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Insulin Secretion with the Effect of Loureirin B through K_{ATP} Channel–Dependent Pathway

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Abstract

The development of new diabetes drugs continues to be explored. Loureirin B, a flavonoid, extracted from *Dracaena cochinchinensis*, has been confirmed to increase insulin secretion and decrease blood glucose levels. For understanding the mechanism, a series of experiments had been employed based on computational methods and cell experiments. The insulin secretion significantly increased with the incubation of 0.01μM loureirin B for 4 hours. The viability of Ins-1 cells showed no significant difference with the treatment of loureirin B. Through computational methods, we hypothesized that loureirin B could interacts with K_{ATP} channels to promote insulin secretion. In cell experiments, it could be found that the current of K_{ATP} channel of Ins-1 cells was inhibited by the effect of loureirin B. After then, the voltage-dependent calcium channels were activated, the increase of Cx43 protein expression might mediate the Ca^{2+} to the intracellular. In summary, it could be concluded that loureirin B promoted insulin secretion mainly through inhibiting K_{ATP} current, the influx of Ca^{2+} to the Intracellular and the expression of Cx43.

Keywords: loureirin B; Ins-1 cells; Insulin secretion; K_{ATP} channel; influx of Ca^{2+}; expression of Cx43

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1. Introduction

Diabetes mellitus is principally characterized by insulin resistance with elevated blood glucose levels [1]. For treating with diabetes mellitus, it is important to understand how to promote the insulin secretion and sequentially decrease the blood glucose levels in diabetic patients. Numerous anti-diabetic drugs have been developed to treat diabetes mellitus. For different type of drugs, they have different target to produce efficacy. Sulfonylureas still remain the most widely used drugs for treating patients with diabetes so far [2].

Sulfonylureas, oral hypoglycemic agents, stimulate pancreatic β cells to secrete insulin. It has been nearly 50 years that sulfonylureas anti-diabetes agent used for the treatment of diabetes so far [3]. However, this agent may cause hypoglycemia due to sustained effect resulting in insulin secretion [4-6]. As research continues, the mechanism of sulfonylureas was gradually understood that it stimulated the release of insulin by inhibiting the flow of potassium through K_{ATP} channels which were formed by SUR1 and Kir6.2 [7]. However, it remains unclear that how sulfonylureas interacts with the K_{ATP} channels at molecular level. With the development of bioinformatics, the possibility of the interaction between ligands and target protein can be obtained. Based on the computational methods, the possible binding sites between sulfonylureas and K_{ATP} channels was predicted in our study.

Loureirin B is a kind of flavonoid extracted from *Dracaena cochinchinensis* [8]. Several efforts have been made to study loureirin B. Among them, it has been reported that loureirin B has the ability to decrease the plasma sugar level and improve the glucose-resistance in glucose-induced hyperglycemic rats [9, 10]. However, the function mechanism of its anti-diabetic activity hasn't established so far. The interaction between loureirin B with K_{ATP} channels was simulated at the binding site which was predicted from the interaction between glimepiride (sulfonylureas) and K_{ATP} channels. Hence, we guessed that loureirin B had the similar mechanism that it could promote insulin secretion through interacting with the K_{ATP} channels.

Meanwhile, several experiments were developed in *vitro*. Several studies revealed that change in the ATP/ADP ratio, closure of ATP-sensitive K^+ channel (K_{ATP}), depolarization of membranes, Ca^{2+} influx to increase ($[Ca^{2+}]i$) all had impacts on insulin secretion [11-13]. Therefore, the change of K_{ATP} current on Ins-1 cells and the intracellular Ca^{2+} levels were detected. What's more, the expression of Cx43 protein was employed because it played an important role in mediating the flow of ions, such as K^+ and Ca^{2+} [14, 15].

Based on computational methods and experiments in *vitro*, we investigated the mechanism of loureirin B how it could promote insulin secretion and proposed the hypothesis that loureirin B had an effect on insulin secretion of pancreatic β-cells, the current of K_{ATP} channel, the increase $[Ca^{2+}]_i$ and the expression of Cx43 protein (Fig.1).

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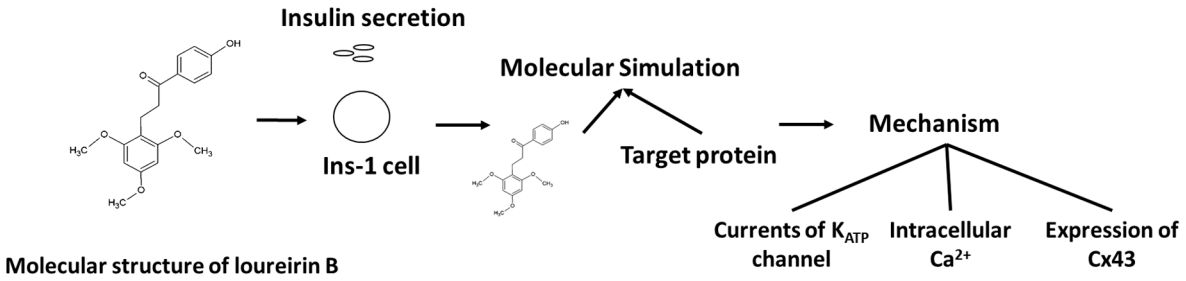

Fig.1 Hypothesis of the effect of loureirin B on Ins-1 cell

2. Materials and methods

2.1 Materials

Loureirin B and glimepiride were purchased from National Institute for the Control of Pharmaceutical and Biological Products (China) and Sigma-Aldrich, respectively. The stock solution of loureirin B and glimepiride was prepared in DMSO. Other reagents were analytical pure, and ultrapure water was used for the experiments.

2.2 Ins-1 cells culture

Rat pancreatic β-cell line, Ins-1 cells purchased from ATCC (USA), was cultured in DMEM high glucose medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100μg/mL streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 50μmol/L b-mercaptoethanol at 37°C in a humidified atmosphere and 5% CO_2. The culture medium was replaced every 2 days, and passaged every four days following trypsinization. The subculture cells were seeded at a density of 1.0-5.0×10^5 cells/mL.

2.3 Measurement of the insulin secretion of Ins-1 cells

To evaluate the effect of loureirin B on insulin secretion, Ins-1 cells were seeded onto 96-well plates and cultured for 48 hours to approximately 80-90% confluence. The cells were washed twice with PBS, and starved for 2 hours in KRB buffer. The cells were incubated in fresh KRB buffer containing different concentrations of loureirin B (0.001, 0.01, 0.1, 1, 10μM) for different reaction time (0.5, 1, 2, 4, and 8h), and then sampled for insulin secretion. Insulin was measured by Rat insulin secretion Elisa Kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.).

2.4 Measurement of the viability of Ins-1 cells

To determine the effect of loureirin B on cell viability, Ins-1 cells were seeded onto 96-well plates and cultured for 48 hours to approximately 80-90% confluence. Then, the cells were starved in a 2% FBS/DMEM for 12h. Control group was cultured in medium without loureirin B, while the positive control group was received fresh medium with glimepiride. After the treatment of loureirin B and GE for 4 and 8 hours,

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the cell viability was measured by Cell Counting Kit-8 (CCK-8) (Sangon Biotech (Shanghai) Co., Ltd.).

2.5 Simulation of the interaction between ligands and ATP-sensitive potassium channel

The structure of ATP-sensitive potassium channel was obtained from the PDB database (http://www.rcsb.org/pdb). The molecular structure of loureirin B and glimepiride were obtained from Pubchem database (https://pubchem.ncbi.nlm.nih.gov/). Discovery Studio 4.1 software was employed to simulate the interaction between ligands and target protein.

2.6 Measurement of electrophysiological recording of Ins-1 cells

The K_{ATP} current was recorded by using whole-cell patch-clamp technique with a multiclamp700B amplifier (Axon Instruments, USA). Data acquisition and analysis were performed *via* Clampex 8.0 system. Currents were recorded with patch pipettes of 2–5MΩ. The seal resistance was greater than 1.0GΩ. The stimulation protocol for the voltage clamp recording of the whole-cell K_{ATP} current in Ins-1 cells was as follows: the composition of the internal pipette solution was: 15 mmol/L NaCl; 92 mmol/L KCl; 33mmol/L KOH;10mmol/L HEPES; 2 mmol/L $MgCl_2$; 1 mmol/L $CaCl_2$; 0.6 mmol/L EGTA; and 0.012 mmol/L MgATP with pH adjusted to 7.2, using deionized water purified by an EPED super purification system (Eped, Nanjing, China). Meanwhile, the extracellular fluid contained 80mmol/L NaCl; 60mmol/L KCl; 10mmol/L HEPES; 2mmol/L $MgCl_2$; and 0.1mmol/L $CaCl_2$ with pH adjusted to 7.4 by NaOH. The above reagents were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China), and were of analytical grade.

To detect the blocking effect of GE on K_{ATP} in Ins-1 cells, Ins-1 cells were continuously stimulated with GE for 12min, and the K_{ATP} current intensity was recorded every minutes. Ins-1 cells were incubated with LB for 10 min to study the effect of loureirin B on K_{ATP} in Ins-1 cells. The results are expressed as current density (I_m/C_m). Current (I_m) is defined as the current intensity, while the capacitance (C_m) is the Ins-1 cell membrane capacitance. All data sampling and analysis were carried out by Clampex 8.0 and Clampfit 8.01 software, respectively. The value of current density was obtained from the following equation:

$$\text{Current density (pA/pF)} = \frac{I_m}{C_m} \qquad (1)$$

2.7 Measurement of the intracellular Ca^{2+} levels of Ins-1 cells

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) were detected by the radiometric fluorescent Ca^{2+} indicator Fura-2, as previously described (Bhavsar, Schmidt et al. 2013). Ins-1 cells were starved in 2% FBS/DMEM medium for 12h. Cells were then incubated with loureirin B for different reaction time. Cells were rinsed with PBS and loaded with 1 μmol/L Fura-2/am (Shanghai Yeason Biotechnology Co., Ltd., Shanghai, China) for 30min at 37°C. Then, the cells were illuminated by excitation with a dual UV light source at 340nm and 380nm, and data were recorded every second with an F-

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7000 fluorescence spectrophotometer (HITACHI, Japan). Changes in $[Ca^{2+}]i$ were deduced from variations in the ratios of fluorescence intensities (F340/F380).

2.8 Western blot analysis

Ins-1 cells were incubated with 10^{-8} M loureirin B for different time intervals, rinsed with PBS, and lysed in RIPA lysis buffer (Beijing ComWin Biotech Co., Ltd., Beijing, China). Protein content was measured by the BCA protein quantification kit. Samples of total extracts and pre-stained protein marker (Multicolour Broad Rang) (TIANGEN BIOTECH Co., Ltd., Beijing, China) were fractionated via electrophoresis using 12% SDS-PAGE. The proteins were transferred onto PVDF membranes (Merck Millipore, USA) (300mA, 1h). After repeated rinsing within TBS-Tween (0.1% Tween 20 in TBS), the PVDF membranes were blocked at room temperature in 5% skimmed milk powder for 1 h. The membranes were then incubated with monoclonal β-actin antibody and polyclonal Cx43 antibody (Proteintech, USA) at a dilution of 1:2000, overnight at 4°C. After washing, the membranes were incubated with goat anti-mouse/rabbit IgG-HRP (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China), diluted at 1:500, at room temperature for 1 h. BeyoECL Plus solution(Beyotime Biotechnology Co., Ltd., Shanghai, China) was added, and the chemiluminescent signals were detected by a gel imaging system ChemiDoc XRS (Bio-Rad, MA, USA).

2.9 Statistical analysis

All statistical analyses were conducted using the software of IBM Spss Statistical 20. Data were analyzed using one-way ANOVA. The limit of statistical significance was set at $P<0.05$. Results are presented as means ± SD.

3. Results

3.1 Effect of loureirin B on insulin secretion in Ins-1 cells

In order to detect the pharmacological efficacy of Loureirin B, the suitable effective concentration and incubation time should be confirmed. Firstly, Ins-1 cells were incubated with different concentration of loureirin B varying from 0.001μM to 10μM for 4 hours. As seen in Fig.2A, the insulin secretion increased gradually while the cells were incubated with 0.01μM loureirin B ($P < 0.01$). Then, the cells were incubated with different time intervals range from 0.5h to 8h. No remarkable changes were observed within 2 hours in the concentration of insulin. However, the insulin concentration increased significantly after 2 hours ($P < 0.01$). The level of insulin secretion reached the highest at the incubation time of 4h, which was about 1.36-fold higher than that of the control group (Fig 2B).

Meanwhile, a comparison between loureirin B and positive drug-glimepiride was made. As seen in Fig.2C, the insulin secretion of both loureirin B and glimepiride group were significantly higher than that of the control group ($P < 0.05$). And the loureirin B -induced insulin secretion was slightly higher than that after the treatment of glimepiride. Furthermore, the viability of Ins-1 cell with loureirin B induced insulin secretion was employed by CCK-8 assay. As seen in Fig.2D, compared with the control

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group, the viability of Ins-1 cells showed no significant difference with the treatment of 0.01μM loureirin B for 4 hours. And then, after incubating with loureirin B for 8 hours, it could be seen that the viability of Ins-1 cell was significantly increasing. It indicated that loureirin B caused no toxicity on cells.

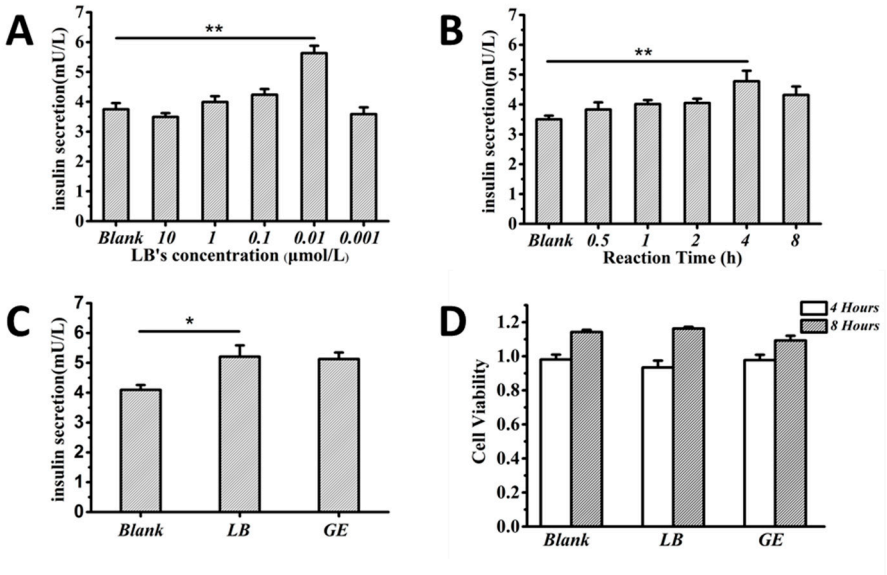

Fig. 2 Effect of loureirin B on insulin secretion in Ins-1 cells

(A)(B) Different concentration of loureirin B and incubation time were applied to search the optimal concentration and incubation time, respectively. **(C)** Comparison of insulin secretion between loureirin B and positive drug-glimepiride. **(D)** The effect of loureirin B on the viability of Ins-1 cells. (n=5, *P < 0.05; **P < 0.01)

3.2 Simulation of loureirin B on ATP-sensitive potassium channel

Molecular dynamics simulation has the advantage of analyzing the interaction between ligand and proteins. Based on the computational methods, significant information of ligand with protein can be obtained to screen out the possibility that whether ligand has the ability to bind target protein or not. As is all known, sulfonylurea medicine has a good therapeutic effect on diabetes through inhibiting the flow of potassium through K_{ATP} channels. However, it is still unclear that how sulfonylurea medicine interacts with K_{ATP} channels at the molecular level. In our experiment, glimepiride was chosen as the positive drug to simulate the interaction with K_{ATP} channels. K_{ATP} channels are made up of two proteins, SUR1 and Kir6.2. Through preliminary screening test, suitable binding site was searched between glimepiride with SUR1 or Kir6.2, respectively. Then, the interaction between loureirin B with SUR1 or Kir6.2 were simulated at the binding site from the previous calculation. Simulation results indicated that loureirin B had the ability to interact with SUR1 or Kir6.2, respectively. Loureirin B interacted with Kir6.2 mainly through hydrogen bond and hydrophobic (Table 1). And it interacted with SUR1 mainly through hydrogen bond, hydrophobic and electrostatic force (Table 2). We hypothesized that insulin secretion was caused by loureirin B due to the interaction with K_{ATP} channels. Therefore, this

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hypothesis will be discussed with the further study.

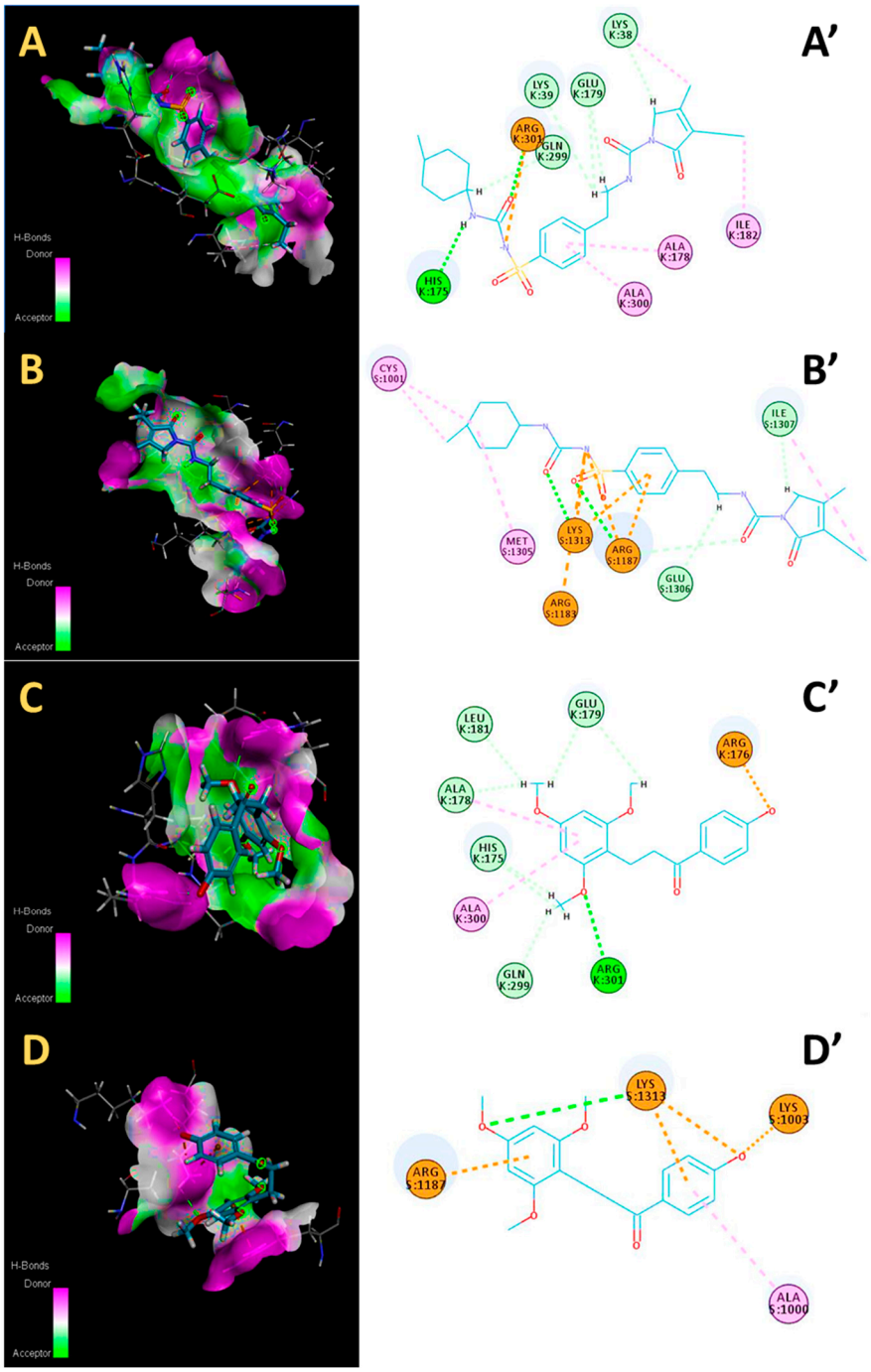

Fig. 3 Simulation of glimepiride and loureirin B on ATP-sensitive potassium channel.

(A) (A') Simulation diagram between glimepiride and Kir6.2; **(B) (B')** Simulation diagram between glimepiride and SUR1; **(C) (C')** Simulation diagram between loureirin B and Kir6.2; **(D) (D')** Simulation diagram between loureirin B and SUR1. **(A-D)** 3D diagram; **(A'-D')** 2D diagram

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Table.1 The interaction parameter between loureirin B (LB) and Kir6.2

Acting Force	Bond length	From	To
Hydrogen Bond	1.86072	KIR6.2:ARG176:HH12	LB:O5
Hydrogen Bond	1.82592	KIR6.2:ARG176:HH22	LB:O5
Hydrogen Bond	2.70856	KIR6.2:ARG301:HH11	LB:O1
Hydrogen Bond	2.93354	LB:H34	KIR6.2:HIS175:ND1
Hydrogen Bond	2.51495	LB:H35	KIR6.2:GLN299:O
Hydrogen Bond	2.65112	LB:H36	KIR6.2:HIS175:ND1
Hydrogen Bond	2.70853	LB:H39	KIR6.2:GLU179:OE1
Hydrogen Bond	2.85464	LB:H40	KIR6.2:ALA178:O
Hydrogen Bond	2.43779	LB:H40	KIR6.2:LEU181:O
Hydrogen Bond	2.64801	LB:H42	KIR6.2:GLU179:OE1
Hydrophobic	5.13651	LB	KIR6.2:ALA178
Hydrophobic	4.96541	LB	KIR6.2:ALA300

Table.2 The interaction parameter between loureirin B (LB) and SUR1

Acting Force	Bond length	From	To
Hydrogen Bond	1.60953	SUR1:LYS1003:HZ3	LB:O5
Electrostatic	3.70871	SUR1:LYS1313:NZ	LB:O5
Hydrogen Bond	2.46844	SUR1:LYS1313:HZ3	LB:O3
Hydrogen Bond	2.30315	SUR1:LYS1313:HE2	LB:O5
Electrostatic	3.30198	SUR1:ARG1187:NH1	LB
Electrostatic	4.40435	SUR1:LYS1313:NZ	LB
Hydrophobic	5.35599	LB	SUR1:ALA1000

3.3 Effect of loureirin B on the current of K_{ATP} channel

To detect the change of currents on ATP-sensitive potassium channel, the whole-cell patch-clamp technique was applied. As seen in Fig.3A, the positive drug-glimepiride had significant effect on K_{ATP} currents. When cells incubated with glimepiride, the K_{ATP} current gradually declined with time and remained stable up to10 minutes. It could be considered that K_{ATP} currents were inhibited. Based on Fig.3A and Equation 1, current density at different time points was obtained (see Fig.3B). The cell current density value with glimepiride for 10 minutes obviously decreased from 82.79±2.8pA/pF to 38.67 ±1.24pA/pF ($P < 0.01$).

Furthermore, the inhibitory effect of loureirin B was studied. The K_{ATP} currents of cells incubated with or without loureirin B were recorded, respectively. Then, the K_{ATP} currents of previous cells were recorded after incubating with glimepiride, it could been found that the K_{ATP} currents of cells incubating with loureirin B previously (23.82±1.69pA/pF) decreased less than that without loureirin B (46.71±3.12pA/pF) ($P < 0.01$). It indicated that loureirin B had caused the decrease of K_{ATP} currents before the effect of glimepiride. The results showed that K_{ATP} current of Ins-1 cells was inhibited by the effect of loureirin B.

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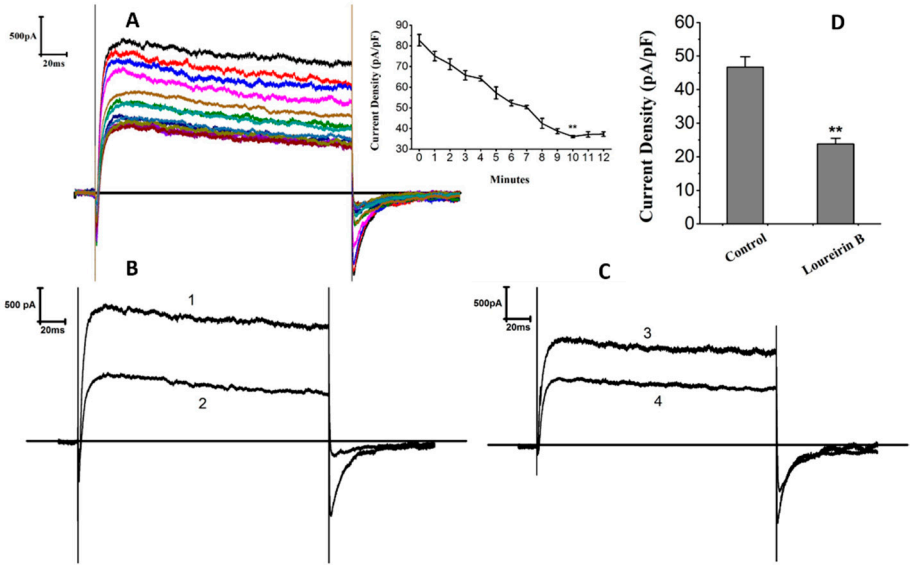

Fig. 3 Effect of loureirin B on the current of K_{ATP} channel

(A) Experiments with positive drug-glimepiride for 12 minutes (the current of K_{ATP} channel was recorded every minutes) **(B)** line 1 (control group): the current of K_{ATP} channel recorded in the absence of loureirin B; line 2: the current of K_{ATP} channel was recorded with the perfusion of glimepiride for 10 minutes **(C)** line 3 (drug-treated group): the current of K_{ATP} channel recorded in the presence of loureirin B for 10minutes; line 4: the current of K_{ATP} channel was recorded with the perfusion of glimepiride for 10 minutes **(D)** Comparison of decrease currents of K_{ATP} channel between control group and drug-treated group.(n=5, **P < 0.01)

3.4 Effect of loureirin B on $[Ca^{2+}]_i$ and the expression of Cx43 in Ins-1 cells

The increase of intercellular free-calcium activity ($[Ca^{2+}]_i$) may promote insulin secretion[13]. Hence, we investigated the effects of loureirin B on $[Ca^{2+}]_i$ in Ins-1 cells. Fluorescence spectroscopy was applied to detect $[Ca^{2+}]_i$ in Ins-1 cells (Fig. 4A). In the presence of loureirin B, $[Ca^{2+}]_i$ was higher than that in the control.$[Ca^{2+}]_i$ increased significantly in an incubation time-dependent manner from 0.5 h to 4 h, and reached the highest level with the incubation time of 4 hours, which were about 1.75-fold higher than that of the control group(Fig. 4B). It could be indicated that the effect of loureirin B might cause and then promote insulin secretion.

As one of the important connexin, Cx43 plays an important role in mediating the flow of ions, such as K^+ and Ca^{2+} [15]. In this study, the expression of Cx43protein was detected (Fig. 4C). It could be found that the expression of Cx43increased with the incubation time of loureirin B within 2 hours, but there was no obvious difference over the incubation of 2h. It inferred that it was the effect of loureirin B that possibly caused the increase of the expression of Cx43 that medicated the influx of Ca^{2+} to the intracellular.

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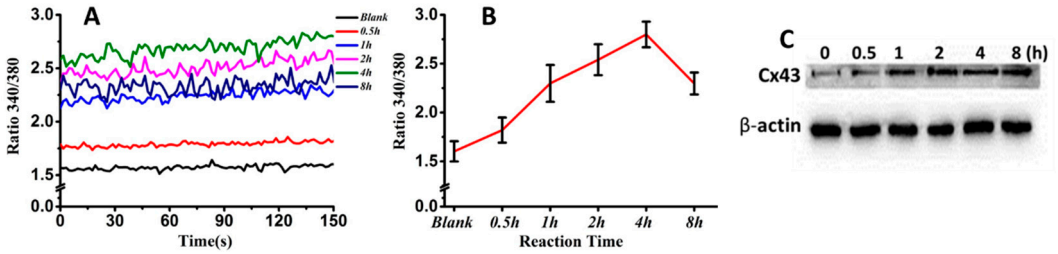

Fig.4 The effect of loureirin B on $[Ca^{2+}]_i$ and the expression of Cx43 protein on Ins-1 cell.

(A) Fluorescence intensity of the Radio 340/380 indicated the intracellular calcium concentration **(B)** The value of fluorescence intensity of the Radio 340/380(Fig.4A) at 75 second was selected. **(C)** The change of expression of Cx43 protein with the different incubation time of loureirin B

4. Discussion

In a healthy body, the insulin secretion is increasing with the increase of blood glucose levels. However, for diabetics, they have not the ability to promote insulin secretion. The characteristic of diabetes mellitus is the sustained decline of pancreatic β-cell function [16, 17]. Therefore, it is important to understand how to promote the insulin secretion and sequentially decrease the blood glucose levels in diabetic patients. With the development of diabetes drugs, a series different type of drug have been demonstrated that they can solve the problem about dysfunction of pancreatic cells. Sulfonylureas drugs have been used for the treatment of diabetes for several years. As a representative drug, sulfonylureas has been reported to inhibitor the K_{ATP} channel to promote insulin secretion [18, 19]. However, sulfonylureas might cause hypoglycemia due to not dependence of blood glucose [20]. Hence, the development of sulfonylurea analogues might improve the efficacy and reduce side effects.

In previous studies, loureirin B has been reported have the ability to decrease the plasma sugar level. However, the mechanism is unclear. Recently, bioinformatics gradually help researchers to solve the deeper biological problems, such as the interaction between ligands and target protein. Based on the molecular simulation of glimepiride with K_{ATP} channel, the potential active sites were chosen. On the chosen active sites, it could be found that loureirin B interacted with K_{ATP} channel like the positive-drug glimepiride. Furthermore, the acting force between loureirin B and target protein was analyzed. We guessed that whether loureirin B have the similar mechanism on pancreatic β-cell like sulfonylurea drugs.

In our study, the mechanism of loureirin B on pancreatic β-cell was mainly illustrated by detecting the currents of K_{ATP} channel, the influx of Ca^{2+} to the intracellular and the expression of Cx43 protein. Firstly, it could be observed that the currents of K_{ATP} channel obviously decreased after the incubation of loureirin B.

K_{ATP} channels, formed by SUR1 and Kir6.2, plays a very important role as a bridge

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between glucose metabolism and the electrical activity in the cell membrane of islet β-cells [21]. When the ratio of cytosolic nucleotides (ATP/ADP) increases due to the rise of blood glucose level, K_{ATP} channel begins to close and the membrane is depolarized. Then, voltage-dependent calcium channel is activated, resulting in an influx of Ca^{2+}. It has been reported that the increase of $[Ca^{2+}]_i$ is the trigger for insulin secretion [22, 23]. Consequently, the intracellular Ca^{2+} was detected after the incubation of loureirin B for different time. The result showed that the content of intracellular Ca^{2+} increased with the effect of loureirin B. And it reached the highest at the incubation of 4 hours. Hence, it could be concluded that loureirin B might promote insulin secretion due to inhibiting of K_{ATP} channels and the influx of Ca^{2+} to the intracellular. As one of the important connexin, Cx43 plays an important role in mediating the flow of ions and small molecules such as K^+ and Ca^{2+} [24]. In our study, the expression of Cx43 protein was also detected. With the effect of loureirin B, the expression of Cx43 increased and remain stable after 2 hours. It could be illuminated that the influx of Ca^{2+} to the intracellular was aroused by the increasing expression of Cx43 protein.

Loureirin B, extracted from its stems of *Dracaena cochinchinensis*, has been proposed to be an active compound. It has numerous pharmacological effects, such as decreasing the plasma sugar level, inhibiting thrombosis and hypertrophic scar formation and promoting blood circulation [25-27]. Since it has a similar mechanism to promote insulin secretion like sulfonylureas, it gives us an opportunity to develop deeper research in *vivo*. As mentioned above, sulfonylureas might cause some side effects, such as hypoglycemia. If loureirin B has a similar effect on the treatment of diabetes with less side effects, it might be a better choice for diabetics.

5. Conclusion

This study shows that loureirin B has the ability to promote insulin secretion on Ins-1 cells with less cytotoxicity. The results showed that the mechanism how loureirin B had effect on insulin secretion is dependent on the K_{ATP} channel pathway (Fig. 5). With the effect of loureirin B, K_{ATP} channel current was inhibited and subsequently increased the influx of Ca^{2+} to the intracellular. Furthermore, we speculated that Cx43 protein might mediate the Ca^{2+} to the intracellular.

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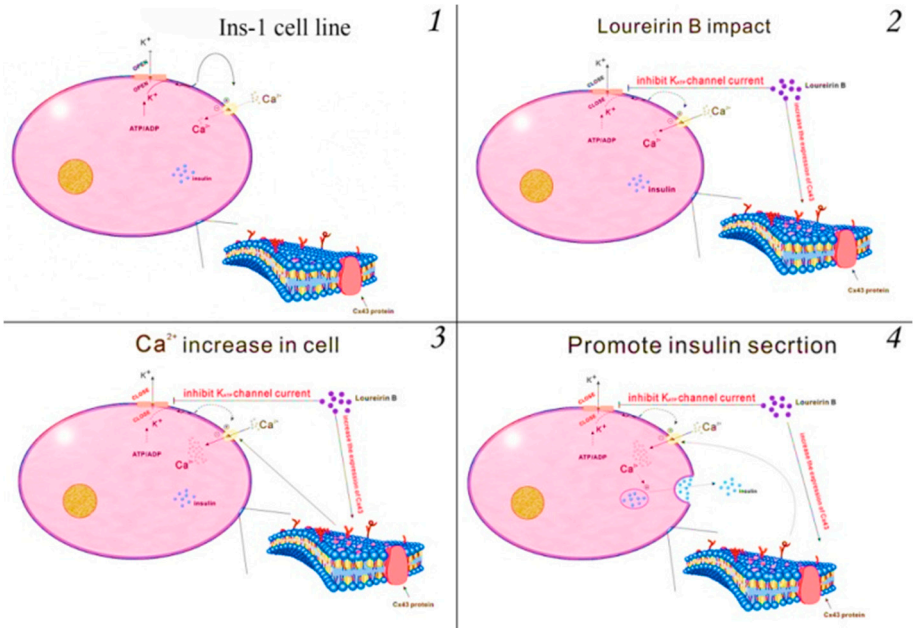

Fig. 5 Mechanism of induction of insulin secretion by loureirin B in Ins-1 cells

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