mM glucose (Table 2). We also compared lactate content under both conditions. The lactate content in the cells cultured in 30 mM glucose were significantly higher than those of cells cultured in 5.5 mM glucose (Table 2). The lactate/pyruvate ratio in 30 mM glucose was also significantly higher than that in 5.5 mM glucose.

Effect of antioxidants on the oxygen consumptions of the cells

We examined the effect of antioxidants on total oxygen consumption of cells. The total oxygen consumption rates were expressed as percentages of the oxygen consumption of cells cultured in 5.5 mM glucose without antioxidant. All antioxidants inhibited the reduction in oxygen consumption rates caused by high glucose. The recovery of the total oxygen consumption was significant for α-tocopherol acetate (vitamin E) and α-lipoic acid (Fig. 4).

Effect of high glucose on the ROS accumulation inside the cells

Accumulation of ROS (in fact we measured the peroxidase production in cells) increased in cells cultured in 30 or 50 mM glucose compared with cells cultured in normal (5.5 mM) glucose (Fig. 5).
DISCUSSION

Prior to the current investigations, there was no direct evidence of a decrease of oxygen consumption in peripheral neurons under high extracellular glucose. Using SH-SY5Y cells as a model of peripheral neurons, we found that total oxygen consumption decreases when neurons are exposed to high glucose environment. Oxygen consumption is thought as an index of mitochondrial function, because mitochondria is the major oxygen consuming organelle in the cell. The fact that mitochondrial respiratory chain inhibitors reduced the oxygen consumptions, confirms the relevance of mitochondrial respiratory chain to the oxygen consumption of the cells. In other words, the decrease in oxygen consumption under high glucose is probably caused by mitochondrial dysfunction.

In this study, we directly demonstrated that a high glucose environment significantly impairs the activities of mitochondrial complex I and complexes III + IV in SH-SY5Y cells. This result is compatible with the previous study of Kaur and Bhardwaj, in which they demonstrated the decrease in oxygen consumptions in complex I, III and in IV, and increase in complex II in the brain of diabetic rats\(^9\), indicating that high glucose can induce mitochondrial dysfunction in the peripheral nerves besides the central nervous system.

High glucose also impaired intracellular ATP production and enhanced anaerobic energy...
metabolism, indicating that the decrease in energy production in high glucose may be a result of mitochondrial dysfunction. Indeed, enhanced anaerobic metabolism has been reported previously in the sciatic nerves of diabetic rats\(^5,6\). Although a decrease in ATP has not yet been observed, the decrease in phosphocreatine, another bioenergy generating metabolite has been reported in sciatic nerves or superior cervical ganglia\(^5,6,15\).

Mitochondrial dysfunction or disorder of aerobic energy metabolism involves four phases, including: transport system of substrates used in TCA cycle; dehydrogenation system (pyruvate dehydrogenase and TCA cycle); electron transport system (complex I–IV); and ATP synthetase (complex V)\(^6,7\). As shown in our study, we confirmed that there is an impairment in part of electron transport system and ATP synthesis under high glucose.

Mitochondrial respiratory dysfunction may be induced by oxidative stress\(^18–21\). In fact, several studies have reported that some antioxidants have a beneficial effect on diabetic complications including diabetic neuropathy\(^6,22–24\). More importantly, our studies with antioxidants and our ROS measurements support this hypothesis. For these reasons, we postulate that oxidative stress in a high glucose environment can cause at least part of mitochondrial dysfunction, which leads to the decrease in ATP production, and eventually the impairment of nerve function.

It seems natural that ATP reduction causes functional deficiency in neuronal cells, because almost all activities in living organism are driven with the energy yielded by the ATP hydrolysis\(^16\). For example, the Na\(^+\), K\(^+\)-ATPase which maintains the membrane potential, various intracellular signaling kinases, neurotransmitter synthesizing enzymes, the axonal transport system, and neurotransmitter exocytosis, all consume ATP\(^16\).

About two thirds of the ATP produced are utilized for the Na\(^+\), K\(^+-\)ATPase activity. So, the decrease in ATP production might be most related to decline in Na\(^+\), K\(^+-\)ATPase activity, which has been reported in diabetic state and thought to play a important role in the pathogenesis of diabetic neuropathy\(^25\). Indeed, Na\(^+\), K\(^+-\)ATPase activities are reduced and nerve conduction velocities are decreased in diabetic patients and diabetic rats\(^26,27\). Similarly, we reported that the activity of Na\(^+\), K\(^+-\)ATPase decreases in SH-SY5Y in the presence of elevated glucose\(^28,29\). Voltage clamp analysis in rat single myelinated nerve fibers suggests that decreased Na\(^+\), K\(^+-\)ATPase activity along with high glucose leads to the reduction in conduction velocity in diabetic neuropathy\(^30\). In addition to these effects on the Na\(^+\), K\(^+-\)ATPase, a recent study showed that ATP increases the activity of capsaicin-activated channel, which helps generate cognitive signals\(^31\). Thus, ATP reduction can cause insufficient cognitive signals.

Recently, several reports indicated that apoptosis plays an important role in the pathogenesis of diabetic neuropathy\(^32,33\), and that mitochondrial dysfunction, or reduction in mitochondrial transmembrane potential causes apoptosis in dorsal ganglia cells in diabetic rats\(^34,35\). We suspect that the mitochondrial complex deficiency we have observed may cause the reduction in mitochondrial membrane potential observed in these studies.

Although SH-SY5Y cells are usually regarded as a model of peripheral nerve cells, they have characteristics of autonomic nerve cells rather than sensory nerve cells\(^36,37\). Given this, we suspect that the mitochondrial dysfunction caused by high glucose that we observed in SH-SY5Y
cells is most relevant to diabetic autonomic neuropathy. Finally, we propose that mitochondrial respiratory dysfunction and a following decrease in energy production under high glucose, mediated by oxidative stress, impairs various neuronal cell functions, and participates in the pathogenesis of diabetic neuropathy.

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