High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal β-cell HIT-T15

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Abstract Prolonged exposure to free fatty acids induces β-cell cytotoxicity. We investigated whether this fatty-acid-induced cytotoxicity is affected by high glucose levels. In clonal β-cell HIT-T15, palmitate-induced cytotoxicity was potentiated depending on elevated glucose concentrations due to increased apoptosis without cytotoxic effects of high glucose per se. This palmitate cytotoxicity was blocked by NO synthase inhibitors, and palmitate actually increased cellular NO production. The potentiation of palmitate cytotoxicity under high glucose was reversed by decreasing superoxide production, suggesting that superoxide overproduction under high glucose enhances NO-mediated cytotoxicity in β-cells, which may explain the mechanism of synergistic deterioration of pancreatic β-cells by free fatty acids and high glucose.

Key words: Pancreatic β-cell; Palmitate; High glucose; Apoptosis; Nitric oxide; Superoxide

1. Introduction

Type 2 diabetes mellitus is a disease characterized by impaired insulin secretion and peripheral insulin resistance. Accumulated evidence suggests that hyperlipidemia enhances β-cell dysfunction in type 2 diabetes [1]. Especially, it has been suggested that chronically elevated concentrations of free fatty acids (FFAs) play an important role in pancreatic β-cell defects [2,3]. Prolonged exposure of β-cells to FFAs causes defective insulin secretion and insulin gene expression [4–6] and induces β-cell apoptosis [7–10]. This FFA-induced β-cell apoptosis is observed both in vivo [7,8] and in vitro [7–10] and has been called 'lipoapoptosis' [8,9].

Our initial question is whether lipoapoptosis in β-cells is enhanced by ambient high glucose. It has been reported that FFA-induced β-cell apoptosis is exaggerated in islets from hyperglycemic Zucker diabetic fatty rats [7–9]. In cell culture study, inhibition of insulin gene expression by long-term exposure of β-cells to palmitate is observed only in the presence of elevated glucose concentrations [11], suggesting that chronic exposure to FFAs and high glucose levels synergistically impairs β-cell function. However, it is still unknown whether fatty-acid-induced β-cell cytotoxicity is directly affected by high glucose. Here, we report that β-cell cytotoxicity caused by palmitate is significantly enhanced depending on extracellular glucose concentrations in the β-cell line HIT-T15.

We furthermore investigated the mechanism of palmitate-induced apoptosis in this cell line and the mechanism of potentiation of palmitate's effects by high glucose. Previously, several mechanisms for the deleterious effects of FFAs on β-cells have been proposed, including cellular triglyceride accumulation [12–14], ceramide formation [9,10], and increased NO formation [7]. However, it remains unclear which of these mechanism makes the greatest contribution. We investigated whether palmitate-induced cytotoxicity is mediated by NO production by observing the effects of simultaneous treatment with N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) on cell viability and measuring the amount of NO metabolite production in cell culture supernatant as an index of cellular NO production.

It has been suggested that diabetic hyperglycemia increases the production of reactive oxygen species, especially superoxide, causing pathological changes in a variety of tissues including pancreatic islets [15,16]. To test the role of high-glucose-induced superoxide overproduction in palmitate-induced cytotoxicity, we treated HIT-T15 β-cells with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of mitochondrial oxidative phosphorylation, and manganese(III)tetrakis(4-carboxyphenyl)porphyrin (MnTBAP), a superoxide dismutase mimetic, both of which are reported to reverse high-glucose-induced superoxide overproduction [15,17], together with palmitate.

In addition to the above, increased hexosamine flux and protein kinase C (PKC) activation have been implicated in high-glucose-induced impairment of β-cell function and viability [18–20]. Thus we investigated the involvement of these pathways in the potentiation of palmitate cytotoxicity by 12.8 mM glucose, respectively by measuring the dose–response curve of palmitate-induced cytotoxicity in the absence and presence of glucosamine under 2.8 mM glucose, and testing whether bisindolylmaleimide, a PKC inhibitor, affects palmitate-induced cytotoxicity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 me-

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Abbreviations: FFA, free fatty acid; L-NMMA, N^G-monomethyl-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MnTBAP, manganese(III)tetrakis(4-carboxyphenyl)porphyrin; PKC, protein kinase C

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dium, fetal bovine serum, and antibiotics were from Gibco (Grand Island, NY, USA). L-NMMA and L-NAME were from Dojindo (Kumamoto, Japan). MnTBAP was from Calbiochem (San Diego, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Preparation of palmitate solution

A concentrated stock solution of palmitate was prepared by dissolving powder in ethanol:H_2O (1:1) at 50°C. This stock solution was added to RPMI 1640 (no glucose) supplemented with 2.8 mM glucose and 1% fatty-acid-free bovine serum albumin (BSA) and incubated at 37°C with gentle shaking to allow complex formation of BSA and palmitate. After filtration, the concentration of albumin-bound palmitate was measured with the NEFA-C kit from Wako (Osaka, Japan).

2.3. β-Cell culture and treatment

HIT-T15 cells, a hamster β-cell line, were routinely maintained in RPMI 1640 (no glucose) supplemented with 2.8 mM glucose, 10% fetal bovine serum, 10 000 U/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B in a humidified incubator with 5% CO_2 at 37°C. For all assays, cells were inoculated into 96-well plates at 3×10^4/well and cultured for several days. Each well was washed with 150 μl of RPMI 1640 (no glucose) twice, and then 100 μl of RPMI 1640 supplemented with 1% fatty-acid-free BSA including various concentrations of palmitate and glucose was added. For glucosamine treatment, 100 μl of RPMI 1640 supplemented with 1% fatty-acid-free BSA including various concentrations of palmitate and glucosamine was added. For L-NMMA, L-NAME, CCCP, MnTBAP, and bisindolylmaleimide treatment, 1 μl of various concentrations of each compound was added directly from each stock solution to give final concentrations. One microliter of the solvent used for the preparation of each compound stock solution was added to control wells. Treated cells were further incubated in the incubator above at 37°C for 24 h.

2.4. MTT assay

A solution of 500 μg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was prepared in DMEM (low glucose) before use. The medium was aspirated, 100 μl of MTT solution added, and incubated at 37°C for 1 h. The medium was removed and 100 μl of dimethyl sulfoxide added to lyse the cells. The absorbance at 490 nm with 650 nm of reference wavelength was measured using a SpectraMax microplate reader from Molecular Devices (Osaka, Japan).

2.5. Measurement of DNA fragmentation

For quantitative determination of apoptotic DNA fragmentation, cytoplasmic histone-associated DNA fragments were measured with the Cell Death Detection ELISA kit (Roche) according to the manufacturer's instructions. After the medium was removed, cells were incubated with lysis buffer at 4°C for 30 min. The lysates were centrifuged at $20\,000\times g$ for 10 min and the obtained supernatant was collected. After 1:10 dilution, 100 μl of each sample was used for the detection.

2.6. Nitrate/nitrite determination

Total nitrate and nitrite formation in cell culture supernatants was measured as an indication of NO production. After palmitate treatment for 24 h, 5 μl of the supernatant was taken and the total amounts of nitrate and nitrite in each sample determined with the Nitrate/Nitrite Fluorometric Assay kit from Cayman (Denver, CO, USA) according to the manufacturer's instructions.

2.7. Statistical analysis

Results are expressed as the mean ± S.E.M. The significance of the difference between two groups was assessed by Student's unpaired *t*-test. *P* values of less than 5% were considered statistically significant.

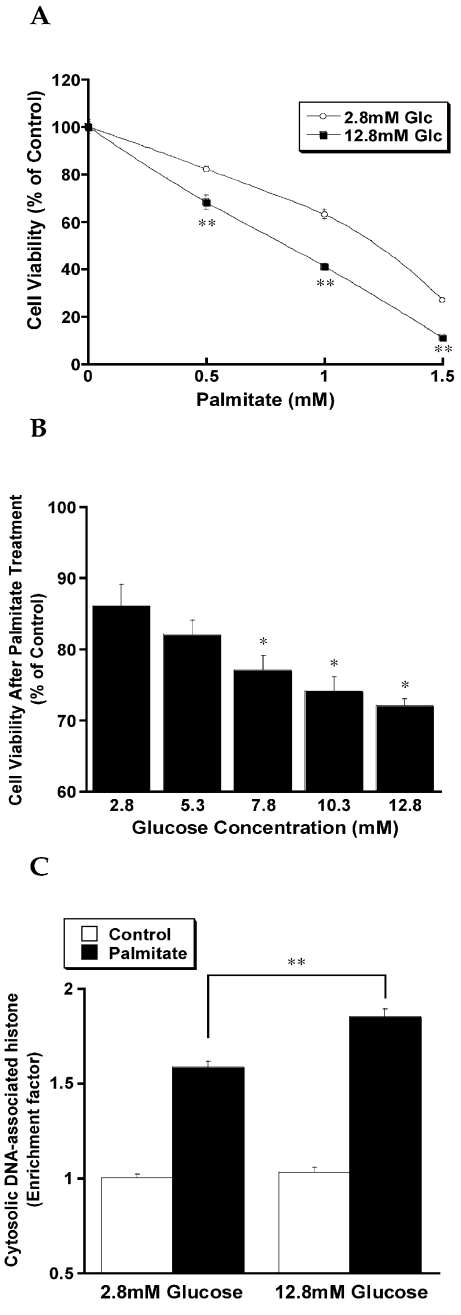

Fig. 1. Potentiation of palmitate-induced cytotoxicity and apoptosis by high glucose levels. A: HIT-T15 β-cells were incubated with the indicated concentrations of palmitate for 24 h under 2.8 mM or 12.8 mM glucose. Cell viability was measured by the MTT assay and represented as percent of control under each glucose concentration. In the control condition, cell viability was measured after 24 h incubation in the absence of palmitate under each glucose concentration. Each bar represents the mean of six replicates ± S.E.M. $^{**}P<0.005$ versus cell viability in the treatment of the same concentration of palmitate under 2.8 mM glucose. B: HIT-T15 β-cells were incubated in the absence (control) or presence of 0.5 mM palmitate for 24 h under each indicated glucose concentration and cell viability was measured by the MTT assay. Cell viability in the treatment of palmitate is represented as percent of control under each glucose concentration. Each bar represents the mean of six replicates ± S.E.M. $^{*}P<0.05$ versus cell viability under 2.8 mM glucose. C: HIT-T15 β-cells were incubated in the absence (control) or presence of 0.5 mM palmitate for 24 h under 2.8 mM or 12.8 mM glucose. Cytosol fractions were prepared and the amount of cytosolic DNA-associated histone was measured with the Cell Death Detection ELISA kit. Each bar represents a relative factor versus the control value under 2.8 mM glucose as the mean of four replicates ± S.E.M. $^{**}P<0.005$ between 2.8 mM and 12.8 mM glucose conditions in the presence of palmitate.

3. Results

In HIT-T15 β-cells, palmitate induced cytotoxicity in a dose-dependent manner between 0.5 and 1.5 mM after 24 h treatment. This cytotoxic effect of palmitate was significantly potentiated at all concentrations tested when ambient glucose concentration was 12.8 mM versus 2.8 mM (Fig. 1A). Cell viability was not affected solely by increasing glucose concentration from 2.8 mM to 12.8 mM in the absence of palmitate (data not shown), therefore 12.8 mM glucose per se does not appear to have any negative effects on cell viability except for enhancing palmitate-induced cytotoxicity. This potentiation of palmitate-induced cytotoxicity was dependent on glucose concentration between 2.8 and 12.8 mM (Fig. 1B).

Palmitate-induced cytotoxicity in HIT-T15 β-cells was caused by cell death due to apoptosis characterized by DNA fragmentation (Fig. 1C). In the absence of palmitate, increasing the glucose concentration from 2.8 mM to 12.8 mM did not increase DNA fragmentation, indicating that at concentrations up to 12.8 mM, glucose per se does not increase apoptosis for 24 h in HIT-T15 β-cells. However, palmitate-induced DNA fragmentation was significantly increased in the presence of 12.8 mM glucose versus that observed with 2.8 mM glucose (Fig. 1C). Therefore palmitate-induced apoptosis was accelerated by 12.8 mM glucose in these cells.

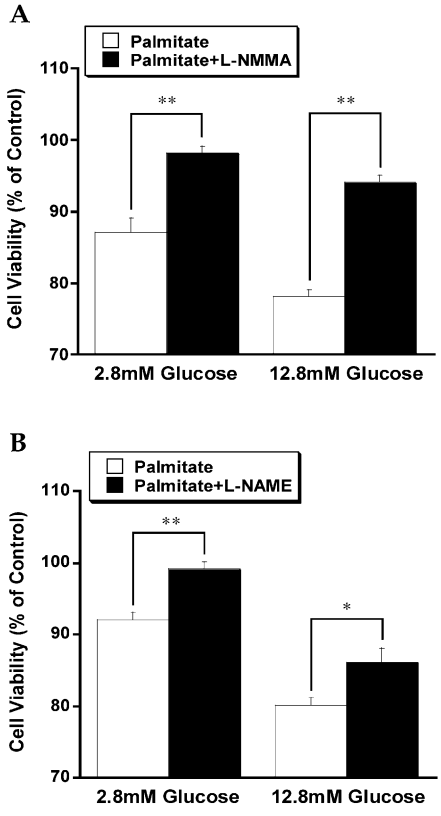

Fig. 2. Blockade of palmitate-induced cytotoxicity by NO synthase inhibitors. A: HIT-T15 β-cells were incubated without palmitate (control), with 0.5 mM palmitate, or with 0.5 mM palmitate+2 mM L-NMMA for 24 h under 2.8 mM or 12.8 mM glucose. Cell viability was measured by the MTT assay and is represented as percent of control under each glucose concentration. Each bar represents the mean of six replicates ± S.E.M. $^{**}P < 0.005$ between treatment with palmitate alone and palmitate+L-NMMA. B: HIT-T15 β-cells were incubated without palmitate (control), with 0.5 mM palmitate, or with 0.5 mM palmitate+2 mM L-NAME for 24 h under 2.8 mM or 12.8 mM glucose. Cell viability was measured by the MTT assay and is represented as percent of control under each glucose concentration. Each bar represents the mean of six replicates ± S.E.M. $^{**}P < 0.005$ between treatment with palmitate alone and palmitate+L-NAME.

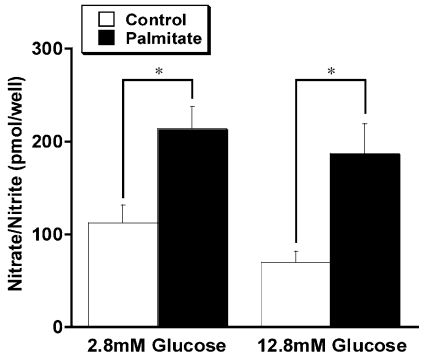

Fig. 3. NO production by palmitate treatment in HIT-T15 β-cells. HIT-T15 β-cells were incubated in the absence (control) or presence of 0.5 mM palmitate for 24 h under 2.8 mM or 12.8 mM glucose. Cell culture supernatant in each condition was collected and total amounts of nitrate and nitrite contained in each supernatant were measured with the Nitrate/Nitrite Fluorometric Assay kit. Each bar represents the mean of four replicates ± S.E.M. $^{*}P < 0.05$ between control and palmitate treatment under each glucose concentration.

Palmitate-induced cytotoxicity in the presence of both 2.8 and 12.8 mM glucose was significantly inhibited by simultaneous treatment with L-NMMA and L-NAME (Fig. 2). Furthermore, palmitate treatment significantly increased the amount of nitrate/nitrite in the supernatant (Fig. 3). However, no difference was observed between nitrate/nitrite in supernatant under 12.8 mM glucose versus 2.8 mM glucose (Fig. 3), indicating that the potentiation of the cytotoxic effect of palmitate under 12.8 mM glucose cannot be explained solely by increased NO production.

In the experiments investigating the effect of treating HIT-T15 β-cells with CCCP and MnTBAP together with palmitate, palmitate-induced cytotoxicity was significantly diminished only under the 12.8 mM glucose condition, suggesting that superoxide is involved in palmitate-induced cytotoxicity when glucose concentration is elevated (Fig. 4).

Glucosamine did not mimic the effect of high glucose on palmitate-induced cytotoxicity up to 10 mM (Fig. 5A), and bisindolylmaleimide did not reverse enhanced palmitate-induced cytotoxicity under 12.8 mM glucose (Fig. 5B).

4. Discussion

Our data clearly show that β-cell cytotoxicity by palmitate is potentiated under high glucose conditions in HIT-T15 β-cells. This indicates that exposure to FFAs and high glucose synergistically damages β-cells in terms of induction of apoptosis. In addition, palmitate-induced cytotoxicity was enhanced by glucose depending on glucose concentration between 2.8 and 12.8 mM, suggesting that the degree of lipoapoptosis may be tightly modulated by glucose concentrations.

Our results do not support the participation of increased hexosamine flux or PKC activation in the enhancement of palmitate-induced cytotoxicity when glucose concentration is

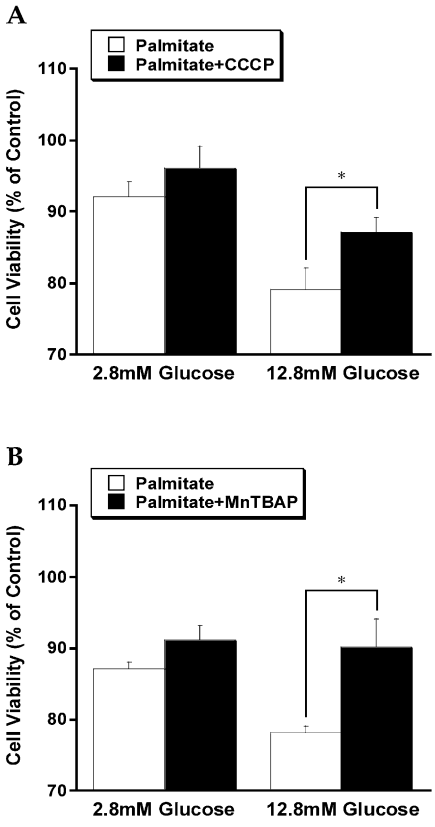

Fig. 4. Reversal of high-glucose-induced potentiation of palmitate cytotoxicity by decreasing superoxide. A: HIT-T15 β-cells were incubated without palmitate (control), with 0.5 mM palmitate, or with 0.5 mM palmitate+3 μM CCCP for 24 h under 2.8 mM or 12.8 mM glucose. Cell viability was measured by the MTT assay and is represented as percent of control under each glucose concentration. Each bar represents the mean of six replicates ± S.E.M. $*P < 0.05$ between treatment with palmitate alone and palmitate+CCCP. B: HIT-T15 β-cells were incubated without palmitate (control), with 0.5 mM palmitate, or with 0.5 mM palmitate+100 μM MnTBAP for 24 h under 2.8 mM or 12.8 mM glucose. Cell viability was measured by the MTT assay and is represented as percent of control under each glucose concentration. Each bar represents the mean of six replicates ± S.E.M. $*P < 0.05$ between treatment with palmitate alone and palmitate+MnTBAP.

elevated. However, palmitate-induced cytotoxicity was blocked by treatment with L-NMMA and L-NAME, both of which are inhibitors of NO synthase. NO is a widely known apoptosis mediator [21] and pancreatic β-cells are susceptible to NO-induced apoptosis [22,23]. In our study, palmitate treatment actually elevated NO production in HIT-T15 β-cells, as indicated by increased amounts of NO metabolites in supernatant, therefore it seems likely that palmitate leads to cell death by increasing NO levels in this cell line. Although it has been reported that FFAs induce the expression of inducible NO synthase (iNOS) in isolated islets [7], palmitate-induced cytotoxicity in HIT-T15 β-cells was not inhibited by the treatment with aminoguanidine, an inhibitor of iNOS (data not shown), and so the contribution of iNOS to palmitate-induced cytotoxicity in this cell line is unclear. However, it has been reported that constitutive NO synthase (cNOS) protein is palmitoylated and cellular distribution of this molecule is controlled by this modification [24]. Therefore palmitate might increase cellular NO production by affecting cNOS. In addition, involvement of NO in FFA-induced β-cell death is still controversial [10,25]. In our study, NOS inhibitors did not inhibit palmitate-induced cytotoxicity completely. Therefore some other mechanisms may be implicated in palmitate-induced cytotoxicity.

NO-mediated cell injury is considered to be mostly due to the generation of peroxynitrite, a reactive oxidant produced from NO and superoxide [26,27]. Peroxynitrite is a potent initiator of DNA breakage, leading to cell apoptosis [28]. In the present study, we demonstrated that palmitate-induced NO-mediated cytotoxicity is significantly potentiated when ambient glucose concentration is elevated although high glucose per se did not increase cell apoptosis at the concentrations and incubation period we used. Under high glucose conditions, the production of superoxide is elevated mainly because of increased glucose oxidation and following increase of superoxide production through the mitochondrial electron transport chain [15]. Superoxide overproduction accelerates the formation of peroxynitrite in the presence of NO and exaggerates the cytotoxic effects of NO [29,30]. Our data show that two distinct pharmacological applications to decrease superoxide, abolition of mitochondrial membrane proton gradient by an uncoupler of oxidative phosphorylation

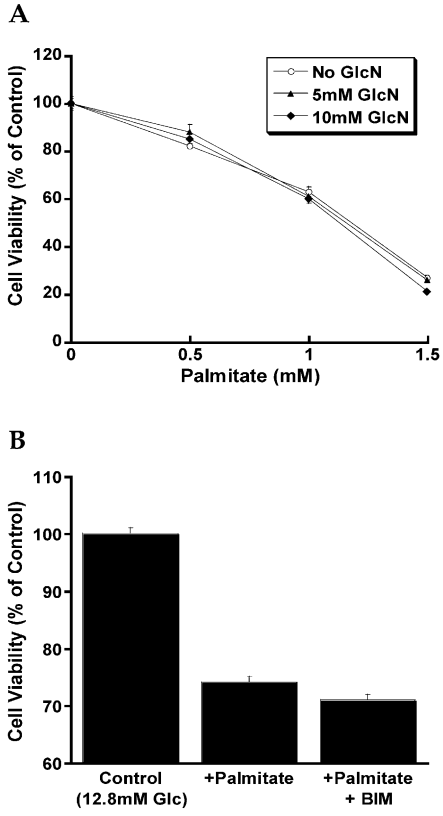

Fig. 5. No participation of increased hexosamine flux or PKC activation in high-glucose-induced potentiation of palmitate cytotoxicity was observed. A: HIT-T15.20 β-cells were incubated with the indicated concentrations of palmitate for 24 h under 2.8 mM glucose supplemented with 0, 5, or 10 mM glucosamine (GlcN). Cell viability was measured by the MTT assay and is represented as percent of control (no palmitate). Each bar represents the mean of six replicates ± S.E.M. B: HIT-T15 cells were incubated without palmitate (control), with 0.5 mM palmitate, or with 0.5 mM palmitate+1 μM bisindolylmaleimide (BIM) for 24 h under 12.8 mM glucose. Cell viability was measured by the MTT assay and is represented as percent of control. Each bar represents the mean of six replicates ± S.E.M.

and direct elimination of superoxide anion by a superoxide dismutase mimetic, significantly lessen palmitate-induced cytotoxicity only when the glucose concentration is elevated. These findings raise the possibility that cytotoxicity of NO generated by palmitate treatment is enhanced through increased peroxynitrite formation caused by superoxide overproduction under high glucose conditions. This potentiation of palmitate-induced NO-mediated β-cell toxicity by high-glucose-induced superoxide overproduction may explain the mechanism of synergistic deterioration of pancreatic β-cells by FFAs and high glucose.

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