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Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line

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Abstract

Glucose metabolism plays a pivotal role in many physiological and pathological conditions. To investigate the effect of hypoglycemia (obtained by glucose deprivation) on PC12 cell line, we analyzed the cell viability, mitochondrial function (assessed by MTT reduction, cellular ATP level, mitochondrial transmembrane potential), and the level of reactive oxygen species (ROS) after glucose deprivation (GD). Upon exposure to GD, ROS level increased and MTT reduction decreased immediately, intracellular ATP level increased in the first 3 hours, followed by progressive decrease till the end of GD treatment, and the mitochondrial transmembrane potential ($\Delta\Psi_m$) dropped after 6 hours. Both necrosis and apoptosis occurred apparently after 24 hours which was determined by nuclei staining with propidium iodide(PI) and Hoechst 33342. These data suggested that cytotoxity of GD is mainly due to ROS accumulation and ATP depletion in PC12 cells.

Keywords: glucose deprivation • ATP depletion • mitochondrial membrane potential • reactive oxygen species • apoptosis • necrosis

Introduction

The pathogenesis of brain damage under conditions of metabolic stress continues to be of great interest, since much still needs to be understood. Many *in vivo* and *in vitro* models were established to mimic the ischemia-like pathological process. Rat pheochromocytoma (PC12) cells, originated from the adrenal medulla, synthesize and release catecholamines, is a well known neuronal model for *in vitro* ischemic studies [1]. Ischemic injury model of PC12 cells (oxygen and glucose deprivation, OGD) has been well established to investigate the mechanism of ischemia-induced cell death, and this model is also widely used for the development of neuroprotective drugs against ischemic insults [2]. However, OGD is a disastrous stimulus, and usually leads to rapid cell death therefore it is difficult to determine the molecular mechanisms of OGD

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induced cell injury which involved vigorous and complicated cellular changes. An alternative approach is to investigate the effect of hypoglycemia and hypoxia respectively. Glucose metabolism is the key point in maintaining the balance between the life and death, and glucose serves as an important regulatory factor in the ischemic injury cascade [3].

The cell death scenarios linked to glucose metabolic inhibition include ATP depletion and oxidative stress which are tightly associated with the mitochodrial function. Glucose deprivation (GD) results in ATP depletion and this in turn triggers the death cascade. Severe ATP depletion usually leads to necrosis since ATP is required for many steps in apoptosis. Glucose deprivation also results in oxidative stress and the alteration of the redox status of cells triggers stress-activated or other signal transduction pathways resulting in cell death [4–6]. In recent years, many reports showed that the loss of mitochondrial potential and the dysregulation of Ca^{2+} are also involved in this process [7–8]. Some studies have elucidated the cytotoxity of glucose deprivation in PC12 cells, but there is no systematic and detailed description for this process yet [9-10]. Therefore, our work presented here is focused on the dynamic changes of mitochondrial function in PC12 cells undergoing glucose deprivation.

Material and methods

Cell Culture

PC12 cells, purchased from American Type Culture Collection (ATCC), were grown routinely in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated horse serum (GibcoBRL), 5% fetal calf serum (GibcoBRL), 50 U/ml penicillin and 100μg/ml streptomycin, maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. The cells were fed every two days and passed twice a week. Cells were plated at 100,000 cells/cm^2 on poly-L-lysine-coated (100μg/ml) 96-well plates (0.1 ml/well) for MTT assay and DCF fluorescence assay, 24-well plates (0.5 ml/well) for Hoechst 33258 staining and ATP measurement, and on 12-well plates (1 ml/well) for mitochondrial potential assay.

Glucose deprivation

The cells were gently washed three times with phosphate-buffered saline and then incubated in glucose-free DMEM medium containing 2% horse serum and 1% fetal calf serum for indicated further time. The control cells were treated in the same way while incubated in high-glucose DMEM medium containing 1% horse serum and 1% fetal calf serum.

Trypan blue exclusion

The culture medium was removed and replaced by 0.1% trypan blue solution in phosphate-buffered saline for 3 min at room temperature. Viable cells in 10 randomly chosen fields were counted at 200-fold magnification using bright-field and phase-contrast microscopy for blue cells and total cells respectively.

Fluorescent staining of nucleus

Apoptosis and necrosis were distinguished using combined staining of chromatin dye, Hoechst dye 33342 and propidium iodide (PI) (Molecular Probes) [11]. Hoechst 33342 (λ_{ex} 360nm, λ_{em}490nm) freely enters living cells and therefore stains the nuclei of viable cells, as well as those that died by apoptosis or necrosis. Apoptotic cells can be distinguished from viable and necrotic cells on the basis of nuclear condensation and fragmentation, PI(λ_{ex} 536nm, λ_{em} 620nm) enters only cells with damaged cell membranes, then viable cells are PI negative while necrotic cells are PI positive. In brief, at the end of the cell culture, Hoechst was added to the culture medium at 1μg/ml for 10 min, and cells were incubated with PI at 1μg/ml for 10 min.

The nuclei were counted according to nucleus morphology and label.

MTT reduction assay

The determination of the mitochondrial function was performed by measuring the MTT reduction ability of PC12 cells, according to the method of Mosmann [12]. MTT, which reacts with dehydrogenases and cofactors of the respiratory chain, is an indicator of mitochondrial activity [13]. In brief, MTT was dissolved in PBS at 5 mg/ml and was added to culture medium at the end of incubated time, the final concentration was 0.5mg/ml.

After an additional 3-hr incubation at 37°C, 0.1 ml isopropanol/HCl was added to each well, and the absorbance at 570 nm, of solubilized MTT formazan products, was measured. Results were expressed as the percentage (%) of MTT reduction, assuming the absorbance of control cells as 100%.

ATP quantification

Intracellular ATP content was determined based luciferin/luciferase method [14] with the use of ATP bioluminescent assay kit (Boehringer). The cells supplemented with glucose or in hypoglycemic conditions, were washed with cold phosphate-buffered saline and lysed with 100 µl of cell lysing buffer. Then, 1 µl of the lysate was diluted to 100 µl with water and mixed with 100 µl of luciferase/luciferin reagent. After 10 s, the light emitted was recorded using a luminometer at 562 nm and integrated over 5s.

Protein quantification

Protein concentrations were determined by the method of Bradford [15], with bovine serum albumin as standard.

Measurement of mitochondrial transmembrane potential with DiOC6(3)

Mitochondrial transmembrane potential of PC12 cells during hypoglycemic treatment was studied with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (λex max 484 nm, λem max 501 nm) (MolecularProbes) [16]. In living cells, this cyanine dye can accumulate in the mitochondrial matrix under the influence of the mitochondrial transmembrane potential. PC12 cells were collected and resuspended with culture medium to 10^6 cells/ml, and incubate at 37°C for further 15min, then $DiOC_6(3)$ fluorescence was immediately recorded with flow cytometry (Becton Dickinson). For each sample, 10,000 cells were acquired for data analysis.

DCF fluorescence measurement

In order to measure the ROS production in PC12 cells under glucose deprivation, we used the DCFH-DA method as described previously [17]. DCFH-DA is membrane permeable, and it can be enzymatically converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. At the end of the hypoglycemic treatment, PC12 cells (10^4 cells per well in a 96-well plate) were loaded with DCFH-DA(final concentration 100µM) for 30 min, fluorescence was monitored on a Cyto Fluor Multi-Well Plate Reader with λ_{ex} 485 nm and λ_{em} 530 nm. The results were expressed as a relative percent of DCF-fluorescence in control cells.

Data Analysis

Data were expressed as means±SEM from at least three independent experiments. Statistical significance analysis was determined by using the Student's *t*-test or analysis of variance (ANOVA; *P* value, 0.05 was considered significant).

Results

Glucose deprivation induced cell death

Previous studies have demonstrated that glucose deprivation can induce apoptosis in PC12 cells. We therefore initiated a series of experiments to investigate the effect of glucose deprivation treatment. Cell viability was determined with trypan blue exclusion assay. Apoptotic or necrotic cells were assessed by the nuclei staining with Hoechst 33342 and PI. After the treatment of glucose deprivation for 24 hours, the percentage of trypan blue positive cells was 19±4.2% (values of the percent survival for three individual cultures), and after 48 hours, the percentage of positive stained cells increased to 69±3.1%, while the cells of control group are almost all negative for trypan blue. Hoechst 33258 and PI stained cells were visualized by fluorescence microscopy. As Figure 1 shows, the cells with condensed chromatin and fragmented nuclear DNA were considered as apoptotic. The percentage of viable cells, apoptotic cells and necrotic cells in each culture was determined. At least 200 cells were counted in each experiment. Upon the exposure to GD for 24 hours, apoptotic cells were 7.8±2.2%, necrotic cell were 8.5±1.9%. After GD treatment for 48 hours, apoptotic cells were 21.6±3.1%, necrotic cells were 59.5±12.8%. In

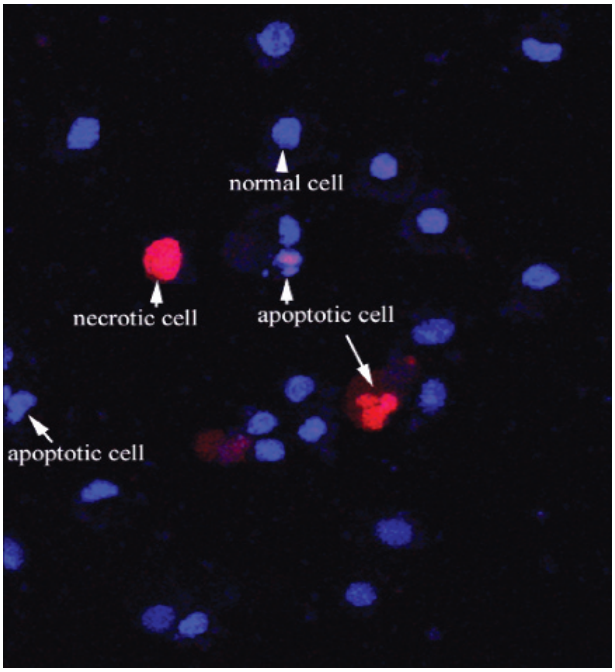

Fig. 1 Representative confocal fluorescence microscopy of PC12 cells stained with H-33342 and PI after GD treatment for 24 hours (x400), cells were grouped as below, (a) normal cells: showed normal morphology of nuclei and negative stained with PI. (b) necrotic cells: showed normal morphology of nuclei and positive stained with PI. (c) apoptotic cells: showed condensation and fragmentation of nuclei, including both PI positive and negative stained cells, nuclei of these cells were condensed and fragmented, demonstrating that apoptosis was primary cause of cell death.

control cells, both necrotic and apoptotic cells were lower than 1%.

Glucose deprivation decreased the MTT reduction in PC12 cells

The capacity of MTT reduction is an index of mitochondrial function, data presented in Figure 2 indicated that the MTT reduction decreased immediately and rapidly at the beginning of glucose deprivation, after 3 hours, it decreased slowly to a level which was about half of the control cells at the time of 24 hours, thereafter, an obvious decrease appeared again, and at the end of GD treatment (48 hours), the MTT reduction ability was less than 20% of control cells. Because the loss of MTT reduction was due to mitochondria dysfunction, then MTT was also an index of cell viability, and the data presented here was in accordance with the cell survival assay.

Effect of glucose deprivation on intracellular ATP level of PC12 cells

Glucose is the main substrate for intracellular ATP generation; PC12 cells are neoplastic in nature and they have a high rate of glycolysis accompanied by a large production of lactate and a low use of glucose through the Krebs cycle [18]. Data presented in Figure 3 shows that, after the first 3 hours of GD, ATP content was increased to a higher level than

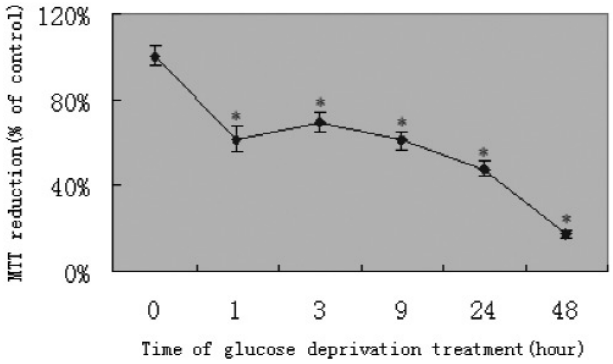

Fig. 2 Time course of the effect of glucose deprivation on PC12 cell MTT reduction. Data, expressed as the percentage (%) of control values, are means ± SEM (n=6) of a representative experiments. **P* <0.01 compared to control cells.

Fig. 3 Time course of the effect of glucose deprivation on intracellular ATP content of PC12 cells. The values expressed as the percentage (%) of control cells, are means ± SEM (n=6) of a representative experiments. **P* <0.05, ** *P* <0.01 compared to control cells.

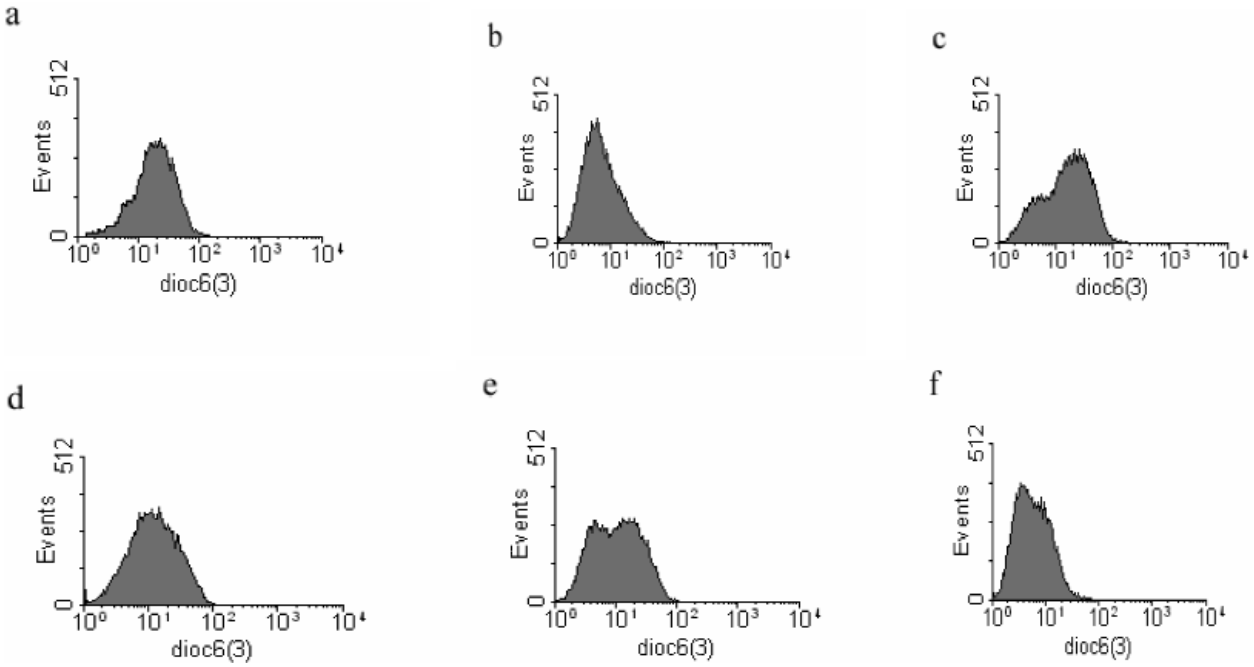

Fig. 4 Effect of glucose deprivation on the mitochondrial transmembrane potential(ΔΨm) of PC12 cells.(a) control cells (b) cells treated with 100μM carbonyl cyanide chlorophenylhydrazone(CCCP) to fully depolarize mitochondria (c) glucose treatment for 3 hours (d) glucose treatment for 6 hours (e) glucose treatment for 24 hours (f) glucose treatment for 48 hours.

control cells and afterwards progressively decreased during the rest time. Previous studies reported that, after glucose depletion, other energy sources, such as amino acids and glutamine in culture medium, could be used to generate ATP. The inhibition of glycolysis may stimulate mitochondria to use substrates other than glucose to maintain ATP level. Thus, an increase of ATP content was observed in the early stage of GD treatment, and even in the condition of hypoglycemia for 24 hours, ATP content was kept at 50% of control cells.

Effect of glucose deprivation on mitochondrial transmembrane potential ($\Delta\Psi_m$)

Recently, mitochondria has been regarded as the key regulator of cell death [19]. Healthy mitochondria maintain a negative membrane potential across the mitochondrial inner membrane, collapse of $\Delta\Psi_m$ lead to a reduction of mitochondrial mass which could activate the cell death cascade in turn. $DiOC_6$ (3), a fluorescent dye that incorporate into mitochondria in a $\Delta\Psi_m$ dependent manner was used to evaluate the changes of mitochondrial potential during GD treatment. As Figure 4 showed, in the first 3 hours, the mitochondrial potential was maintained well, the decrease of $\Delta\Psi_m$ became obvious only after 6 hours. Nearly half of cells were depolarized after 24 hours of GD and at the end of GD period (48 hours), cells were totally depolarized similarly to the cells treated with

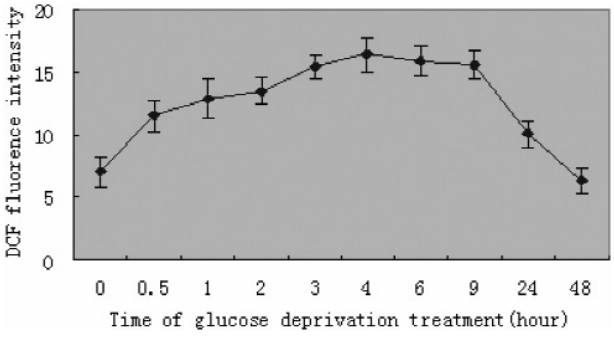

Fig. 5 Time course of the effect of glucose deprivation on ROS accumulation of PC12 cells. The values presented are means ± SEM (n=6) of a representative experiments.

carbonyl cyanide chlorophenylhydrazone (CCCP) to fully depolarize mitochondria.

Glucose deprivation induced a rapid increase of ROS in PC12 cells

It is well known that the mitochondria are the site of pro-oxidant production, and ROS are by-products of oxidative phosphorylation, and it has been suggested that mitochondria are the main source of ROS after cell damage. In our study, DCFH-DA method was used to monitor the ROS level during glucose deprivation insult. Our data indicated that the accumulation of ROS was a quick response to the GD treatment. ROS level increased nearly 2 fold within 30 min, and increased steadily to the peak at the time of 4 hours for GD, after which it followed a progressive decline to normal ROS level.

Discussion

In recent years, the pathogenesis of brain damage under conditions of impaired energy metabolism has been extensively investigated. PC12 cells oxygen-glucose deprivation is a well established and widely used *in vitro* model for ischemic studies, while the investigations for glucose deprivation alone in PC12 cells are less used than the OGD system due to its limitation in modeling ischemia. In our point, the elucidation of the cellular and molecular process in PC12 cells undergoing glucose deprivation will contribute to understand the pathogenesis of ischemic injury. Furthermore, some neuronal degenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) [20-22] seen to associate with the glucose deprivation induced insult and it has been reported that amyloid β-peptide (Aβ) inhibits glucose transport in neurons by a mechanism involving oxidative injury of the neuronal glucose transporter, GLUT3. Therefore, the decrease of glucose transport may trigger a cell death cascade similar to the process occurred in glucose deprivation models.

Cell damages induced by glucose deprivation depend greatly on the cell type and the method of GD treatment. We have reported the cytotoxical effect of GD on CHL cells which were much more susceptible than PC12 cells upon glucose deprivation [23]. Our unpublished data showed that PC12 cells could sustain growth in glucose-free medium (containing 10% serum) for more than 60 hours even in the presence of 2-deoxyglucose (glycolysis inhibitor) cell damage became obvious only at after the serum concentrations lower than 2%. PC12 cells were reported to undergo apoptosis upon the GD treatment, our data demonstrated that both apoptotic and necrotic cell death were involved in this insult, and the necrosis was the predominant form of cell death in our system. This phenomenon is consistent with the ischemia-reperfusion induced insult *in vivo* which is also composed of necrosis and apoptosis [24]. For an individual cell undergoing glucose deprivation, the choice between apoptosis and necrosis is settled by apoptogenic factors which can or cannot act before the bioenergetic catastrophe disrupt the plasma membrane integrity.

However, it can be debated about what triggered the cell death cascade in response to hypoglycemic stress. When cellular ATP stores were severely depleted to a level incompatible with maintenance of basal metabolism and activity of membrane transport pumps, cells were deemed to die due to the energetic catastrophy. In our study, cell death occurred even though intracellular ATP content was maintained at more than 50% of control cells. Thus, there might be other initiator of cell death, possibly ROS levels. Glucose metabolism results in the formation of not only ATP but also the redox potential NADPH. Therefore, in addition to its well known role in energy production, glucose metabolism appears to be related to the metabolic detoxification of intracellular hydroperoxides formed as byproducts of oxidative metabolism in mitochondria.

It was reported that glucose deprivation led to accumulation of pro-oxidants, presumably superoxide and hydrogen peroxide, as a result of the metabolic shift to oxidative phosphorylation, and the glucose deprivation-induced oxidative stress has been shown to activate signal transduction pathways leading to apoptosis in a breast carcinoma cell line (MCF-7/ADR) [25]. Our results also demonstrated that ROS accumulated immediately after the onset of glucose deprivation which was followed by the decreased MTT reduction ability of mitochondria. This phenomenon indicated a failure in elimination of oxidants which is probably due to inadequate production of NADPH via the pentose phos-

phate pathway. After 4 hours for GD treatment, ROS level reached the highest point, then declined afterwards to the normal level. This might be due to two reasons. First, the increased level of ROS would stimulate the cellular defensive system such as glutathione/glutathione and peroxidase/glutathione reductase system to decrease the intracellular hydroperoxide level. Second, the further depletion of substrate of TCA cycle such as amino acids would inhibit the oxidative phosphorylation, and in turn block the ROS accumulation.

Both oxidative stress and ATP depletion were reported to be involved in the dysfunction of mitochondria which was marked with the collapse of mitochondrial transmembrane potential($\Delta\Psi_m$), and the drops of $\Delta\Psi_m$ induced Ca^{2+} release, increased production of ROS, and precipitated release of proapoptotic factors such as cytochrome *c* from mitochondria. Therefore, we detected the alteration of $\Delta\Psi_m$ during GD treatment. The changes of $\Delta\Psi_m$ upon GD is controversial because many groups have observed an early increase in $\Delta\Psi_m$ followed by a further decline. Our data showed that there was no apparent alteration of $\Delta\Psi_m$ during the first 3 hours, and the decline of $\Delta\Psi_m$ became obvious after 6 hours.

In summary, glucose deprivation induced cytotoxity and mitochondria dysfunction mediated by oxidative stress and by ATP depletion. Our present study was focused on the dynamic changes of mitochondria function, while the relationship between these phenomena and the underlying molecular mechanism needs further investigation.

Acknowledgments

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