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Effects of High Iron and Glucose Concentrations over the Relative Expression of Bcl2, Bax, and Mfn2 in MIN6 Cells

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Abstract Type 2 diabetes is characterized by hyperglycemia and oxidative stress. Hyperglycemia is linked to mitochondrial dysfunction and reduced β-cell mass due to the reduced expression of genes such as Mfn2 as well as the participation of the Bcl2 gene family, responsible for increased apoptosis. The purpose of this study was to describe the effect of different iron and/or glucose concentrations over Mfn2, Bax, and Bcl2 expressions in a β-pancreatic cell line (MIN6 cells). MIN6 cells were pre-incubated with different iron and/or glucose concentrations, and the relative mRNA abundance of the Bcl2/Bax ratio and of Mfn2 genes was measured by qRT-PCR. Heme oxygenase (HO) activity, iron uptake, superoxide dismutase activity, and glutathione content were also determined. The Bcl2/Bax ratio increased and Mfn2 expression decreased in MIN6 cells after glucose stimulation. These effects were higher when glucose and iron were incubated together. Additionally, treatment with glucose/iron showed a higher HO activity. Our study revealed that high glucose/Fe concentrations in MIN6 cells induced an increase of the Bcl2/Bax ratio, an indicator of increased cell apoptosis.

Keywords Iron · Glucose · Oxidative stress · Mitofusin 2 · Bax · Bcl2 · Apoptosis

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Introduction

In type 2 diabetes (T2D), the progressive damage of pancreatic β-cells results in decreased insulin secretion, impairment in insulin responsiveness (as insulin resistance), glucose toxicity, and increased oxidative stress (OS) [1]. OS has been defined as the disturbance in the pro-oxidant–antioxidant balance, resulting in potential cell damage [2]. It is widely accepted that OS plays a key role in the development and progression of T2D. Iron contributes to OS by donating and capturing electrons, acting as a catalyst in redox reactions. In the presence of iron, hydrogen peroxide and superoxide anion can be converted into a hydroxyl radical, which is a reactive oxygen species (ROS). On the other hand, an excess of glucose is also toxic for the cell since ROS are produced through glucose auto-oxidation [3–6]. Under these conditions, a cell possesses two types of antioxidant defense mechanisms: enzymatic and non-enzymatic. Superoxide dismutase (SOD), glutathione peroxidase, catalase, and heme oxygenase 1 (HO1) activities are all enzymatic defense mechanisms. HO1 protects against cell death, regulating levels of intracellular redox-active iron [7]. Examples of non-enzymatic defenses are glutathione (GSH), ascorbate, α-tocopherol, β-carotene, and bilirubin concentrations.

The mitochondrion is the main source of ROS in cells when defective coupled electron transport is taking place. Furthermore, the mitochondria play a central role in many cellular functions which include apoptosis, cell proliferation and differentiation, bioenergetics, as well as amino acid and lipid metabolism [8, 9]. The mitochondria frequently form

tubular structures or networks that are crucial for mitochondrial DNA inheritance. This mitochondrial network is a highly dynamic structure that is regulated through changes in the rates of mitochondrial fission and fusion. Mitochondrial fusion depends on the mitochondrial transmembrane GTPase proteins mitofusin 1 and 2 (Mfn2) [9, 10]. Mfn2 is highly expressed in skeletal muscle, heart, and brain tissues and regulates mitochondrial metabolism. Its repression reduces glucose oxidation, mitochondrial membrane potential, and cell respiration. In addition, alterations in Mfn2 expression in muscle cells cause a parallel change in the expression of subunits of the oxidative phosphorylation complexes I, II, and IV [8, 10, 11].

Apoptosis constitutes a common mechanism of cell replacement [12] and has been associated with increased levels of ROS and decreased levels of antioxidants such as GSH [13]. Additionally, it has been associated with diseases such as cancer, immunological disorders, and neurodegenerative disorders [14]. The Bcl2 gene family (Bax and Bcl2) participate in apoptosis regulation, where Bax promotes apoptosis and Bcl2 has an anti-apoptotic function [15, 16]. The Bcl2/Bax ratio has been used to determine cell apoptosis susceptibility [15, 17].

T2D is characterized by hyperglycemia and mitochondrial function impairment. Hyperglycemia elicits an increase in ROS production; moreover, high glucose levels lead to dynamic changes in mitochondrial morphology due to prolonged ROS overproduction, causing changes in mitochondrial gene expression such as the downregulation of Mfn2 [9, 10, 18]. A link between hyperglycemia and apoptosis was found in the development of T2D in animal models. This was due to the overproduction of ROS that increased β-cell apoptosis with a progressive decrease of these cells in the pancreas [19, 20]. In this article, we analyzed the impact of high iron and/or glucose concentrations over mRNA relative abundance of Mfn2, Bax, and Bcl2 in MIN6 cells.

Methods

Cell Cultures

MIN6 cells were used as a β-pancreatic cell line model [21]. MIN6 cells were cultured in RPMI-1640 (with L-glutamine and without glucose; Invitrogen) containing 10 % fetal bovine serum (Hyclone) and 10 % antibiotic–antimicotic (Gibco). Cells were kept at 37 °C, 5 % CO_2, and 80 % relative humidity. Cells were cultured in six-well culture plates at 1.5×10^5 cells per well.

Fe and Glucose Treatment and ^{55}Fe Uptake Experiments in MIN6 Cells

MIN6 cells were pre-incubated with increasing iron and glucose concentrations for a week. Control cells (Cn) were cultured in a medium supplemented with 0.5 μM of $FeCl_3$ and 1.1 μM of sodium nitrilotriacetate (NTA; Sigma-Aldrich) for complexing ferrous iron with NTA as Fe(III)–NTA. Experimental conditions corresponded to cells incubated with (1) 5 and 20 μM iron, (2) 5 and 20 mM glucose, and (3) 5 μM Fe/5 mM glucose or 20 μM Fe/20 mM glucose using the same ratio of Fe/NTA as used above. On the day of the experiment, MIN6 cells were challenged with 10 μM ^{55}Fe for 1 h at 37 °C. A protein extract was prepared, diluted with Ultra Fluor, and radioactivity (in counts per minute) was determined in a liquid scintillation counter (Perkin Elmer). Treatments were performed in triplicate in three independent experiments.

HO1 Activity Measurement

MIN6 cells were incubated with different iron and/or glucose concentrations as aforementioned, were trypsinized, and afterwards centrifuged (1,500×*g* at 4 °C). The pellet was homogenized with a non-denaturing lysis buffer (20 mmol KH_2PO_4 per liter, 135 mmol KCl per liter, and 0.1 mmol EDTA per liter, pH 7.4) and centrifuged for 20 min at 10,000×*g* at 4 °C. A volume of 100 μL of cell extract was incubated for 1 h at 37 °C with the following mix: 100 μL hemin 15 mM (Sigma-Aldrich), 100 μL biliverdin reductase (extracted from rat liver), and 600 μL dilution buffer (100 mM KH_2PO_4). The reaction was initiated by adding 100 μL of 1 mM NADPH (Sigma-Aldrich). Finally, the yielded bilirubin was measured at 530 nm using a spectrophotometer (Shimadzu). This analysis was run in triplicate.

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated using Trizol reagent (Gibco). MIN6 cells were lysed directly in each well by adding 1 mL of Trizol. RNA was separated using chloroform, precipitated by mixing with isopropyl alcohol, washed with 75 % ethanol, and finally dissolved in H_2O-DEPC. RNA was quantified at 260 nm and its purity determined by the 260:280 nm OD ratio. RNA was reverse-transcribed into cDNA using Affinity Script QPCR cDNA Synthesis Kit (Stratagene) according to product instructions. This kit is a genetically engineered reverse transcriptase obtained from the Moloney Murine Leukemia Virus Reverse Transcriptase. This reaction was performed using Thermal Cycler 2720 (Applied Biosystems).

Bax, Bcl2, and Mfn2 Relative mRNA Abundance

Real-time PCR reactions were carried out to determine the levels of mRNA relative abundance. The PCR mix for Bax contained 2 μL cDNA, 6 μL Brilliant II SYBR Green

QPCR mix (Stratagene), 1.2 μL 10× bovine serum albumin (BSA), 1.8 μL H_2O, and 2 μM of sense and antisense primers. The PCR mix for Bcl2 contained 2 μL cDNA, 10 μL Brilliant II SYBR Green QPCR mix (Stratagene), 2 μL 10× BSA, 2.6 μL H_2O, and 2 μM of sense and antisense primers. cDNAs were amplified in a Light Cycler System (Roche, 3.5). The specific primer pairs were: for Bax—forward 5-CGAGTGGCAGCTGACATGTTTT-3′, reverse 5-TGAGGCAGGTGAATCGCTTG-AA-3; for Bcl-2—forward 5-CAGTTGGGCAACAGAGAACCAT-3, reverse 5-AGCCCTTGTCCCCAATTTGGAA-3; and for Mfn2—forward 5-AACTGTCTGGGACC-TTTGCTCA, reverse 5-TTTCTGGCATCCCCTGTGCTTT-3. β-actin was used as a housekeeping gene to normalize gene expression (forward: 5-TGGCACCCAGCACAATG-AAGA-3, reverse: 5-GAAGCATTTGCGGTGGACGAT-3). Quantitative RT-PCR data were analyzed using the comparative threshold (C_t) method; treated samples were compared with the untreated samples. Gene expression levels were calculated as described by Yuan et al. [22]. The results were expressed using the following formula: $C_t = \{C_t\ (genes) - C_t\ (\beta - actin)\}$. To determine the relative expression levels, the following formula was used: $C_t = \{C_t\ (treated) - C_t\ (control)\}$.

SOD Activity and GSH Content

SOD activity was measured in cell lysates using an ELISA CuZn SOD commercial kit (BioVendor). The amount of colored product obtained was proportional to the amount of CuZn SOD present in the sample. Absorbance was measured at 450 nm. Cell lysate GSH content was measured by fluorescence using a GSH Detection Kit (Chemicon) according to product protocol (excitation=380 nm, emission=461 nm).

Statistical Analyses

Data are presented as the mean±SEM. Statistical significance was determined using one-way ANOVA and Dunnett's multiple comparison as a post hoc test. The analysis was performed using GraphPad Prism software, version 5.0. Significance was accepted at $p<0.05$.

Results

Iron uptake decreased in pre-incubated MIN6 cells with increased iron concentration (5 and 20 μM) in the media and challenged with 10 μM ^{55}Fe, as expected (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: $p<0.01$ and $p<0.001$, respectively; Fig. 1). In cells pre-incubated with increased glucose concentration, iron uptake did not change and remained constant and similar to control cells (0.5 μM Fe in the media). However, in cells pre-incubated with the Fe/glucose mix (5 μM Fe/5 mM glucose and 20 μM Fe/20 mM glucose), iron uptake was higher than Cn (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: both $p<0.001$; Fig. 1).

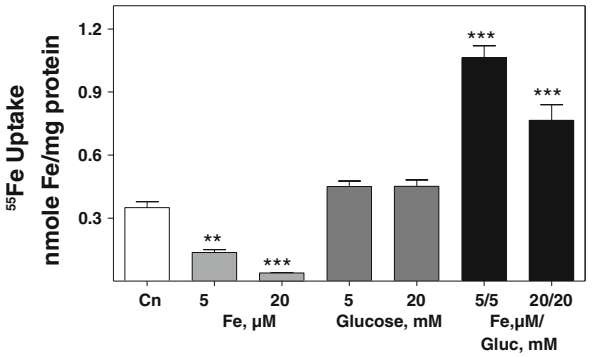

Fig. 1 Iron uptake analysis in MIN6 cells. Pre-incubated MIN6 cells with different Fe and/or glucose concentrations were challenged with 10 μM ^{55}Fe for 1 h. A protein extract was prepared and the counts per minute counted in a liquid scintillation counter. Data are the mean± SEM of three independent experiments (one-way ANOVA: $p<0.0001$; Dunnett's post hoc test: $^{**}p<0.01$, $^{***}p<0.001$)

HO activity showed a similar behavior toward iron uptake. HO activity was higher in cells pre-incubated with low iron compared to high iron concentrations, but these activities were not different amongst each other (one-way ANOVA: $p<0.0001$; Dunnett's post hoc test: NS for 5 or 20 μM Fe; Fig. 2). Also, HO activity tends to decrease in MIN6 cells pre-incubated with different glucose concentrations. However, in cells pre-incubated with the Fe/glucose mix (5 μM Fe/5 mM glucose and 20 μM Fe/20 mM

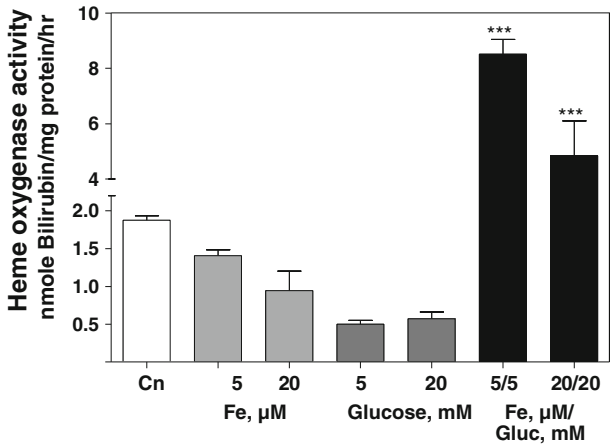

Fig. 2 Heme oxygenase activity in MIN6 cells challenged with different iron and glucose concentrations. A cellular extract was prepared with MIN6 cells pre-incubated with different iron and glucose concentrations. Heme oxygenase activity was determined by measuring bilirubin concentration at 530 nm. Data are presented as the mean±SEM of three independent experiments in triplicate (one-way ANOVA: $p<0.0001$; Dunnett's post hoc test: $^{***}p<0.001$)

glucose), HO activity increased (one-way ANOVA: $p<0.0001$; Dunnett's post hoc test: both $p<0.001$; Fig. 2).

To examine the effects of high Fe and/or glucose concentrations over the expression of apoptosis-related genes Bcl2 and Bax, MIN6 cells were exposed to different Fe, glucose, or Fe/glucose concentrations. The treatment of MIN6 cells with different Fe concentrations showed an induction of the Bcl2/Bax ratio to over 3.5- and 4.5-fold compared to basal conditions (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: both $p<0.001$; Fig. 3). Pre-incubation with 5 or 20 mM glucose induced a significant decrease of the Bcl2/Bax ratio (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: both $p<0.001$; Fig. 3). A similar effect was observed only in cells pre-incubated with 20 μM Fe/20 mM glucose (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: $p<0.01$; Fig. 3). Both treatments with glucose and Fe/glucose showed a lower Bcl2/Bax ratio than control cells.

Mfn2 mRNA relative abundance tends to increase in MIN6cells pre-incubated with Fe, but not significantly (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: NS for 5 or 20 μM Fe; Fig. 4). However, Mfn2 mRNA abundance decreased when cells were incubated with high glucose (20 mM) or high iron/glucose concentrations (one-way ANOVA: $p<0.001$; Dunnett's post hoc test: $p<0.05$ for 20 mM glucose and $p<0.001$ for 20 μM Fe/20 mM glucose, respectively; Fig. 4).

No differences were observed in SOD activity or GSH content in both cell types incubated with different iron or glucose concentrations.

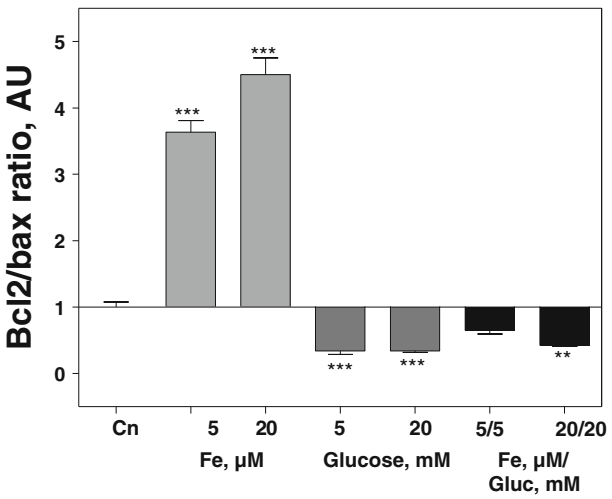

Fig. 3 Bcl2/Bax mRNA ratios in MIN6 cells. Total RNA was isolated from pre-incubated MIN6 cells and the relative abundance of Bcl2 and Bax genes was determined by qRT-PCR. Data are presented as the mean±SEM of three independent experiments (one-way ANOVA: $p<0.0001$; Dunnett's post hoc test: $^{**}p<0.01$, $^{***}p<0.001$)

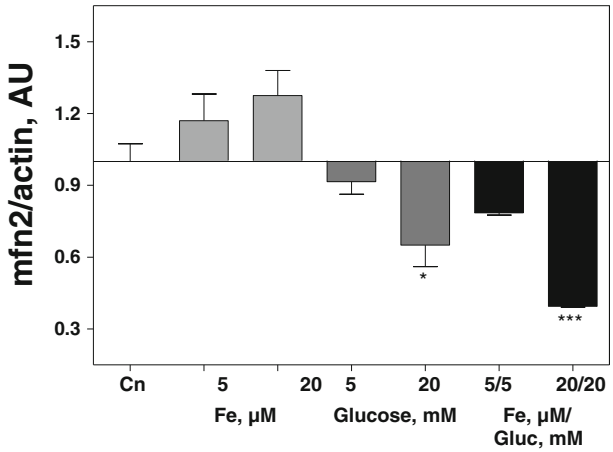

Fig. 4 Relative abundance of Mfn2 mRNA. Total RNA was isolated from pre-incubated MIN6 cells and the relative abundance of Mfn2 mRNA determined by qRT-PCR. Data are represented as the mean± SEM of three independent experiments (one-way ANOVA: $p<0.0001$; Dunnett's post hoc test: $^{*}p<0.05$, $^{***}p<0.001$)

Discussion

The aim of this work was to evaluate the relationship between iron and the Bcl2/Bax ratio since previous evidence has shown that iron could be a risk factor for β-cell dysfunction and apoptosis [23, 24]. Bax and Bcl2 are genes that participate in apoptosis regulation [15, 16]. A lower ratio of Bcl2/Bax mRNA expression has been used to determine the susceptibility of cells toward apoptosis. In this study, the Bcl2/Bax ratio decreased when cells were incubated either with low or high glucose and high Fe/glucose concentrations. Hasnan et al. [25] showed that Bax protein expression increased in the blood vessels of diabetic patients and at elevated glucose levels. Also, Podesta et al. [26] showed a slightly increased but significant level of Bax in postmortem retinas of diabetic patients compared to the levels present in non-diabetic subjects. These studies showed that in the presence of elevated glucose concentrations, an increase in Bax expression is observed, and hence the increased apoptosis in human and animal tissues [27]. We found that chronic exposure to high levels of glucose decreases the Bcl2/Bax ratio, which suggests that the cell may be in an apoptotic pathway.

Iron is broadly recognized as an oxidative stress inducer; the time of exposure to this metal, as well as subsequent ROS production, could trigger cell damage and diminished insulin secretion [28]. However, iron might have an indirect protective mechanism against apoptosis, which could be related to HO enzymatic activity. Previously, we showed that HO activity was increased in diabetic patients with increased iron storage [29]. Fang et al. [30] proposed that HO1 may block apoptosis through three mechanisms: by decreasing intracellular pro-oxidant levels, increasing bilirubin levels, and through CO production. Cho et al. [31]

observed that when HO1 was overexpressed in Jurkat cells, they became resistant to Fas-mediated apoptosis. In addition, the presence of exogenous iron in the media also prevented Fas-mediated apoptosis. Our results indicated that HO activity is not modified in the presence of high Fe, but it is increased at high iron/glucose concentrations, probably partially demonstrating that HO is highly activated under oxidative stress, as it is when exposed to high glucose concentrations.

Studies in animal models and humans have shown that the Mfn2 protein plays an important role in mitochondrial integrity since it maintains membrane potential, thus allowing adequate glucose and lipid oxidation. Bach et al. [10] found a reduced expression of Mfn2 in the skeletal muscle of obese patients compared to lean subjects. In addition, Mfn2 was increased in morbidly obese patients under bile-pancreatic diversion [32]. In our results, Mfn2 relative mRNA abundance was significantly decreased at the highest levels of glucose as well as in high-glucose plus iron conditions. As shown in previous studies, there was a strong relationship between both the reductions of the Bcl2/Bax ratio as well as the decrease of Mfn2 expression at high glucose concentrations. This might be linked to β-cell dysfunction, which consequentially develops diabetes and its complications [10, 32]. Also, Nauespiel et al. [33] observed that when Cos-7 cells possessed a mutation in Mfn2, apoptosis was increased; when Mfn2 was activated, Bax activation and cytochrome c were both repressed, thus preventing apoptosis. These results demonstrate that mitochondrial integrity and activity are closely related to apoptosis. In this work, we were able to demonstrate that glucose at high levels is a pivotal factor in Mfn2 decreased expression, suggesting mitochondrial dysfunction.

In summary, we observed that glucose itself had deleterious effects over β-pancreatic cells, overcoming the oxidative effect of iron and inducing a decrease in Bcl2/Bax ratio and Mfn2 expression. This result is in agreement with evidence in humans and animal models. However, further studies are required after observing that iron surprisingly increased the Bcl2/Bax ratio and the expression of Mfn2. Therefore, our results show that glucose is a factor that causes β-cell failure at molecular and cellular levels, as observed in diabetes development.

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