Original Research Article

Hyperglycemia-induced intramitochondrial glycogen granules: A potential mechanism of glucose cytotoxicity in brain of mice

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Abstract

Aim: The main objectives of this work were to prove that subcutaneous injection of high doses of glucose can lead to occurrence of glycogen granules inside ultrastructurally changed mitochondria of mouse cerebral cortex and to check whether blocking of mitochondrial permeability transition pore (MPTP) by cyclosporine A would diminish occurrence of these granules inside some mitochondria. By this, we aimed to explore if hyperglycemia-induced intramitochondrial glycogen granules (HIMG) may represent a molecular pathway through which hyperglycemia may lead to dysfunction of brain mitochondria.

Materials and Methods: Electron microscopic studies and histopathological investigations have been carried out. We then incubated samples of brain cortex of mouse injected with high doses of glucose in alpha-amylase solvent or disolvent alone before being subjected to microscopic examination.

Results: Electron microscopy experiments established that the observed granules are built of glycogen. It has been also demonstrated that blocking of MPTP by cyclosporine A diminished occurrence of glycogen inside some mitochondria in cerebral cortex, thus inhibiting hyperglycemia-induced apoptotic signaling that results from increased vulnerability of mouse brain mitochondria. Concurrently, cyclosporine A partially suppressed the histopathological changes of brain cortex of these animals.

Conclusions: Taken together, this study indicates that cytotoxicity of hyperglycemia might occur through HIMG and we postulate this as a key molecular pathway through which hyperglycemia may lead to dysfunction of brain mitochondria. This is the first report showing HIMG as a cytotoxic molecular mechanism in mouse model.

Keywords: Cyclosporine A, glycogen, hyperglycemia, mice, mitochondria.

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INTRODUCTION

During the last decade, the functional and metabolic roles of glycogen in the brain have been constantly suggested.[1-3] In the view of involvement of this polysaccharide in learning and memory and its implication in neurodegenerative diseases (NDDs),[4] it is important to explore further

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molecular insight into the potential deleterious effects of glycogen in brain, focusing on mitochondrial targets.

Concomitantly, diabetes mellitus and insulin resistance have been shown to impair brain and peripheral neuronal mitochondria by diminishing the activity of complexes III, IV, and V of the respiratory chain and adenosine triphosphate synthesis with increased oxidative and nitrosative stress.[5] In addition, *in vitro* study demonstrated that exposure of cultured dorsal root ganglion neurons to elevated glucose concentrations caused fragmentation/fission of mitochondria and activation of Bim and Bax followed by apoptosis.[6] More *in vitro* studies showed that diabetes-induced mitochondrial dysfunction is mainly attributed to hyperglycemia rather than to insulin resistance.[7,8] Further, calorie restriction has been demonstrated to improve brain mitochondrial respiratory capability and to avoid loss of neurons.[9]

Our previous study demonstrated, for the first time, the occurrences of glycogen-like granules inside some mitochondria of hippocampus and cortex following administration of high doses of glucose.[10] It is well established that, under normal conditions, glycogen can be solely stored in the cytoplasm, as both glycogen synthase and glycogen phosphorylase are found therein, i.e., glycogen should not be found inside mitochondria.[11] Most strikingly, we observed that morphology of all mitochondria containing glycogen granules was significantly altered and partly damaged.[10] For that reason, we hypothesized that elevated intracellular glucose might devastate the integrity of mitochondrial membrane and consequently opening of mitochondrial permeability transition pore (MPTP), through which glucose and cytoplasmic enzymes transport into the mitochondria and within synthesis glycogen. Noteworthy, as far as we know, our previous work was the first *in vivo* study that evaluated the impact of high doses of glucose on the ultrastructure of animals' brain. Opening of MPTP is believed to be linked with pathogenesis of necrosis and apoptosis which result in reversible or irreversible cell loss;[12] for example, neurodegeneration.[13] Indeed, a number of studies have found that chronic NDDs are mediated through mitochondrial dysfunction, i.e., impairment of mitochondrial morphology has been considered as a causal feature in NDD such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.[14,15]

If correct, our mentioned hypothesis may represent a novel unreported mechanism of hyperglycemia cytotoxicity in the brain.[10] Therefore, our major aim in the present study was to validate our research hypothesis. Specifically, we intended first to confirm whether the observed intramitochondrial granules are really built of glycogen. Next, we examined the preventive effect of cyclosporine A, a potent blocker of MPTP opening by binding to cyclophilin D,[16-18] against abnormal occurrences of intramitochondrial glycogen granules and ultrastructurally changed mitochondria of mouse brain cortex induced by high doses of glucose. We utilized ultrastructural studies and histopathological analysis using animal models to address the aim of this study.

MATERIALS AND METHODS

Study approval

All research works were approved by the Institutional Review Board college of medicine University of Hail, Saudi Arabia. Approval NO: (EC0023).

Animals

The experiments were conducted on male BALB/c mice weighing 20–25 g. The animals were kept in colony cages under steady temperature and humidity and on a regular light-dark cycle. All experimental procedures were preapproved by Hail university animal care and use committee. Mice were randomly assigned to the experimental or control groups consisting of 4–6 animals each.

Administration of glucose alone or with other substance

Glucose solution in the dose of 12g/kg was daily administrated subcutaneous to the animals for 3 days (after 1 h of the last dose, all animals were decapitated for microscopic examination) as follows:

- Freshly prepared glucose solution was administerd, then one block of each hemispheres cortex were maintained. The first sample was incubated in a freshly prepared α-amylase solution (5% in 0.1M phosphoric buffer, pH 7, and 0.02M sodium chloride) for 1 h. The second control sample was incubated in the dissolvent for the same period. Both samples were then tested by electron microscope
- Coadministration of a freshly prepared cyclosporine A intraperitoneally (IP) (dissolved in cremophor) at a dose of 50 mg/kg/day for 3 days with the glucose solution. A sample of brain cortex was then tested by electron microscope
- Coadministration of cremophor to control group IP (for cyclosporine A) at the same volume for 3 days with the glucose solution. A sample of brain cortex was then tested by electron microscope.

Electron microscope

For electron microscopic experiments, the animals were anesthetized IP with 0.2–0.4 ml of 20% urethane. The brains were submitted to *in situ* fixation. After a transcardiac

perfusion with 0.1 m cacodylate buffer (10 s), 2.5% glutaraldehyde in 0.1 m cacodylate buffer (2 ml/min) was applied. After removing the brains, blocks of cerebral cortex were maintained in the same fixative solution at 4°C for 2h and postfixed in 2% osmium tetroxide in 0.1M cacodylate buffer for 2h at 4°C. After overnight rinsing in 0.1 m, cacodylate buffer blocks of tissue were dehydrated in increasing gradients of ethanol and propylene oxide and finally embedded in Epon. Ultrathin sections were prepared on apparatus ultrathome (LKB Nova), picked up on copper grids, poststained with uranyl acetate and lead citrate, and examined under electron microscope (JOEL 1200 EX).

Histology and light microscope

The brain was fixed in 10% neutralized formalin. Tissue samples were processed through ethanol and xylene and embedded in paraffin block. Sections were cut at 6 μm in thickness to be stained with hematoxylin eosin. The specimens were examined under Olympus/3H light microscope-Japan.

RESULTS

Electron microscopic observations

The observations of this study showed that administration of high doses of glucose, 12 g/kg/day for 3 days, led to occurrences of glycogen-like granules inside few mitochondria of some astrocytes and synapses of the brain cortex. In randomly chosen 50 mitochondria, 26 of them contained glycogen granules. These mitochondria were ultrastructurally changed, i.e., with severe abnormalities showing condensation of mitochondrial matrix, lack of mitochondrial cristae, and fragmented mitochondrial membrane. Such phenomena were not observed in the brain of the control animals [Figure 1].

Verification of the nature of intramitochondrial granules

As presented in Figure 1, the cortex block incubated in α-amylase solution results in disappearing of the intramitochondrial granules under the electron microscope. These intramitochondrial granules were observed, however, in the control cortex block of the same tested animals, confirming the glycogen nature of these granules.

Involvement of mitochondrial mega-channels

To verify the involvement of mitochondrial mega-channels and thus the necrotic and apoptotic character of the observed phenomenon, cyclosporine A, which blocks specifically activation of mitochondrial mega-channels, was coadministered with 12 gm/kg/day glucose for 3 days. As shown in Figure 1, this treatment inhibited both the pathology of mitochondria as well as the accumulation of glycogen granules inside these mitochondria. The treatment reduced the number of impaired mitochondria from 26 to 18 out of 50 mitochondria.

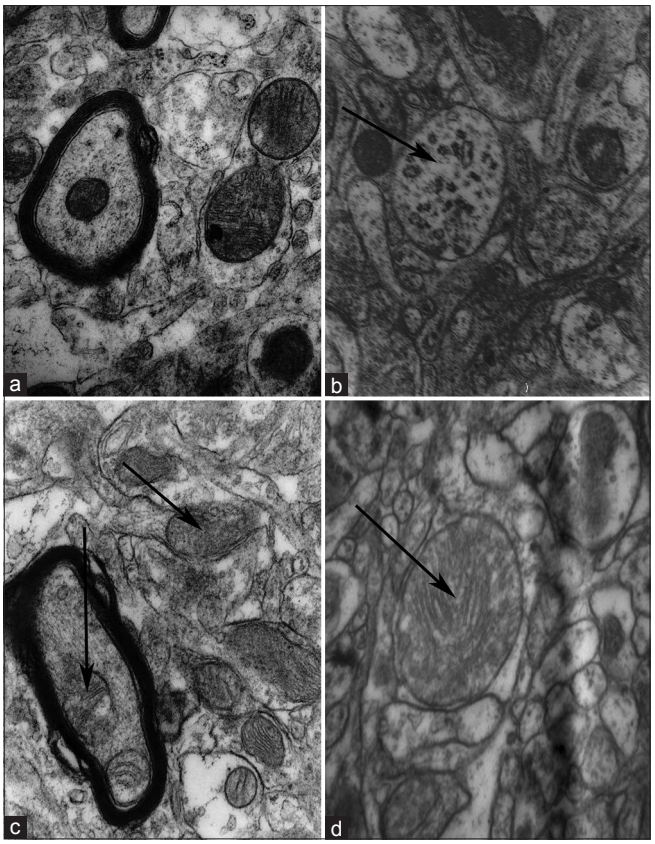

Figure 1: Electron micrographs of brain cortex of mice. (a): Mouse injected with normal saline showing normal mitochondria inside and outside the axons. (b): Mouse injected with glucose (12 g/kg/day for 3 days) showing accumulation of glycogen granules in structurally altered mitochondria (arrow). (c): Mouse injected with glucose and incubated in α-amylase solution for 1 h showing disappearance of intramitochondrial glycogen granules inside and outside the axons (arrows). (d): Mouse injected with glucose and with intraperitoneal dose of 50 mg/kg cyclosporine A showing inhibition of intramitochondrial glycogen in nearly normal mitochondria (arrow)

Histopathology

Glucose injection alone led to shrinkage of some nerve cells with little perineural vacuolation. When cyclosporine A was injected with glucose, it reduced the number of degenerated neurons with very little perineural vacuolation as shown in Figure 2.

DISCUSSION

Our present ultrastructural study, utilizing electron microscope and α-amylase, has substantiated that previously detected intramitochondrial granules are in fact glycogen, as the enzyme α-amylase dissolved these granules.[10] Since distribution of glucose into the brain is independent of insulin, glucose entry into this organ is increased during hyperglycemia, making brain vulnerable

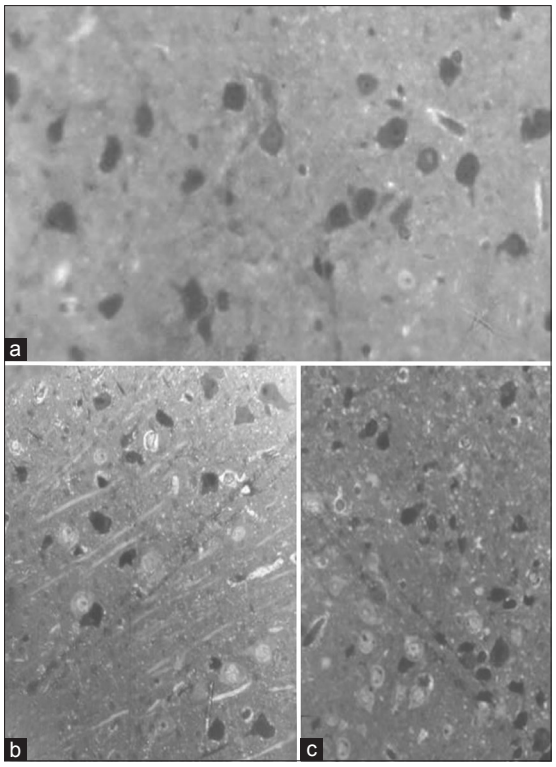

Figure 2: Brain cortex of mice showing hyperglycemia-induced neurodegeneration. (a): Control showing normal structure of brain cortex, (b): effect of glucose injection, some nerve cells appear with small sizes accompanied with perineural vacuolation. (c): Effect of cyclosporine A injected with glucose showing reduced number of degenerated neurons with very little perineural vacuolation H and E, ×400

to overload of glucose supply.[19] Glucose penetration across the astrocyte plasma membrane is also independent of insulin.[20] In parallel, it has been recommended that a huge part of accessible glucose is temporarily converted to glycogen.[21] Therefore, it could be argued that the observed intramitochondrial glycogen granules in this model are a result of excess intracellular glucose. This indicates a specific key role of excess intracellular glucose in causing this phenomenon. A similar intramitochondrial granules were observed in few different cell types, for example, myocardium of patients with congenital heart disease,[22] cerebella,[23] and substantia nigra[24] of homozygous Gunn rats and cardiac muscle of postnormothermic cardiac arrest.[25] However, no study examined the involvement of hyperglycemia in such phenomenon or noticed abnormal morphology of mitochondria-containing glycogen granules. Furthermore, no experiments investigated brain cortex or hippocampus in this context.

A large body of evidence showed that mitochondrial dysfunction appears very early in the course of the NDD and acts causally in their pathogenesis.[14,26] Interestingly, our histological studies of the same samples demonstrated a significant morphological alteration in the brain cortex of mouse treated with high doses of glucose. This is the first *in vivo* study to assess the histology of brain cortex of mouse that received high doses of glucose.

To determine the mechanism of our findings, we evaluated the potential contribution of MPTP (mitochondrial megachannel), and glycogen granules in the mechanism of hyperglycemia-induced intramitochondrial glycogen granules (HIMG), utilizing cyclosporine A. Cyclosporine A is known to specifically block activation of mitochondrial megachannels, thus preventing the release of proapoptotic cytochrome c from mitochondria to cytoplasm and hence prohibiting cell death.[27] In our study, this agent given in the dose of 50 mg/kg IP which is sufficient to pass through blood-brain barrier[28] inhibited occurrences of glycogen inside mitochondria of cerebral cortex, the abnormal pathological changes of this organelle, and the histopathological changes of brain cortex of these animals. These results confirm our previous hypothesis that elevation of intracellular glucose concentrations may defect the integrity of mitochondrial membranes. This in turn could facilitate glucose and cytoplasmic enzymes to enter the mitochondria which leads to synthesis of glycogen.[10] Our results are in line with current experiments that implicate diabetes-induced rising of intracellular glucose concentrations in brain mitochondrial dysfunction.[7]

CONCLUSIONS

Taken together, the results obtained in this study indicate that HIMG can lead to cytotoxicity in brain cells. We postulate this as a key novel molecular pathway through which hyperglycemia may induce dysfunction of brain mitochondria, most likely leading to NDD, and might serve as a new therapeutic strategy for these diseases. To the best of our knowledge, this is the first report demonstrating HIMG as a cytotoxic molecular mechanism in mouse model.

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Dr. Mohd Alaraj is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflicts of interest

There are no conflicts of interest.

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