

Original Article

Effect of glucagon on insulin secretion through cAMP signaling pathway in MIN6 cells

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Abstract: Objective: To explore the direct regulation effects and mechanisms of glucagon in insulin secretion of MIN6 cells that in the kind of the islet β cells. Methods ICUE3 and PCDNA3.1 plasmid were transfected to the MIN6 cells by electroporation transfection, and then treated with different concentrations of glucagon (Glg) and glucose (Glu). Biosensor technology that based on the fluorescence resonance energy transfer (FRET) was used to monitor the change of cAMP quantitatively and real-time. The level of cAMP and insulin were measured by the enzyme-linked immunosorbent assay (ELISA). Results: The receptor of Glg was mainly located on the cell membrane in MIN6 cells. Compared with the 0 ng/L Glg group in the Glu-free state, the average value of CFP/YFP increased $4\% \pm 0.02$ in the 500 ng/L Glg group, and the value in the 1000 ng/L Glg group increased $6\% \pm 0.03$ ($P > 0.05$). While in the high-Glu (16.7 mmol/L) state, the value increased $11\% \pm 0.02$ in the 500 ng/L Glg group, and increased $23\% \pm 0.06$ in the 1000 ng/L Glg group when compared with the 0 ng/L Glg group ($P < 0.01$). The levels of the cAMP of 1000 ng/L and 500 ng/L Glg group were higher than those of the 100 ng/L and 0 ng/L Glg group in the condition of Glu-free (81.27 ± 6.29 , 76.73 ± 2.10 , 39.45 ± 2.83 , 40.36 ± 4.20 ; $P < 0.01$). The levels of the cAMP of 1000 ng/L, 500 ng/L and 100 ng/L Glg group were higher than those of the 0 ng/L Glg group, at the meanwhile, the levels of the cAMP of 1000 ng/L and 500 ng/L Glg group were also higher than 100 ng/L Glg group in the condition of low-Glu (2.8 mmol/L) (92.91 ± 7.35 , 90.36 ± 3.15 , 65.82 ± 10.49 , 46.73 ± 1.05 ; $P < 0.01$). And this trend in the condition of high-Glu was almost to the low-Glu (106.75 ± 7.26 , 94.18 ± 2.99 , 83.09 ± 1.16 , 55.60 ± 5.51 , $P < 0.01$). The levels of the insulin of 1000 ng/L, 500 ng/L and 100 ng/L Glg group were higher than those of the 0 ng/L Glg group. While 1000 ng/L Glg group was higher than that of the 500 ng/L and 100 ng/L Glg group in the condition of Glu-free (1844.02 ± 200.93 , 1387.94 ± 483.12 , 1251.817 ± 60.30 , 787.33 ± 81.72 ; $P < 0.01$). The levels of the insulin of 1000 ng/L and 500 ng/L Glg group were higher than those of the 100 ng/L and 0 ng/L Glg group, and the 1000 ng/L and was also higher than 500 ng/L Glg group in the condition of low-Glu (1552.31 ± 81.20 , 1285.62 ± 131.67 , 1020.85 ± 42.60 , 762.89 ± 26.94 , $P < 0.01$). And this trend in the condition of high-Glu was almost to the low-Glu (1898.337 ± 169.03 , 1399.30 ± 148.66 , 1061.735 ± 9.13 , 972.89 ± 22.19 ; $P < 0.01$). The levels of cAMP and insulin secretion of MIN6 cells had a positive correlation in different Glu conditions ($r^2 = 0.559$, $P < 0.01$). Conclusion: Glg may stimulate insulin secretion by increasing cAMP levels in the way of concentration gradient within the islet β cell lines—MIN6 cells. And the increasing trend was Glu dependent.

Keywords: Glucagon, camp, insulin, islet β cells, diabetes mellitus

Introduction

Diabetes (diabetes mellitus, DM) is a frequently occurring disease worldwide. According to statistics [1], the global number of 20-79 year-old adult DM patients had more than 285 million, and is still in growth. DM and its variety of complications may involve multiple body organs, and lead tremendous physical and mental damage on patients, and a heavy financial burden on families and society. In recent years,

many researchers have begun to focus on the DM.

Some studies have shown that there is a close relationship between the incidence of DM and insulin insufficient of islet β cells [2, 3]. The secretion of insulin is also affected by a variety of hormones and neurotransmitters except for a variety of energy materials, such as glucagon (Glucagon, Glg), glucagon-like peptide (GLP-1), epinephrine (E) and somatostatin (SS) and

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Table 1. Different Groups for measuring the cAMP by FRET

Classification	Groups	Plasmid	Treatment
Treatment group	A (0 ng/L Glg)	ICUE3	0 mmol/l Glu, 16.7 mmol/l Glu
	B (500 ng/L Glg)	ICUE3	0 mmol/l Glu, 16.7 mmol/l Glu
	C (1000 NG/L Glg)	ICUE3	0 mmol/l Glu, 16.7 mmol/l Glu
Negative control group	D (1000 NG/L Glg)	PCDNA3.1	0 mmol/l Glu, 16.7 mmol/l Glu
	E (1000 NG/L Glg)	No	0 mmol/l Glu, 16.7 mmol/l Glu

Table 2. Different Groups for detecting the cAMP and Insulin by ELISA

Glucagon (ng/L)	Glucose (mmol/L)		
	0 (No)	2.8 (Low)	16.7 (High)
0 (No)	(1)	(2)	(3)
100 (Low)	(4)	(5)	(6)
500 (Middle)	(7)	(8)	(9)
1000 (High)	(10)	(11)	(12)

other hormones and peptides. They do not directly caused insulin secretion [4], but it may play a role in regulating the insulin secretion by adjusting the concentration of some of the second messenger pathways [5].

Glg is a hormone secreted by islet α cells. It mainly targets on the liver, on the other hand, Glg may regulate the insulin secretion directly by the regulation of adjacent β cells through the paracrine action. And the effect may only strengthen glucose-induced insulin secretion, not stimulate the generation of insulin directly [6]. Second messenger signaling molecules may be a key point involved in the regulation of insulin secretion [7, 8]. But the mechanism is not clear yet. In this study, biosensor technology that based on the FRET was used to monitor the change of cAMP in MIN6 cells quantitatively and real-time after the stimulation of different concentrations of glucagon and glucose, and the enzyme-linked immunosorbent assay (ELISA) was used to detect the level of cAMP and insulin intracellular. Our study directly explored the specific role of Glg on regulating insulin secretion of islet β cells, and its possible mechanisms through second messenger cAMP pathway.

Materials and methods

Materials

One mg glucagon powder was dissolved in the 10 ml PBS which with glacial acetic acid (10%)

to make the stock solution of Glg, and then diluted to 100 ng/L (low-concentration) step-wise, 500 ng/L (middle-concentration), 1000 ng/L (high-concentration) with Krebs as the Glg working fluid. Glucose solution was added as needed to the Glg working fluid made a final concentration of 2.8 mmol/L (low-concentration) and 16.7 mmol/L (high-concentration) of glucose fluid.

Cell culture

MIN6 cell is a kind of islet β cell lines. It was cultured in a medium of DMEM (4.5 g/L glucose, 15% FBS, 2% penicillin-streptomycin, 1% L-glutamine, and 1% β - mercaptoethanol).

Electroporation transfection

After completion of MIN6 cells were trypsinized, 1000 rpm centrifuged at room temperature for 5 minutes, then added 100 μ l electroporation buffer and 10 μ g ICUE3 or PCDNA3.1 empty plasmid. The suspension was moved to electric rotor and transfected into electrical instrument for transfection. After transfection, cells were cultured in dishes, for ready to use.

Measurement of cAMP production by biosensor technology that based on the FRET

The transfected and treated cells were divided into five groups-A, B, C, D, and E. All the treatment groups and the negative control group were added 30 μ mol/L forskolin as a positive control. We observed the changes of CFP/YFP colors with the changes of intracellular cAMP concentration, which indicated that the changes of cAMP concentration directly reflected by the results of the FRET.

Measurement of cAMP and insulin secretion by ELISA -The MIN6 cells were divided into 12 groups according to the treating factors (n = 3 wells) (Table 2). After the cell grew well, we starved them, treated them differently, and then measured the cAMP concentration and insulin secretion by ELISA.

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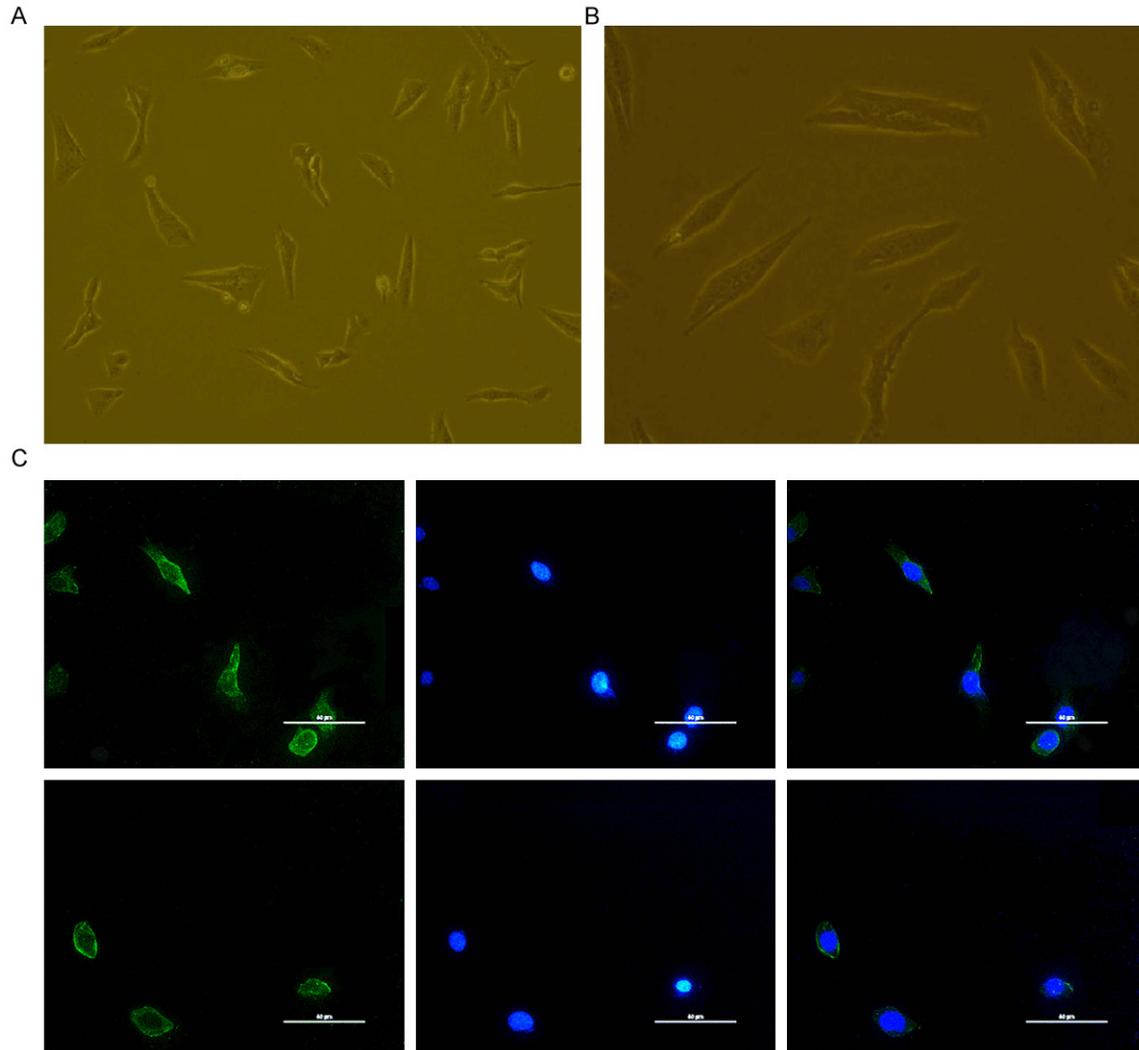


Figure 1. Expression of glucagon receptor on MIN6 cells (200 \times). A, B. The forms of the cultured MIN6 cells (A: 100 \times , B: 200 \times); C. Expression of glucagon receptor on MIN6 cells.

Statistical analysis

Data were analyzed by the SPSS 17.0 software and presented as the mean \pm standard deviation. To compare the mean of the groups, analysis of variance was used. Pearson correlation test was performed to determine the associations of cAMP and insulin. $P < 0.05$ was considered to indicate a statistically significant difference exist.

Results

Expression of glucagon receptor on the MIN6 cells

MIN6 cells were irregularly shaped tile-like adherent growth (**Figure 1A, 1B**). By immunofluorescence staining, we can see the expres-

sion of Glg receptor in the MIN6 cells, and the expression mainly exist on the cell membrane (**Figure 1C**).

cAMP biosensor expression and function in MIN6 cells

To demonstrate whether the biosensor was working, we used the FSK as a positive control and found changed color of fluorescence and a increasing of CFP/YFP when stimulating the MIN6 cells (**Figure 2**). It indicated that the system was working well.

CFP/YFP in different group measured by biosensor technology based FRET

After transfection, MIN6 cells from the treated groups (A, B, C. (**Table 1**)) were placed in a con-

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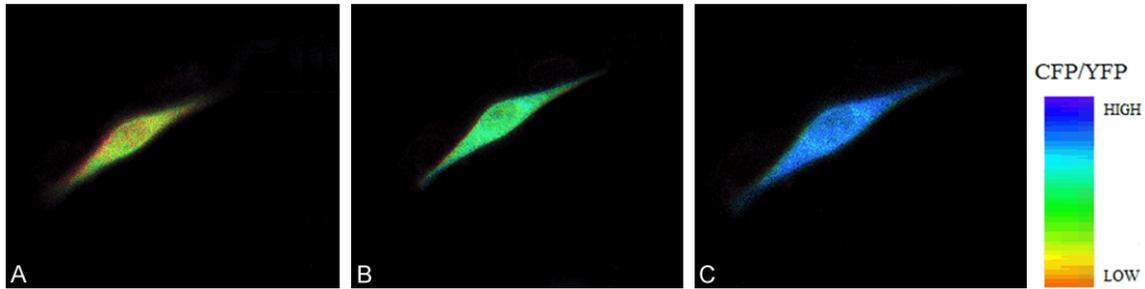


Figure 2. cAMP biosensor expression and function in MIN6 cells. The fluorescence color was changed after FSK treatment. Early cells are yellow (A), with the processing time, cells turn green (B), but soon become cyan-blue (C). The changes of color in MIN6 cells indicate the concentration of cAMP increasing.

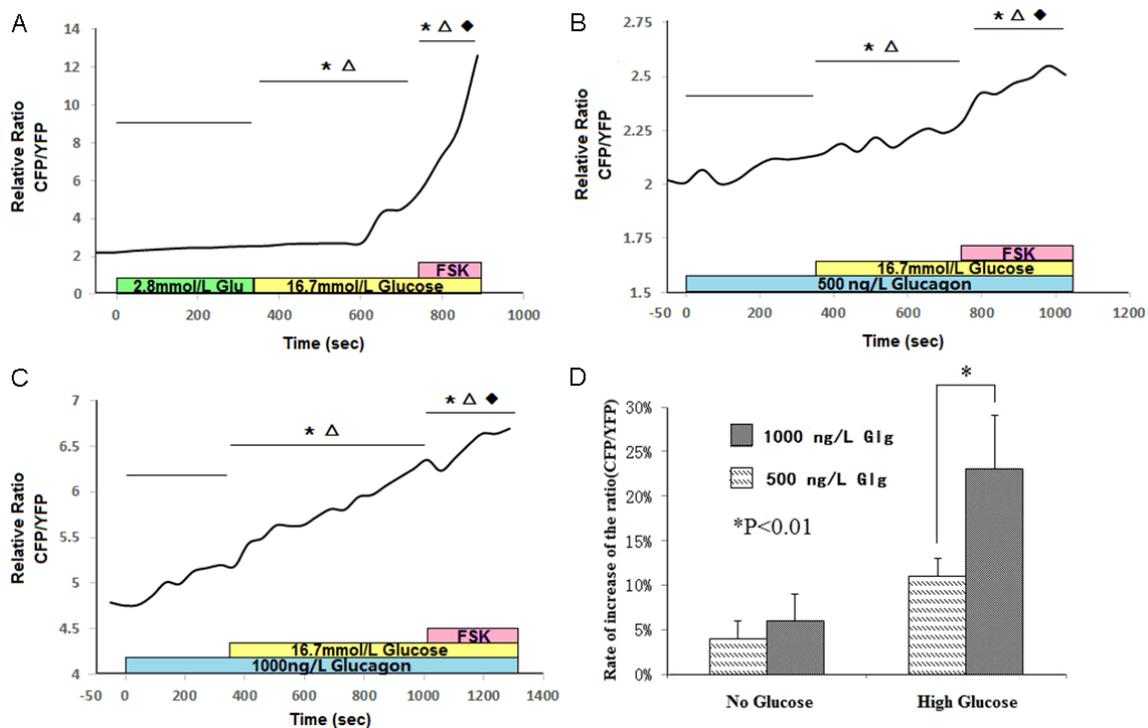


Figure 3. Change of CFP/YFP in MIN6 cells after different treatments. A-C. CFP/YFP change in a single MIN6 cell following application of glucose (0, 2.8, 16.7 mmol/L) and glucagon (0, 500, 1000 ng/L), compared with base level (the values measured 50 s before stimulation), the ratio (CFP/YFP) is higher ($*P < 0.01$), compared with the previous stage, the ratio is higher ($\Delta P < 0.01$, $\blacklozenge P < 0.01$). The increase in the ratio (CFP/YFP) indicates an increase in cAMP concentration. D. Quantity data of different concentrations of glucagon on cAMP formation. When high-glucose condition, cAMP concentration is higher than those in in 500 ng/L glucagon group. Every experiment was repeated three times.

focal microscope, then the previously prepared stimulation fluid was added (Table 1). The results showed that in glucose-free state the average value of CFP/YFP increased $4\% \pm 0.02$ in the 500 ng/L glucagon group when compared with the 0 ng/L glucagon group. And this value in the 1000 ng/L glucagon group was 6%

± 0.03 ($P > 0.05$). While in the high-glucose state, the value increased $11\% \pm 0.02$ of the 500 ng/L glucagon group, and $23\% \pm 0.06$ of the 1000 ng/L glucagon group when compared with the 0 ng/L glucagon group ($P < 0.01$) (Figure 3). This result means that the level of cAMP is increasing following the Glg concentra-

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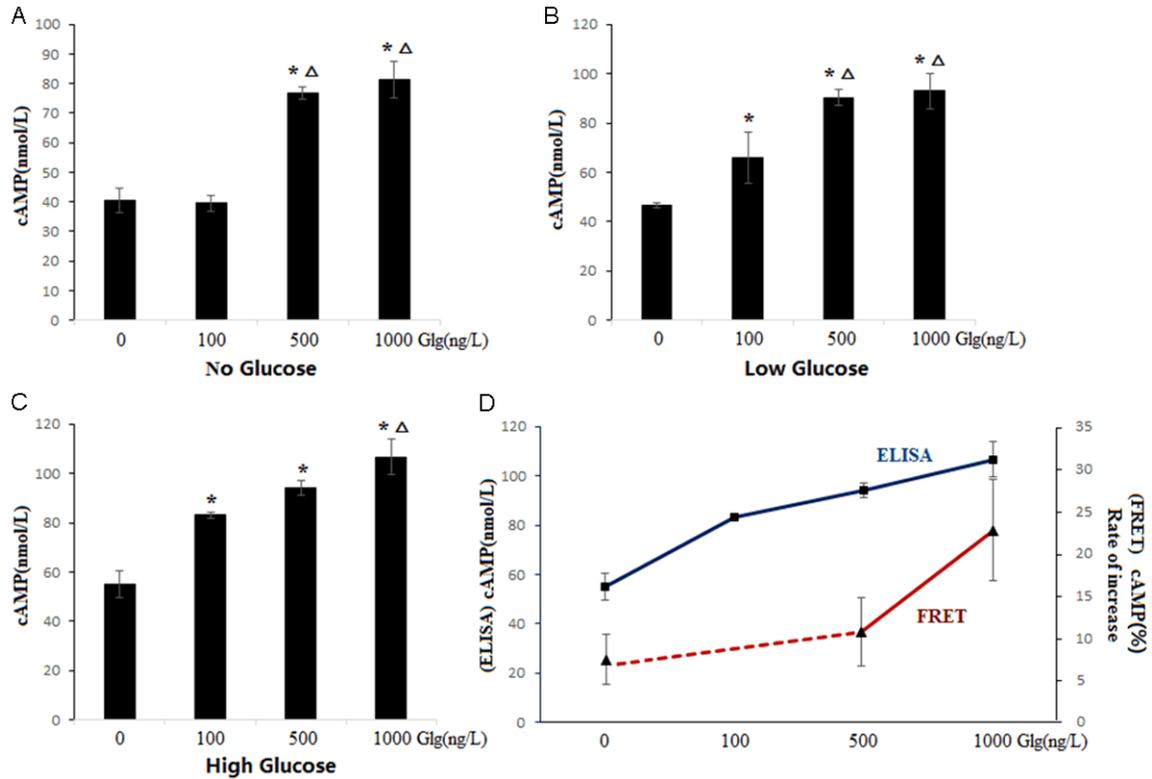


Figure 4. Comparison of different groups of cAMP formation Measured by ELISA. A-C, gradients of cAMP concentration following application of glucose (0, 2.8, 16.7 mmol/L) and glucagon (0, 100, 500, 1000 ng/L) respectively, whichever glucose concentration, compared with groups (0 ng/L Glg), * $P < 0.05$; compared with groups (100 ng/L Glg), $\Delta P < 0.01$. D, comparison of the two methods (FRET and ELISA) of cAMP concentration, there was a same increasing trend of them.

tion going up in high-glucose condition. But in glucose-free condition, there is no significant difference ($P > 0.05$).

Change of cAMP in different groups measured by ELISA

The level of the cAMP in MIN6 cells was detected by ELISA, the results showed that the levels of the cAMP of 1000 ng/L and 500 ng/L glucagon group were higher than those of the 100 ng/L and 0 ng/L glucagon group in the condition of glucose-free ($P < 0.01$). The levels of the cAMP of 1000 ng/L, 500 ng/L and 100 ng/L glucagon group were higher than those of the 0 ng/L glucagon group, at the meanwhile, the levels of the cAMP of 1000 ng/L and 500 ng/L glucagon group were also higher than 100 ng/L glucagon group in the condition of low-glucose (2.8 mmol/L) ($P < 0.01$). Otherwise, the levels of the cAMP of 1000 ng/L, 500 ng/L and 100 ng/L glucagon group were higher than those of the 0 ng/L glucagon group and 1000 ng/L was

higher than 100 ng/L glucagon group in the condition of high-glucose (16.7 mmol/L) ($P < 0.01$). In a word, with the increasing of glucose concentration, the concentration of the cAMP stimulated by Glg has increased more. In different concentrations of Glu, the level of cAMP was increasing following the Glg concentration going up, which showing some of the concentration gradient and the trend was more of obviously following the Glg concentration. This result was also similar to the results observed by the FRET method dynamical (**Figure 4D**).

Comparison of different groups of insulin secretion measured by ELISA and correlation analysis between the level of cAMP and insulin

The results showed that the levels of the insulin of 1000 ng/L, 500 ng/L and 100 ng/L glucagon group were higher than those of the 0 ng/L glucagon group. While 1000 ng/L glucagon group was higher than that of the 500 ng/L and 100 ng/L glucagon group in the condition of

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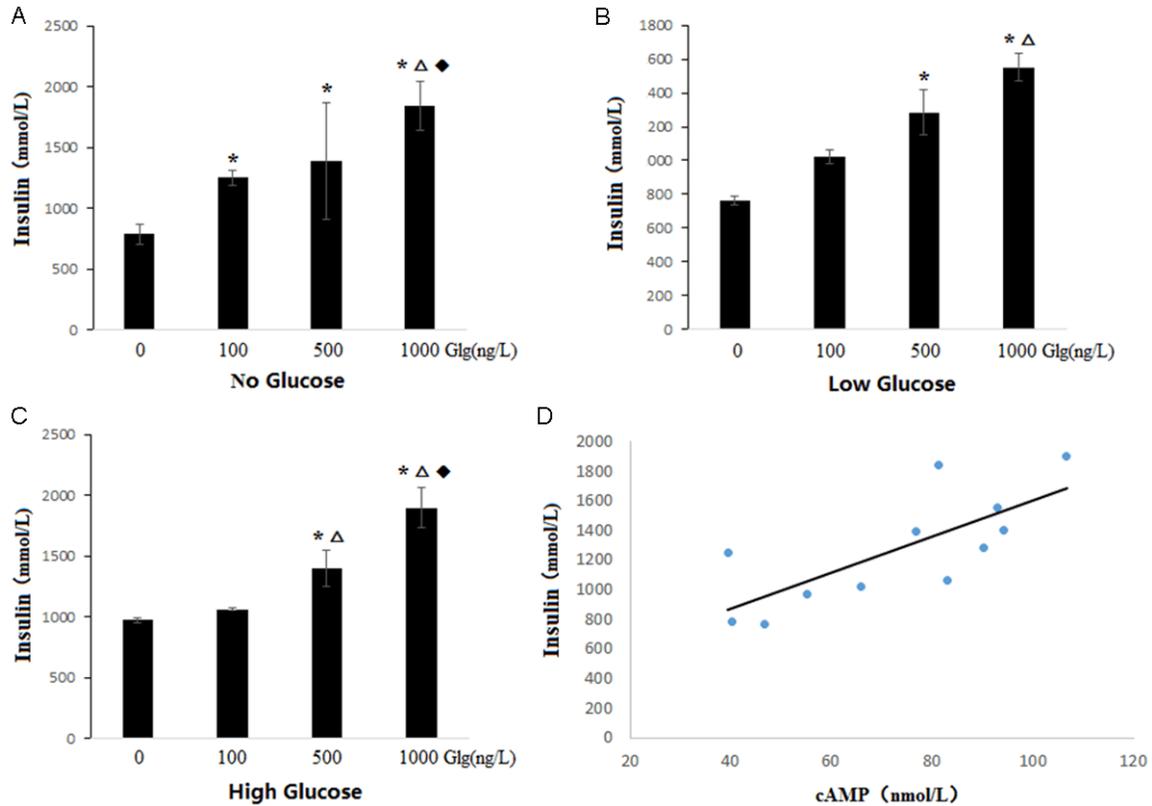


Figure 5. Comparison of different groups of insulin secretion measured by ELISA and correlation analysis between the level of cAMP and insulin. A-C, gradients of the concentration of Insulin secretion following application of glucose (0, 2.8, 16.7 mmol/L) and glucagon (0, 100, 500, 1000 ng/L), respectively. Compared with 0 ng/L Glg groups, * $P < 0.05$; compared with 100 ng/L Glg groups, $\Delta P < 0.01$, compared with 500 ng/L Glg groups, $\blacklozenge P < 0.01$. D. correlation analysis between cAMP and insulin ($r = 0.748$, $P = 0.005$).

glucose-free ($P < 0.01$). The levels of the insulin of 1000 ng/L and 500 ng/L glucagon group were higher than those of the 100 ng/L and 0 ng/L glucagon group, at the meanwhile, the 1000 ng/L and was also higher than 500 ng/L glucagon group in the condition of low-glucose ($P < 0.01$). Otherwise, the levels of the insulin of 1000 ng/L and 500 ng/L glucagon group were higher than those of the 100 ng/L and 0 ng/L glucagon group and 1000 ng/L was higher than 500 ng/L glucagon group in the condition of high-glucose ($P < 0.01$).

The effect of Glg to insulin secretion showed a concentration gradient, especially in the condition of high-glucose (Figure 5A-C). In addition, when we made the correlation analysis between the level of cAMP and insulin with the treatment of different concentrations. The results showed a positive correlation between the level of cAMP and insulin ($r = 0.748$, $P < 0.01$; Figure 5D).

Discussion

According to the report [9], Glg receptors are coupled with seven transmembrane G protein. In addition to distribution in the liver, Glg receptors are also widely distributed on the kidneys, brain, intestine, spleen, thyroid, pancreatic α and β cells and other tissues or cells. In islet β cells, Glg may regulate the insulin secretion through the second messenger-cAMP.

In this study, MIIN6 cells were treated with different concentrations of Glg at different concentrations of Glu. The biosensor technology based on the FRET method was used to monitor the change of cAMP in MIN6 cells quantitatively and real-time with the stimulation of different concentrations of glucagon and glucose. The results showed in the high-glucose state, the increasing Glg can stimulate the cAMP level increasing. So we speculate that it may correlate with the threshold value, reaction speed and the Glu-dependent of Glg effects on insulin

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secretion in MIN6 cells. The level of the cAMP and insulin in MIN6 cells was detected by ELISA. The results also showed that the level of cAMP and insulin secretion was going up following the Glg increasing. This situation presented a concentration gradient, and was consistent with the results of biosensor method. On the other hand, the trend is more obviously in Glu-existed state. For the detecting of Ins secretion, the glycemic effect of Glg *in vivo* can be excluded because of the basis of islet β cells cultured *in vitro*. So our results directly reflect the insulin secretion in β cells by the regulation of Glg. When we made the correlation analysis between the level of cAMP and Ins with the treatment. The results showed a positive correlation ($r = 0.748$, $R^2 = 0.559$, $P < 0.01$). We supposed that cAMP signaling pathway plays an important role in the process of insulin secreting in the islet β cells.

In summary, glucagon may stimulate the insulin secretion by increasing cAMP levels in the way of concentration gradient within the islet β cell lines-MIN6 cells. And the increasing trend was glucose dependent. And there is a positive correlation between the level of cAMP and insulin. So, the study results will provide a experimental basis for the mechanism of insulin secretion and lay a foundation for the further development of new DM therapeutic drugs.

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Disclosure of conflict of interest

None.

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