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EFFECTS OF GLUCOSE AND ADVANCED GLYCATION END PRODUCTS ON OXIDATIVE STRESS IN MIN6 CELLS

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Abstract – To explore the mechanism of oxidative stress induced by glucose and advanced glycation end products (AGEs) in MIN6 cells. The MIN6 cells were exposed to various concentrations of glucose or AGEs for some time, MTT assay was used to evaluate the cell viability, reactive oxygen species (ROS) was monitored using intracellular ROS capture Dihydroethidium (DHE) and dihydrorhodamine123 (DHR123). The signal was quantified using flow cytometry by measuring the mean fluorescent intensity (MFI). The NADPH oxidase activity was measured by chemiluminescence with lucigenin. Treatment of high glucose or AGEs decreased cell viability in a dose- and time- dependent fashion. Exposure of MIN6 cells to high glucose or AGEs significantly increased intracellular ROS production in a concentration- and time-dependent manner. In parallel with the results of ROS production, the NADPH oxidase in MIN6 cells was activated due to increased glucose or AGEs concentration. High glucose and AGEs stimulated ROS production via the activation of NADPH oxidase. The oxidative stress may consequently impair pancreatic β-cell function and contribute to diabetes mellitus as a result.

Key words: Glucose, advanced glycation end products, oxidative stress, reactive oxygen species, MIN6, NADPH oxidase, diabetes.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia, resulting from absolute or relative insulin deficiency. Recent studies have confirmed that hyperglycemia is the central initiating factor for all types of diabetic complications. Constant hyperglycemia exerts deleterious effects on β-cell function, the cells in turn undergo hypertrophy and loss of differentiation, then insulin secretion decreased, as a result the level of blood glucose is even higher. In a pathophysiological condition, persistent elevation of the glucose concentration impairs β-cell function and induces β-cell apoptosis, so-called glucose toxicity. One potential mechanism for glucose toxicity is that of excessive formation of reactive oxygen species (ROS) in β-cells. Recent research has proved that oxidative stress is increased in pancreatic β-cells in diabetic animal models and diabetic patients (2). Oxidative stress is a state of ROS overproduction. Hyperglycemia leads to the formation of advanced glycation end products (AGEs), and is accompanied by an accelerated rate of AGEs formation. Therefore it is important to evaluate whether glucose or AGEs increase ROS production in β-cells and to explore the molecular mechanism. In this study, we examined the dose- and time- dependent effects of glucose or AGEs on cell viability, ROS production and NADPH oxidase using the pancreatic β-cell line MIN6.

Abbreviations: **AGEs**, advanced glycation end products; **MFI**, mean fluorescent intensity; **ROS**, reactive oxygen species; **DM**, Diabetes mellitus; **FCS**, Fetal calf serum; **DHE**, Dihydroethidium; **DHR123**, dihydrorhodamine123; **RLU**, relative light units.

MATERIALS AND METHODS

Reagents

DMEM, Fetal calf serum (FCS) and 2-mercaptoethanol were purchased from Gibco Company (Gibco, USA). Dihydroethidium (DHE), dihydrorhodamine123 (DHR123), lucigenin, D-glucose and NADPH were purchased from Sigma Company (Sigma, USA).

Cell culture

Pancreatic β-cell line MIN6 cells were kindly provided by professor Xiao-ying Li of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The cells were cultured on 25cm dishes and propagated in DMEM medium supplemented with 10% heat-inactivated FCS, 50μM 2-mercaptoethanol, 100U/mL penicillin, 100μg/mL streptomycin and 25mmol/L glucose. Cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2/ 95% air. The culture medium was removed and replaced with fresh media every 24 h.

MIN6 cells in growth medium were equally seeded into 6-well clusters or 96-well clusters at a density of 5×10^5 /well and 1×10^3/ well respectively. The growth medium was replaced with DMEM containing 0.1% serum and 5.5mmol/L glucose when cells had grown to approximately 90% confluence for 24 h. The subconfluent cells were then exposed to different glucose or AGEs medium for 24, 48, 72 or 96h respectively.

The MIN6 cells were grouped as follows: A. Effects of glucose concentration: (1) normal glucose medium (5.5mM); (2) high glucose medium (12.5mM); (3) high glucose medium (25mM); (4) high glucose medium (50mM); and (5) osmotic control was assured by incubating cells with 25mM mannitol.

B. Effects of AGEs concentration: (1) normal medium group (5.5mM glucose); (2) medium with different AGEs concentration（50、100、200、400mg/L）; and (3) control group with 200mg/L BSA medium was assured.

Prepare of AGE-BSA in vitro

AGE-BSA was prepared as previously reported. Briefly, 0.5g BSA was dissolved in 10ml of 0.2mol/L sodium phosphate buffer (PBS) (pH 7.4) with 0.9g D-glucose. The samples were filter-sterilized by using a 0.22μm Millipore filter and incubated at 37℃ for 120d under sterile and dark conditions, dialyzed against PBS in order to eliminate unconjugated glucose. While non-glycated BSA was prepared simultaneously as the same method the solution lacked glucose. AGE-BSA was identified by fluorescence spectrophotometer.

MTT Assay for cellular activity

The MTT assay is conventionally used for measuring cell proliferation. Because of the convention of the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolinum bromide) mediated by active mitochondrial dehydrogenases of living cells, this assay can also be used for assessing cell viability (4). That is, after exposure to high glucose or AGEs, the cells were treated with MTT (0.5mg/mL final concentration in culture medium) for 4 h at 37℃. The formazan product generated was solubilized by the addition of DMSO. Absorbance of the converted dye was recorded at 570nm with a reference wavelength of 690nm.

Analysis of cellular ROS levels

Fluorescent microscopy

ROS production was assessed using DHE probe. Cells were incubated in the presence of 10μmol/L DHE for 1 hour at 37℃ after their treatment with various high glucose or AGEs for 48h, and then were washed three times with PBS. The fluorescence of MIN6 cells was observed by fluorescent microscopy (excitation 520nm, emission 610nm).

Flow cytometry

DHR123 was used as ROS capture, and the cells were incubated together with 5μmol/L DHR123 in culture medium for 1 hour. Blank controls were set, in which DHR123 incubation was omitted. DHR123 was a nonfluorescent compound that freely permeated cells and interacted with intracellular oxidants to form fluorescence compound rhodamine123 (Rh123) which was maintained there. After DHR123 incubation, MIN6 cells were trypsinized, harvested, washed twice with PBS and directly collected before an immediate detection of mean fluorescence intensity (MFI) of Rh123 by flow cytometry. The results were expressed as relative light units (RLU) per 10^5 cells. It was made to measure cellular ROS levels.

Measurement of NADPH oxidase activity

NADPH oxidase was measured using lucigenin chemiluminescence. Cells were trypsinized, pelleted by centrifugation, and resuspended at 1×10^6cells/mL with cold Krebs-Hepes buffer containing NaCl 119mmol/L, Hepes 20mmol/L, KCl 4.6mmol/L, $CaCl_2$ 1.2 mmol/L, Na_2HPO_4 0.15mmol/L, KH_2PO_4 0.4mmol/L, $MgSO_4$ 1.0mmol/L, $NaHCO_3$ 25.0mmol/L and glucose 5.5mmol/L (pH7.4). 300μl cellular suspensions were put into a 96-well white plate in a luminescence reader and dark-adapted lucigenin (10μmol/l) was added to start the reaction. Chemiluminescence was recorded every 15s for 10min. The lucigenin chemiluminescence was expressed as counts per minute per 10^6cells. NADPH (final concentration 100μmol/L) was added after measurement of background lucigenin chemiluminescence and measurements were performed for another 10min. The difference between the values obtained before and after adding NADPH was calculated and it represented the activity of NADPH oxidase.

Statistical analysis

Each experiment was carried out in duplicate or triplicate and three independent experiments were performed. Results were expressed as means ±standard deviation (SD) and analyzed with SPSS 11.5 software. Groups were compared using analysis of variance (ANOVA). Statistical significance was set at $P<0.05$.

RESULTS

Cellular activity detected by MTT assay

First, we examined the effect of glucose or AGEs on MIN6 cells growth. To evaluate this effect on MIN6 cell viability, MTT assay was used. Together, these data demonstrated that high glucose or AGEs caused injury to MIN6 cells.

The effect of glucose on MIN6 cells (Tab1)

Cell viability with glucose treatment at 24h was significantly decreased above 50mM compared with mannitol treated control, 48h later, this effect was obvious at any high glucose concentration. As illustrated in Tab1, this cytotoxic effect was time- and concentration-dependent.

Table 1. Cell viability of MIN6 measured by MTT（n=5, OD, $\bar{\chi}$ ±s）

Group	24h	48h	72h	96h
control	0.549±0.021	0.539±0.038	0.591±0.068	0.699±0.074
mannitol	0.551±0.032	0.529±0.033	0.578±0.101	0.575±0.119
Glu12.5mM	0.455±0.103	0.406±0.054**	0.388±0.066*	0.356±0.051**
Glu25.0mM	0.437±0.097	0.376±0.043**	0.352±0.096**	0.349±0.021**
Glu50.0mM	0.343±0.050**	0.305±0.030**	0.279±0.083**	0.273±0.042**

Versus control: *P<0.05; **P<0.01.

Table 2. Cell viability of MIN6 measured by MTT（n=5, OD, $\bar{\chi}$ ±s）

Group	24h	48h	72h	96h
control	0.549±0.021	0.539±0.038	0.591±0.068	0.699±0.074
BSA	0.542±0.032	0.548±0.102	0.601±0.121	0.598±0.073
AGE50mg/L	0.426±0.165	0.429±0.063	0.410±0.076	0.395±0.050**
AGE100mg/L	0.403±0.100	0.384±0.111*	0.374±0.022*	0.352±0.061**
AGE200mg/L	0.315±0.043*	0.317±0.031**	0.282±0.090*	0.270±0.129**
AGE400mg/L	0.312±0.121*	0.289±0.017**	0.269±0.027**	0.187±0.114**

Versus control: *P<0.05; ** P<0.01.

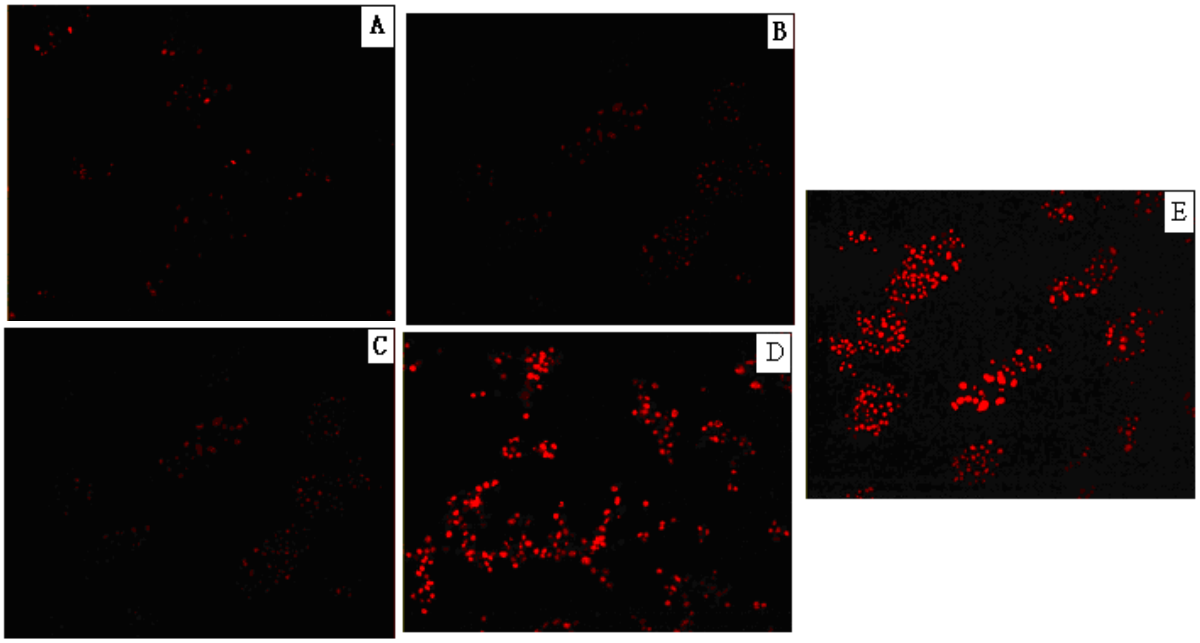

Figure 1. ROS of MIN6 detected with probe HE by fluorescent microscopy ×200
(A) MIN6 with normal glucose concentration **(B)** MIN6 with 25mM mannitol **(C)** MIN6 with 200mg/L BSA **(D)** MIN6 with 25mM glucose **(E)** MIN6 with 200mg/L AGEs

The effect of AGEs on MIN6 cells (Tab2)

MIN6 cells, which were exposed to AGEs above 200mg/L had significantly decreased cell viability compared with that in BSA and normal control groups at 24h (P<0.05); this effect could been seen under lower AGEs concentration along with the prolonged time (Tab2).

Effects of glucose and AGEs on intracellular ROS production in MIN6 cells measured by fluorescent microscopy

After the cultured MIN6 cells were exposed to various high glucose or AGEs concentrations for 48h, the intracellular fluorescence was intensified as compared with normal glucose concentration group, mannitol or BSA control group (Fig 1).

The intracellular ROS levels measured by flow cytometry

Effects of glucose on intracellular ROS production in MIN6 cells

The effect of glucose concentration on ROS production

As shown in Fig 2, exposure of the cells to glucose at the concentration of 12.5、25、50mM for 24h induced a significant increase in ROS production compared with 5.5 mM glucose control (P<0.05). These stimulatory effects were observed obviously above 25mM glucose concentration (P<0.01). In contrast, ROS generation in cells exposed to mannitol did not differ from that in control cells.

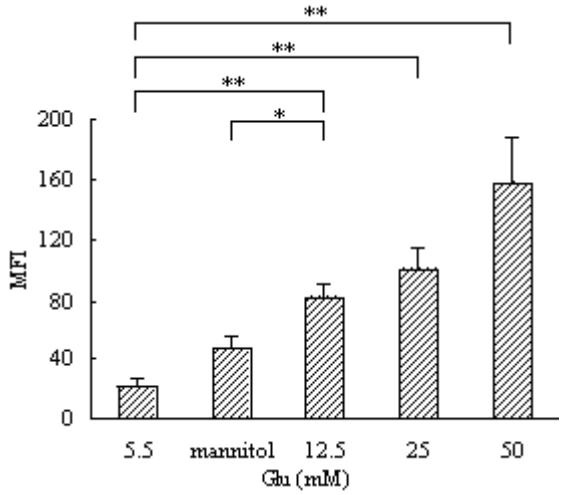

Figure 2. MFI of MIN6 treated with different glucose concentrations (mM) or mannitol for 24h
The MFI is applied to flow cytometry data, the fluorescent intensity was divided by the total number of counts being considered over the data range collected, and expressed as relative light units (RLU) per 10^5 cells. (*P<0.05, **P<0.01)

The effect of time course on ROS production

As shown in Fig 3, exposure of the MIN6 to glucose at the concentration of 25mM for 24, 48, 72h induced a significant increase in ROS production compared with 5.5 mM glucose and mannitol control (P<0.05). This effect was time-dependent. The ROS production of MIN6 cultured with 25mM glucose for 72h was almost 3-fold to that of the cells cultured for 24h. Referred to the mannitol group or 5.5mM glucose group, there is no statistical significance between the different culturing times.

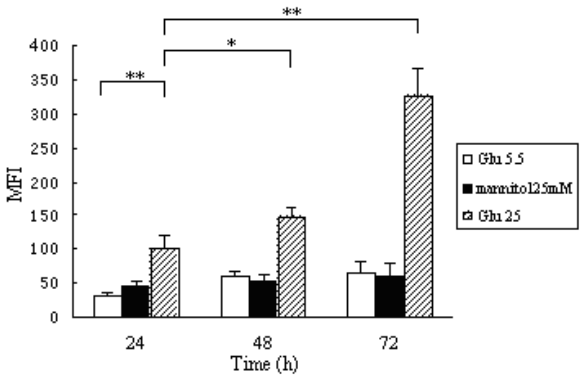

Figure 3. MFI of MIN6 treated with 25mM glucose for different time (*P<0.05, **P<0.01)

Effects of AGEs on intracellular ROS production in MIN6 cells

The effect of AGEs concentration on ROS production

As shown in Fig 4, exposure of the cells to AGEs at the concentration of 100, 200, 400mg/L for 48h induced a significant increase in ROS production compared with normal culture (P<0.01). These stimulatory effects were AGEs concentration dependent and were observed obviously above 100mg/L AGEs concentration (P<0.01). In contrast, ROS generation in cells exposed to BSA did not differ from that in normal control cells.

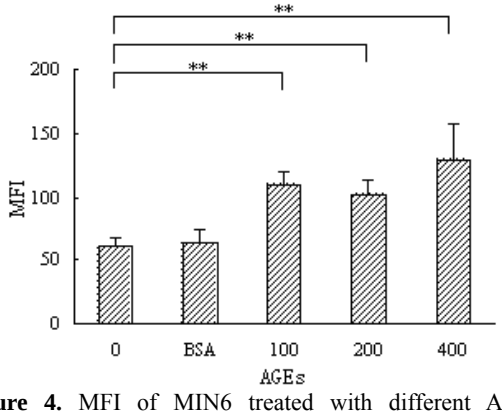

Figure 4. MFI of MIN6 treated with different AGEs concentrations (mM) or mannitol for 48h.

The effect of time course on ROS production

As shown in Fig 5, exposure of the MIN6 to AGEs at the concentration of 200mg/L for 24, 48, 72, 96h induced a significant increase in ROS production compared with normal culture and BSA control (P<0.05). This effect was time-dependent (P<0.05).

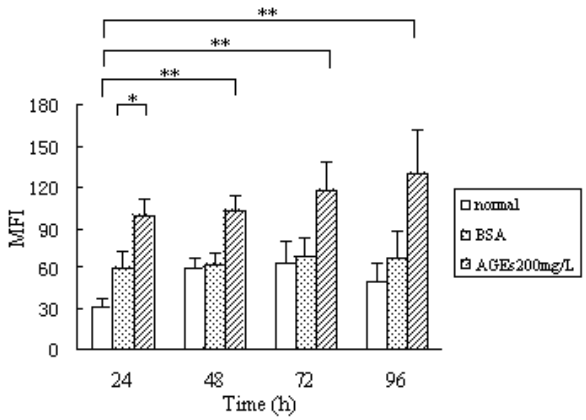

Figure 5. MFI of MIN6 treated with 200mg/L AGEs for different time
*P<0.05, **P<0.01

The NADPH oxidase activity

The effect of glucose on MIN6 cells

As shown in Tab3, exposure of the cells to glucose at the concentration of 50mM for 24h increased the NADPH oxidase activity compared with normal glucose culture and mannitol control groups (P<0.01). 48h later, glucose at the concentration above 12.5mM induced a significant increase in the NADPH oxidase activity (P<0.05). 48h later, the NADPH oxidase activity was almost 3-fold to that of the cells for 24h incubation. These stimulatory effects were glucose concentration- and time- dependent (P<0.01). In contrast, NADPH oxidase activity in MIN6 cells exposed to mannitol did not differ from that in control cells. These data implied that activations of NADPH oxidase participated in high glucose induced ROS production in MIN6 cells.

The effect of AGEs on MIN6 cells

As shown in Tab4, the NADPH oxidase activity was increased in MIN6 cells acted by AGEs above 200 mg/L at 24, 48, 72, 96h compared with normal and BSA control groups. These effects were time- and concentration-dependent (P<0.01). These data implied that activations of NADPH oxidase participated in AGEs induced ROS production in MIN6 cells.

Table 3. NADPH oxidase activity of MIN6 treated with high glucose（n=3, $\bar{\chi} \pm s$）

group	24h	48h	72h
control	53.95±17.21	39.78±11.13	49.15±16.23
mannitol	45.11± 9.79	41.57±10.04	52.96±14.77
Glu12.5mM	43.93±13.03	76.93±20.61*	117.53±23.21*
Glu25mM	78.68± 13.16	116.75±31.22**	209.05±17.24**
Glu50mM	107.33±29.11**	204.33±17.99**	331.09±40.32**

Versus control: *P<0.05; **P<0.01.

Table 4. Activity of NADPH oxidase treated with different concentrations of AGEs for different time（n=3, $\bar{\chi} \pm s$）

group	24h	48h	72h	96h
control	53.95±17.21	39.78±11.13	49.15±16.23	42.32±13.33
BSA	56.77±7.96	42.64±15.50	44.91±17.02	50.75±15.46
AGE100mg/L	84.05±22.24	99.78±21.07*	99.55±33.72	86.43±26.43
AGE200mg/L	103.10±22.39*	119.73±41.38*	116.55±42.18*	125.10±43.64*
AGE400mg/L	106.33±32.23*	126.45±39.69**	153.00±39.29**	180.00±51.62**

Versus control: *P<0.05; **P<0.01.

DISCUSSION

Type 2 diabetes, the more prevalent form of diabetes, is considered a heterogeneous disease resulting from the combination of insulin resistance and/or a β-cell secretory defect. Hyperglycemia resulting from uncontrolled glucose regulation is widely recognized as the causal link between diabetes and diabetic complications. Chronic exposure of the β-cell to supraphysiologic concentrations of glucose causes defective insulin gene expression accompanied by marked decreases in insulin content and abnormal insulin secretion (14). Hyperglycemia-induced superoxide production is an important aspect in β-cell glucose toxicity (3). It has been well demonstrated that AGEs progressively accumulated on the tissues and organs that develop chronic complications due to the long-time hyperglycemia such as micro- and macro vasculopathy. Increased AGEs formation is another key metabolic pathway contributed to hyperglycemia induced cell damage. It is important to understand how glucose and AGEs may cause β-cell dysfunction. In this article, we observed the possible deleterious effects of various glucose and AGEs.

In this study we employed a cellular model in which pancreatic β-cells MIN6 were exposed to different glucose or AGEs concentration. Preliminary results showed that MIN6 cells viability were decreased obviously resulting from treatment with high glucose or AGEs.

Oxidative stress is widely accepted as playing a key mediatory role in the development and progression of diabetes and its complications due to increased production of free radicals and impaired antioxidant defenses (5, 6, 12). Several reports have shown that persistent exposure of β-cells to elevated glucose induces β-cell apoptosis, which was also mediated by excessive ROS production (7, 11). In a normal cellular environment, ROS is essential to life, while in case of overproduction or exhaustion of antioxidants it might become deleterious. It is of extreme importance that ROS generation has been implicated in the etiology of several diseases. The ROS designation comprehends not only free radicals, such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), but also non-radicals, namely hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Of all the ROS, $O_2^{\cdot -}$ is the most important one and it is the source of other ROS. The generation of ROS is one major factor in the development of diabetes and its complications (8, 15).

ROS possesses one or more unpaired electrons, its short lifetime and instability make it difficult to be detected. The fluorescence methodology, associated with the use of suitable probes, is an excellent approach to measure ROS because of its high sensitivity, simplicity in data collection, and high spatial resolution in microscopic imaging techniques (17, 18). DHE has been used as a fluorescent probe for detecting $O_2^{\cdot -}$ due to its reported relative specificity for this ROS (1, 21). The product is a red fluorescent compound (probably ethidium). DHR123 is a non-fluorescent molecule that diffuses across cell membranes and is oxidized by ROS to the fluorescent rh123 within cells.

In the present paper, our observations demonstrated that high glucose and AGEs significantly increased cellular ROS. In our study, the higher the glucose or AGEs level, the more ROS the MIN6 produced. This result suggested that prolonged hyperglycemia may induce more severe damage in pancreatic β-cells. Unlike high glucose and AGEs, mannitol or BSA did not induce cellular ROS in MIN6. So it was

not the high osmolality or albumin required for high glucose- or AGEs- induced ROS generation in MIN6 cells. It also suggested that oxidative stress might play an important role in the progressive deterioration of β-cell function. Our result is consistent with previous studies that oxidative stress is increased in pancreatic β-cells in diabetic animals and diabetic patients (2, 16, 19).

As for the source of ROS production, the mechanisms for glucose- or AGEs- induced ROS production are still a matter of investigation. It has largely been established that the mitochondria respiratory chain is an important site of ROS production within cells, including β-cells (9). In contrast, superoxide-producing enzymes including NADPH oxidase have received increasing attention as one of the most important source of ROS production in vascular cells (10). Recently, several reports have shown the presence of NADPH oxidase in pancreatic β-cell and demonstrated the expression of phagocyte-type NADPH oxidase components in pancreatic islet (13, 20). Our study showed that the ROS formation induced by high glucose or AGEs in MIN6 cells was mainly mediated by NADPH oxidase activation. NADPH oxidases activity in the mannitol- or BSA- treated groups showed no statistical significance as compared with the control groups. So it is more likely that glucose itself or its reaction product plays the role. The activation of NADPH oxidase was also glucose or AGEs concentration dependent and time course dependent.

In summary, the present study showed that high glucose and AGEs could cause injury to MIN6 cells, they decreased the cells viability, induced ROS production via the activation of NADPH oxidase in MIN6 cells. This may, at least in part, explain the mechanism of pancreatic β-cell dysfunction. It helps to understand β-cell failure in the type 2 diabetic conditions. So these data implied that cellular ROS might be potential therapeutic targets in progressive accumulation of AGEs in diabetes.

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