Stimulation of β-adrenergic receptors plays a protective role via increased expression of RAF-1 and PDX-1 in hyperglycemic rat pancreatic islet (RIN-m5F) cells

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Abstract

Introduction: It is a widely held view that a progressive reduction of beta-cell mass occurs in the progression of diabetes. RAF-1 kinase and pancreas duodenal homeobox 1 (PDX-1) are major factors that promote survival of cells and maintain normal insulin functions. In this study we investigated the effect of a β-adrenergic receptor agonist and antagonist on RAF-1 and PDX-1, and their respective effects on apoptosis and insulin release in RIN-m5F cells.

Material and methods: RIN-m5F cells were cultured in normal (5 mM) and high (25 mM) glucose to mimic diabetic conditions, followed by treatment with 5 µM, 10 µM and 20 µM of isoproterenol and isoproterenol + propranolol for 6, 12 and 24 h. Western blotting and reverse transcription analysis were performed to examine the expression of RAF-1 and PDX-1. Annexin-V-FITC and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays were used to investigate apoptosis. ELISA was used to measure insulin levels. Reverse transcription polymerase chain reaction was conducted to investigate the expression of genes.

Results: Stimulation of β-adrenergic receptors with isoproterenol significantly induced RAF-1 and PDX-1 genes in a concentration-dependent and time-independent manner. Changes were significant both at protein and mRNA levels. Up-regulation of RAF-1 and PDX-1 was accompanied by improved insulin levels and reduced apoptosis. Concentrations of 10 µM and 20 µM for 12 and 24 h were more effective in achieving significant differences in the experimental and control groups. Propranolol reversed the effect of isoproterenol mostly at maximum concentrations and time periods.

Conclusions: A positive effect of a β-adrenergic agonist on RAF-1 and PDX-1, reduction in β-cell apoptosis and improved insulin contents can help to understand the pathogenesis of diabetes and to develop novel approaches for the β-cell dysfunction in diabetes.

Key words: β-adrenergic receptors, v-raf-leukemia viral oncogene 1, pancreas duodenal homeobox 1, hyperglycemia, apoptosis, insulin.
Introduction

Diabetes is one of the most common metabolic disorders [1, 2], characterized by defective secretion of insulin. Progressive β-cell failure is the hallmark of both type 1 and type 2 diabetes. In both forms of the disease, apoptosis is probably the main form of β-cell death. In rodent models, it has been shown that β-cell apoptosis causes a gradual β-cell depletion in type 1 diabetes [3, 4]. In type 2 diabetes, studies have shown a significant reduction in β-cell mass [5, 6] and a threefold increase in β-cell apoptosis [6], a major underlying mechanism thereof being β-cell apoptosis [7]. Secondary to increased rates of β-cell apoptosis, these observations suggest that β-cell mass is decreased in type 2 diabetes [8]. Recent studies have revealed that chronic exposure to high glucose and free fatty acids are toxic to pancreatic β-cells and impair cellular functioning [9–11]. However, the exact mechanisms of β-cell dysfunction and apoptosis have not been fully elucidated [12, 13].

Beta-adrenergic receptors such as β1, β2 and β3-adrenergic receptors are G protein coupled receptors (GPCRs), located at the transmembrane region, which allow ligand binding and elicit a range of cellular actions such as phosphorylation and activation of various signaling pathways [14]. Recent reports have shown that stimulation of β-adrenergic receptors can prevent many of the deleterious changes associated with diabetic complications in retinal endothelial cells [15, 16]. Furthermore, activation of β-adrenergic receptors has been implicated in the preservation of cardiac function by inhibiting apoptosis and cardiac remodeling [17, 18].

Several kinases such as phosphoinositide 3-kinase (PI3K) and Akt (v-akt murine thymoma viral oncogene, a serine-threonine protein kinase) have been investigated for their possible roles in β-cell survival [19, 20]. Experiments on transgenic mice with overexpressed Akt in their β-cells have revealed a protective role against streptozotocin-induced diabetes [21, 22]. However, mice with reduced Akt activity did not show increased apoptosis in islet cells, which indicates the likelihood of other kinases that may contribute to β-cell survival [23]. RAF-1 (v-raf-leukemia viral oncogene 1), a critical target for various growth factors that promote proliferation and survival of many cell types, including pancreatic β-cells, is another multifunctional protein with serine and threonine kinase activity [24, 25]. Knockout studies in mice have shown that RAF-1 and b-RAF are imperative in developmental cell survival [26]. Whole-body deletion of RAF-1 causes embryonic lethality, making glucose intolerance and adult islet function impossible in those knockout mice [27]. Similarly, homeobox transcription factor PDX-1 is another regulator of the morphogenesis and survival in multiple cell types [28, 29]. Gunton et al. reported decreased levels of insulin receptor and Irs2 mRNA in islets isolated from human type 2 diabetics [30]. Insulin functions as a growth factor as well as a hormone regulating energy metabolism. Several studies have shown that PDX-1 and insulin regulate the survival of the primary human and mouse β cells. Johnson et al. reported that insulin can act as a master regulator of islet survival by regulating PDX-1 [31]. These findings suggest the possibility that PDX-1 may mediate the effects of insulin.

In this study we investigated the effect of a β-adrenergic receptor agonist and antagonist on two cell survival responsible genes, RAF-1 and PDX-1, in hyperglycemic rat pancreatic islet cells (RIN-m5F). To correlate the agonistic and antagonistic effects of isoproterenol and propranolol respectively, we also carried out measurements of insulin and apoptosis in these cells.

Material and methods

Cell culture

RIN-m5F (ATCC CRL11605) cells were cultured in ATCC-formulated RPMI-1640 Medium (Catalog No. 30-2001) supplemented with 10% serum and 1% penicillin streptomycin. Cells were grown in normal (5 mM, 0.9008 g/l) and high (25 mM, 4.5 g/l) glucose. Cells were divided into 3 main groups: cells treated with glucose, glucose + isoproterenol and glucose + isoproterenol + propranolol. The drugs

| Gene   | Forward primer | Reverse primer |
|--------|----------------|----------------|
| PDX-1  | F-CGGGCGTATGAGTGGAAAAA | R-AGCCAAACACAACAGCCAAATC |
| RAF-1  | F-CGGGCCGAGAGTCTTAATCG | R-CCCCTCACCTTGAGTGCTTT |
| β1-AR  | F-GGCCCTGCTATTTCTGTCC | R-TCTGGACCAGTTCTGCCTCT |
| β2-AR  | F-ACAACACTACCCACCGAGATGAAGG | R-AGCCGGGGTGATGCGAATGATG |
| β3-AR  | F-CGCCCTTTCCTCCGTTTGT | R-GAGTTTGACAGACGGTGAGG |
| α1-AR  | F-TGACATCATCCCCACGACC | R-TGACTGTCCGGTCCTTGGAGG |
| α2-AR  | F-TAGCCTGGCTATTCCCTT | R-TCTCAAGCAGGTCCTTGGTC |
isoproterenol and propranolol were used to treat the cells for 6, 12 and 24 h in concentrations of 5 µM, 10 µM and 20 µM. When cells reached 80% confluence, they were used for the experiments. The cells were grown in 5% CO₂ at 37°C.

**Annexin-V-FITC assay**

Early and late apoptosis was studied using the Annexin-V/PI staining assay. Briefly, RIN-m5F cells (1 × 10⁶) were treated with a β-adrenergic receptor agonist and antagonist. Cells were harvested and washed twice with PBS. Following the manufacturer’s instructions cells were re-suspended in Annexin-V binding buffer (BD Biosciences, San Jose, CA, USA) and stained with Annexin-V-FITC (BD) and PI (Sigma). The fluorescence intensity of RIN-m5F cells was then analyzed by flow cytometry (BD FACSCanto II, San Jose, CA, USA) through quadrant statistics for necrotic and apoptotic cell populations. Annexin-V was used to detect the early stage and late stage apoptosis, while PI was used for detection of the late stage apoptosis and necrosis.

**Western blot analysis**

RIN-m5F cells were lysed and centrifuged and proteins were extracted according to the manufacturer’s protocol (Sigma). The Bradford assay (Bio-Rad Laboratories) was used to quantify the protein concentrations in the supernatants. Equal amounts of protein (30 µg) from the cell extracts were separated on the pre-cast tris-glycine gel (Precast gels, Bio-Rad, cat. no. 456-1093) and blotted onto a nitrocellulose membrane. After blocking in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, the membrane was incubated overnight at 4°C with primary antibodies (1 : 3000) against RAF-1 and PDX-1 (Santa Cruz). Anti-β-actin (Santa Cruz, sc-7210) antibodies were used to ensure the quality of protein separation and loading contents. After extensive washes, the membranes were incubated with HRP-conjugated IgG secondary antibodies (Santa Cruz, sc-2004) and visualized with enhanced chemiluminescence (Amersham Life Sciences, UK) using a gel imaging system (Biospectrum 410, UVP).

**Reverse transcription polymerase chain reaction**

Total RNA from RIN-m5F cells was isolated using an RNeasy commercial kit (Qiagen, cat. no. 74104) according to the manufacturer’s protocol and quantified spectrophotometrically. CDNA was synthesized using 1 µg of DNA free RNA along
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Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

A colorimetric apoptosis detection kit with TUNEL staining in a 96-well format was used to examine DNA damage according to the manufacturer’s protocol (Titer TACS; R&D System). Briefly, RIN-m5F cells were cultured in medium containing 5 mM (normal) and 25 mM (high) glucose. High glucose treated cells were further treated with 5 µM, 10 µM and 20 µM isoproterenol (β-adrenergic receptor agonist) and propranolol (β-adrenergic receptor antagonist) for 6, 12 and 24 h. Cells were then transferred into a 96-well plate (1 × 10^5 cells/well) and fixed with 3.7% buffered formaldehyde for 5 min. After washing with PBS, cells were subjected to permeabilization with 100% methanol for 20 min (at room temperature) and again washed with PBS. According to the manufacturer’s instructions, the labeling procedure was carried out and the reaction was stopped with 0.2 N HCl after 30 min. A nuclease-treated control was used to check genomic DNA carryover in RNA. Reaction conditions for cDNA synthesis were 25°C for 5 min, 42°C for 50 min and 85°C for 5 min. Equal amounts of cDNA were subsequently used for amplification in a 20 µl polymerase chain reaction (PCR) using EmeraldAmp PCR Master Mix (Takara, Japan), containing dNTPs, Taq polymerase, buffer and primers PDX-1 and RAF-1 genes. PCR was performed with an initial denaturation step at 94°C for 3 min, followed by denaturation at 94°C for 30 s, annealing (62°C for PDX-1 and 59°C for RAF-1) for 30 s and extension at 72°C for 60 s. β-actin was used as a quantitative control in each PCR reaction. The PCR products were separated on 1.5% agarose gel and visualized using ethidium bromide staining under UV transillumination. For the characterization of β- and α-adrenergic receptors, we used the same conditions with variable annealing temperatures for each gene. Primer sequences are given in Table I.
was used to confirm the permeabilization and labeling reaction. The absorbance was measured at 450 nm with a microplate reader.

Measurement of insulin content

Insulin content was determined by treatment of $5 \times 10^5$ RIN-m5F cells with isoproterenol ($\beta$-adrenergic receptor agonist) and propranolol ($\beta$-adrenergic receptor antagonist). The cells were centrifuged and the supernatant was removed. The cell pellet was re-suspended in PBS and sonicated. The cell lysate was used to estimate the quantity of insulin by ELISA (Crystal Chem Inc, USA). Insulin content was normalized to the total protein concentration.

Statistical analysis

Each test was performed in triplicate. The results were expressed as the mean value ± SD. One-way ANOVA test was used to determine statistical significance. A value of $p < 0.05$ was considered as significant. Statistical analysis was performed using the SPSS-17.0 package (IBM Corporation, Armonk, NY, USA) and GraphPad prism (version 4.0 GraphPad software Inc, San Diego, CA, USA).

Results

Characterization of $\beta$- and $\alpha$-adrenergic receptors

We characterized the RIN-m5F cell line first. Reverse transcription PCR was conducted to check the expression of $\beta$- and $\alpha$-adrenergic receptors in the said cell line. The RIN-m5F cell line showed expression of $\beta$- and $\alpha$-adrenergic receptors. Hyperglycemia showed no significant effect on the expression patterns of these genes except $\alpha_1$-adrenergic receptor ($p < 0.05$), as shown in Figure 1.

Beta-adrenergic receptor stimulation protects pancreatic cells from death

RIN-m5F cells were treated with glucose alone, glucose + isoproterenol ($\beta$-adrenergic receptor antagonist) and glucose + propranolol ($\beta$-adrenergic receptor antagonist) for 24 h. Beta-adrenergic receptor stimulation by isoproterenol reduced the early and late apoptosis as compared to the control (Figures 2 A–C). In this preliminary experiment, we used 20 µM of each isoproterenol and propranolol for 24 h. Glucose treatment increased the apopto-

Figure 3. Stimulation of $\beta$-adrenergic receptors with isoproterenol induced the expression of RAF-1 in hyperglycemic RIN-m5F cells (A and B). Isoproterenol treated cells were then subjected to propranolol. Increase in the expression of RAF-1 was dependent on concentration but independent of time periods. The isoproterenol generated effect on RAF-1 protein was reversed when the cells were treated with isoproterenol + propranolol (C and D).

Data are presented as mean ± standard deviation and compare glucose treated vs. glucose + isoproterenol treated vs. glucose + isoproterenol + propranolol treated (*$p < 0.05$; **$p < 0.01$).
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sis and treatment of 20 µM isoproterenol for 24 h significantly \((p < 0.05)\) reduced the apoptosis. This effect was reversed by the antagonist propranolol.

**Beta-adrenergic receptor stimulation induced the expression of RAF-1 in rat pancreatic islet cells under hyperglycemic conditions**

RIN-m5F cells were treated with normal (5 mM) and high glucose (25 mM) for 24 h, followed by treatment with 5 µM, 10 µM and 20 µM of isoproterenol and propranolol for 6, 12 and 24 h. Isoproterenol (β-adrenergic agonist) increased the protein level of RAF-1 in a concentration-dependent but time-independent manner as shown in Figures 3 A and B. Protein levels were higher at 20 µM of isoproterenol followed by 10 µM and 5 µM. A significant effect was observed when cells were treated with 5 µM agonist for 24 h \((p < 0.01)\), 10 µM for 12 and 24 h \((p < 0.05)\), and 20 µM for 6, 12 and 24 h \((p < 0.01)\). At 5 µM concentration of isoproterenol, both time- and concentration-dependent changes were seen under hyperglycemic conditions. The agonistic effect of isoproterenol on RAF-1 was confirmed by the reverse effect of propranolol. The agonist-induced effect on RAF-1 was significantly reduced in the cells treated with 10 µM \((p < 0.05)\) and 20 µM \((p < 0.05)\) of propranolol for 6, 12 and 24 h, but at 5 µM, no significant effect was observed. To confirm the effect of the same agonist and antagonist on mRNA level, we extracted RNA from the cells treated with 20 µM agonist for 24 h. Reverse transcription PCR demonstrated the same pattern of effect on the RAF-1 gene, as shown in Figures 4 A and B.

**Beta-adrenergic receptor stimulation induced the expression of PDX-1 in rat pancreatic islet cells under hyperglycemic conditions**

RIN-m5F cells were treated with normal (5 mM) and high glucose (25 mM) for 24 h, followed by treatment with 5 µM, 10 µM and 20 µM of isoproterenol and propranolol for 6, 12 and 24 h. Protein levels of PDX-1 were higher in isoproterenol (β-adrenergic agonist) treated cells. The PDX-1 gene showed approximately the same pattern of expression as RAF-1, which was concentration dependent but time independent, as shown in Figures 5 A and B. A gradual increase was observed in the protein levels of PDX-1 as the concentration of isoproterenol was increased from 5 to 20 µM. A significant effect was observed when cells were treated with 5 µM agonist for 6,
12 and 24 h \( (p < 0.01) \), 10 µM for 6 h \( (p < 0.05) \), 12 and 24 h \( (p < 0.01) \), and 20 µM for 6, 12 and 24 h \( (p < 0.01) \). The agonistic effect of isoproterenol on PDX-1 was confirmed by the reverse effect of propranolol. The agonist-induced effect on PDX-1 was significantly reduced in the cells treated with 10 µM \( (p < 0.05) \) and 20 µM \( (p < 0.05) \) of propranolol for 6, 12 and 24 h, but at 5 µM, no significant effect was observed on PDX-1 protein level. mRNA level of PDX-1 was confirmed by reverse transcription PCR which demonstrated approximately the same pattern of effect on the PDX-1 gene, as shown in Figures 4 A and B.

**Effect of \( \beta \)-adrenergic receptor agonist and antagonist on insulin release and apoptosis**

After we found that RAF-1 and PDX-1 genes are up-regulated at both protein and mRNA levels on \( \beta \)-adrenergic receptor stimulation, we further investigated its effect on insulin and \( \beta \)-cell apoptosis. RIN-m5F cells treated with normal (5 mM) and high glucose (25 mM) exhibited low and high apoptosis respectively. High glucose cells, treated with different concentrations (5 µM, 10 µM and 20 µM) of isoproterenol and propranolol for different time periods, showed different apoptosis and insulin levels. There was a significant increase in the insulin contents when cells were treated with 5 µM agonist for 12 and 24 h \( (p < 0.05) \), 10 µM for 12 \( (p < 0.05) \) and 24 h \( (p < 0.01) \), and 20 µM for 6, 12 and 24 h \( (p < 0.01) \), as shown in Figures 6 A–C. Propranolol significantly reversed the isoproterenol-induced insulin release only at a concentration of 10 µM for 24 h \( (p < 0.05) \) (Figure 6 C). Exposure to high glucose induced apoptosis in RIN-m5F cells. The agonist of \( \beta \)-adrenergic receptor significantly reduced the glucose-induced apoptosis when treated with 5 µM agonist for 12 and 24 h \( (p < 0.05) \), 10 µM for 6, 12 \( (p < 0.05) \) and 24 h \( (p < 0.01) \), and 20 µM for 6 h \( (p < 0.05) \), 12 and 24 h \( (p < 0.01) \) (Figures 7 A–C). Propranolol \( (p < 0.05) \) was significantly effective only at 20 µM for 24 h.

**Discussion**

Diabetes mellitus (DM) is a serious global health issue. According to the World Health Organization, approximately 180 million people worldwide currently have type 2 DM, and this number is expected to double by 2030 \[32, 33\]. Diabetes is a disease that is strongly associated with vari-

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**Figure 5.** Stimulation of \( \beta \)-adrenergic receptors with isoproterenol induced expression of the PDX-1 gene in hyperglycemic RIN-m5F cells (A and B). Isoproterenol treated cells were then subjected to propranolol. Agonist-induced expression of PDX-1 was dependent on concentration but independent of time periods. The isoproterenol generated effect on PDX-1 protein was reversed when the cells were treated with isoproterenol + propranolol (C and D).

Data are presented as mean ± standard deviation and compare glucose treated vs. glucose + isoproterenol treated vs. glucose + isoproterenol + propranolol treated \( (*p < 0.05; ^{*}p < 0.01) \).
Stimulation of β-adrenergic receptors plays a protective role via increased expression of RAF-1 and PDX-1 in hyperglycemic rat pancreatic islet (RIN-m5F) cells. Our findings suggest a mechanism by which β-adrenergic receptor stimulation induces the expression of RAF-1 and PDX-1, which in turn promote cell survival. Preliminary we investigated the effect of 20 µM of isoproterenol and propranolol on apoptosis, treated for 24 h. This opened up the possibility to hypothesize that RAF-1 and PDX-1 genes might be involved in the β-adrenergic receptor induced effects on apoptosis and insulin contents in hyperglycemic RIN-m5F cells. Cells treated with 10 µM and 20 µM concentrations of agonist (isoproterenol) and antagonist (propranolol) of β-adrenergic receptors showed significant effects on apoptosis and insulin contents. Increasing the time period (6–24 h) did not show any consistent increase in the expression of RAF-1 and PDX-1, but increasing the concentration caused a steady increase in their protein levels.

It is well established that RAF-1 interacts with apoptosis signal-regulating kinase 1 (ASK1) and other critical regulatory proteins such as the cell-cycle modulators Cdc25 and Rb [36–38]. Chen et al. reported that RAF-1 may promote cell survival via protein-protein interactions in addition to its established mitogen-activated protein kinase (MAPK) function. RAF-1 interaction with ASK1 may allow a functional cross-talk between two antagonistic signaling pathways, which is likely to be important for signal incorporation. Chen et al.

**Figure 6.** RIN-m5F cells were treated with glucose alone, glucose + isoproterenol (Iso) (β-adrenergic receptor antagonist) and glucose + isoproterenol (Iso) + propranolol (Pro) (β-adrenergic receptor antagonist). Stimulation with different concentrations (5 µM, 10 µM and 20 µM) of isoproterenol and propranolol for 6 h (A), 12 h (B) and 24 h (C) resulted in increased and decreased insulin release respectively.

Data are presented as mean ± standard deviation and compare glucose treated vs. glucose + isoproterenol treated vs. glucose + isoproterenol + propranolol treated (*p < 0.05; #p < 0.01).
further suggested that inhibition of the pro-apoptotic function of ASK1 by RAF-1 may be the reason for maintaining survival [39]. In our results, β-adrenergic receptor stimulation clearly induced the expression of RAF-1 (Figures 2, 5 A and B) and stabilized the insulin contents (Figure 6). This demonstrates that the increased expression of RAF-1 might have antagonized and suppressed ASK-1, with consequently reduced apoptosis and increased insulin contents. Studies have revealed that RAF-1 regulates the anti-apoptotic and mitogenic effects of insulin on cultured β-cells [39, 40]. In β-cell lines and rat islets, blockade of RAF-1 signaling also reduced insulin secretion [41, 42]. In our study, increased insulin secretion after β-adrenergic receptor stimulation may not be a direct effect of the agonist, but an indirect effect via RAF-1 signaling.

The homeodomain transcription factor PDX-1 is another key regulator of insulin and β-cell survival. Mutations in this gene are widely associated with the development of diabetes; therefore understanding the expression levels of PDX-1 is important in the study and treatment of diabetes [43]. Several studies have demonstrated that PDX-1 plays an essential role in pancreatic β-cell survival and insulin secretion [44–46]. In another study, it was reported that if PDX-1 is suppressed in MIN6 cells, survival will be reduced, with a higher apoptosis rate [47]. These studies validate the importance of PDX-1 in regulating the cell survival and insulin release. Our findings which suggest that PDX-1 is up-regulated as a result of β-adrenergic receptor stimulation are parallel to these studies. Our results demonstrate that β-adrenergic receptor stimulation through isoproterenol in-

Figure 7. RIN-m5F cells were treated with glucose alone, glucose + isoproterenol (β-adrenergic receptors antagonist) and glucose + isoproterenol + propranolol (β-adrenergic receptors antagonist). Stimulation with different concentrations (5 µM, 10 µM and 20 µM) of isoproterenol for 6 h (A), 12 h (B) and 24 h (C) resulted in decreased apoptosis in the cells. Antagonist propranolol was treated with the same concentrations for the same time periods.

Data are presented as mean ± standard deviation and compare glucose treated vs. glucose + isoproterenol treated vs. glucose + isoproterenol + propranolol treated (*p < 0.05; **p < 0.01).
creases the protein and mRNA levels of PDX-1 and consequently the agonist induced PDX-1 protects the cell from hyperglycemia-induced apoptosis.

In conclusion, a positive effect of β-adrenergic agonist on RAF-1 and PDX-1, reduction in β-cell apoptosis and improved insulin contents can help to understand the pathogenesis of diabetes and to develop novel approaches for the treatment of diabetes.

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Conflict of interest

The authors declare no conflict of interest.

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