Glucose plus metformin compared with glucose alone on β-cell function in mouse pancreatic islets

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Abstract. Metformin is currently the first drug of choice for treatment of type II diabetes. The primary function of metformin is to decrease hepatic glucose production mainly by inhibiting gluconeogenesis. The aim of the present study was to investigate the effects of glucose alone (control groups) and glucose and metformin (treatment groups) on pancreatic islets functions. Pancreatic islets were isolated by collagenase digestion and incubated for 24 or 48 h in RPMI-1640 containing 5 mmol/l glucose (control groups 1 and 2, respectively) or 24 h with 25 mmol/l glucose (control group 3) and 15 µmol/l metformin (treatment groups 1, 2 and 3, corresponding to the control groups, respectively). Subsequently, the rate of insulin output from islets, pancreatic and duodenal homeobox 1 (Pdx-1) and insulin genes expression and islet viability were assayed. The rate of insulin secretion in a 5 mmol/l glucose concentration in the 48 h treatment group increased significantly compared with that of the 24 h treatment group (P<0.05). An increase of the glucose concentration (25 mmol/l) caused insulin secretion to increase compared with that of 5 mmol/l glucose. Pdx-1 gene expression in treatment group 2 significantly decreased compared with the control group 2 (P<0.05). The the Pdx-1 gene expression in treatment group 2 decreased compared with that of the treatment group 1. The expression of the insulin gene in treatment group 1 increased compared with control group 1, and in treatment group 2, there was a 2-fold increase in insulin gene expression compared with control group 2. The insulin gene expression in treatment group 2 increased compared with treatment group 1. The percentage of islet cell viability was increased in treatment group 3 by ~40% compared with the islet cells of treatment groups 1 and 2 (P<0.05). These data indicate that glucose and metformin have direct effects on β-cell function.

Introduction

Diabetes mellitus is a disease affecting ~5% of people worldwide. Type I diabetes is mainly due to autoimmune damage of pancreatic β-cells and patients are dependent on exogenous insulin, and this accounts for 5-10% of all the diabetic cases. Type II diabetes develops more slowly and the intensity of metabolic disturbances is much lower and accounts for 90-95% of all diabetic cases (1,2). In type II diabetes, the reduction in β-cell mass and insulin secretion was observed (3-5).

Metformin is the first choice for the treatment of type II diabetes. In type II diabetes, metformin lowers blood glucose concentrations without causing overt hypoglycemia, and therefore is an antihyperglycemic agent. Metformin is also described as an insulin-sensitizer causing reduction in insulin resistance and the plasma fasting insulin level. The positive effects of metformin on insulin receptor expression and tyrosine kinase activity could be improving the insulin sensitivity (6,7). However, the primary function of metformin is to decrease hepatic glucose production (8), mainly by inhibiting gluconeogenesis (9,10).

Studies on the rat pancreas (11) and isolated human islets (12) have shown that metformin improved insulin release in response to glucose. A previous study has demonstrated that metformin can restore the normal secretory pattern in isolated rat islets that were incubated in the presence of elevated glucose or free fatty acid concentrations (13).

In the adult pancreas, pancreatic and duodenal homeobox 1 (Pdx-1) regulates the genes associated with pancreatic cell differentiation and maturation, which includes the glucose transporter 2, insulin, glucokinase and amyloid precursor protein genes (14). The decision to test metformin was based on these considerations and the results showed that the drug can reverse the majority of the alterations found in type II diabetes islets.

The aim of the present study was to investigate the direct effects of metformin on pancreatic β-cells by incubation of pancreatic islets cells undergoing glucose stress to assess the expression of Pdx-1 and insulin genes, β-cell function and viability.

Materials and methods

Animals. A total of 56 male NMRI mice (25-30 g) (9 mice in each group) were obtained from the Animal House of Ahvaz
Table I. Sequence information on the primers used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5’→3’)</th>
<th>Product size, bp</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdx-1</td>
<td>F: CCGAGAGACACATCAAAAAATCTGG R: CCCGCTACTACGTTTCTTTACTTCCC</td>
<td>80</td>
<td>NM_008814.3</td>
</tr>
<tr>
<td>Insulin</td>
<td>F: AGGACCCCAAGTGGCACA R: GAGGGGTAGGCTGGTATGG</td>
<td>184</td>
<td>NM_001185084.1</td>
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<tr>
<td>Glut-2</td>
<td>F: TTGACTGGACCCCTTTGTAG R: CACCTCGTCCAGCAATGATGA</td>
<td>73</td>
<td>NM_031197.2</td>
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<tr>
<td>β-actin</td>
<td>F: GGCCAACCGTTGAAAAAGATGA R: CACACCTCGTGCTAGTACGT</td>
<td>79</td>
<td>NM_007393.3</td>
</tr>
</tbody>
</table>

qPCR, quantitative polymerase chain reaction; bp, base pairs; F, forward; R, reverse.

Jundishapur University of Medical Sciences (Ahvaz, Iran). The mice were housed in cages (22±2°C, under a standard 12-h light:12-h dark cycle) and allowed ad libitum feed access. All the experimental protocols were performed according to the Standards for Animal Care, and they were approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences.

Isolation of mice pancreatic islets. Pancreatic islets were isolated from overnight-fasted male NMRI mice by the Lacy and Kostianovsky-modified collagenase digestion method (13). In brief, following cervical dislocation, the abdomen of the animals was opened. The common bile duct was occluded at the distal end, close to the duodenum. Hanks’ balanced salt solution (HBSS; 5 ml) [115 mmol/l NaCl, 10 mmol/l NaHCO₃, 5 mmol/l KCl, 1.1 mmol/l MgCl₂, 1.2 mmol/l NaH₂PO₄, 2.5 mmol/l CaCl₂, 25 mmol/l HEPES and 5 mmol/l D-glucose (pH 7.4), as well as 1% bovine serum albumin (Merck KGaA, Darmstadt, Germany)] containing 1.4 mg/ml of collagenase IV (Sigma, St. Louis, MO, USA) was injected into the duct (14,15).

After removal of the pancreas, it was placed into a 50 ml conical tube and incubated for 15 min in a 37°C water bath. Subsequently, 15 ml of cold HBSS was added to the tube to dilute the collagenase concentration and stop a further digestion process. For washing the collagenase from the islet tissues, the tube was centrifuged for 2 min at 1,200 rpm and the supernatant has discarded. The washing procedure of islets followed 2 times and for 30 min at 37°C, and the islets were separated by handpicking under a stereomicroscope (Euromex Microscopen BV, Arnhem, The Netherlands) and were cultured overnight in RPMI-1640 medium (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 5 mmol/l D-glucose and was gassed with 95% O₂:5% CO₂ atmosphere. Subsequently, the pancreatic islets were divided into 6 groups, including 3 control and 3 treatment groups: Control group 1: Incubated for 24 h in RPMI-1640 containing 5 mmol/l glucose (Sigma); control group 2: Incubated for 48 h in RPMI-1640 containing 5 mmol/l glucose; and control group 3: Incubated for 24 h in RPMI-1640 containing 25 mmol/l glucose; and treatment group 1: Incubated for 24 h in RPMI-1640 containing 5 mmol/l glucose and 15 μmol/l metformin; treatment group 2: Incubated for 48 h in RPMI-1640 containing 5 mmol/l glucose and 15 μmol/l metformin; and treatment group 3: Incubated for 24 h in RPMI-1640 containing 25 mmol/l glucose and 15 μmol/l metformin.

Insulin secretion measurement. Islet function was determined by monitoring insulin release following a static incubation glucose challenge assay. Function was determined by exposing the islets to glucose and metformin for different concentrations of glucose and for different periods, and determining how much insulin was secreted in response.

After in vitro treatment, 5 sets of 4 islets were incubated in HBSS containing 5 or 25 mmol/l glucose at 37°C for 1 h. At the end of the incubation period, the supernatant was collected and frozen at -20°C for later insulin radioimmunoassay (Bio-Rad, Hercules, CA, USA) using a mouse insulin standard (17).

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from mouse pancreatic islets using the RNeasy plus mini kit (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA (200 ng) was used to generate cDNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific) and amplified by qPCR using a QuantiTect SYBR-Green kit (Qiagen), the ABI StepOnePlus qPCR instrument and software (Applied Biosystems, Foster City, CA, USA). Primer sequences used are provided in Table I. All the quantifications were performed with mouse β-actin as an internal standard. The PCR was performed for 40 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec (16,17).

Iset viability. Islet cell viability was tested by a colorimetric assay that detected the conversion of MTT (Sigma) into the formazan by the mitochondrial enzyme succinate dehydrogenase in viable cells (18,19). After in vitro treatment, islets dissociated into single cells by incubation in HBSS containing 0.25 mg/ml trypsin for 10 min at 37°C with gentle agitation and were resuspended in RPMI-1640. Islet cells were cultured in a 96-well plate supplemented with 0.5 mg/ml MTT. After
4 h incubation, the insoluble formazan crystals within islet cells were extracted by dimethyl sulfoxide and absorbance was measured by a microplate reader (Bio-Rad) at a wavelength of \(\lambda_{\text{test}} = 570\,\text{nm}\) and \(\lambda_{\text{reference}} = 650\,\text{nm}\).

**Statistical analysis.** Data were expressed by SPSS software (SPSS, Inc., Chicago, IL, USA) as mean ± standard error of the mean. One-way analysis of variance was used for comparison of the data from different groups, followed by the Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**In vitro islet function.** The rate of insulin secretion (ng/ml/60 min/islet) from isolated islets in 5 mmol/l glucose in the 48 h treatment group (glucose plus metformin) increased significantly compared with that of the 24 h treatment group (P<0.05). The differences between the control groups compared with the treatment groups were not significant (Fig. 1).

To determine the effect of different concentrations of glucose on insulin secretion in the glucose alone and glucose plus metformin groups, insulin secretion was examined at different extracellular glucose concentrations. Treated mice islets were incubated for 60 min in the presence of 5 or 25 mmol/l glucose, and the results demonstrated that in the glucose compared with the glucose and metformin group, insulin secretion increased but this difference was not significant. Data demonstrated that insulin secretion increased with the increase of glucose concentration (Fig. 2).

**qPCR.** The *Pdx-1* and insulin gene expression was analyzed by qPCR to demonstrate whether metformin has a significant effect on \(\beta\)-cell function during different time exposures to glucose or glucose plus metformin.

The *Pdx-1* gene expression in the glucose plus metformin group 1 decreased compared with that of the control group 1, and *Pdx-1* gene expression in glucose plus metformin group 2 decreased significantly compared with the control group 2 (P<0.05), as well as during different time exposures to glucose plus metformin group 2 (48 h) compared with the glucose plus metformin group 1 (24 h) (Fig. 3).

To investigate the effect of metformin on insulin gene expression during different time exposures of metformin and glucose, the same experiment was performed. The expression of the insulin gene in treatment group 1 increased compared with that of control group 1, and in glucose plus metformin group 2 there was a 2-fold increase in insulin gene expression compared with that of control group 2. The expression of the insulin gene in the glucose plus metformin group 2 increased compared with that of glucose plus metformin group 1 (Fig. 4).

**Islet viability.** Incubations of pancreatic islet cells with MTT demonstrated that reduction of this compound to formazan differed in islet cells of different treatment groups. Formazan formation was increased in treatment group 3 (25 mmol/l glucose and metformin) by ~40% compared with the islets of treatment groups 1 and 2 (P<0.05) (Fig. 5).
In conclusion, the present study indicates that metformin has a profound effect on insulin release, transcriptional regulation in pancreatic islets and islet cell viability, and these effects are dependent on the presence of glucose. These data indicate that metformin has direct effects on islet function and suggests that this widely prescribed antidiabetic drug may play a previously unrecognized role in the direct regulation of pancreatic β-cell function. Further studies are required to fully delineate the cell signaling mechanisms regulating these events.

Acknowledgements

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References