Supplement of TCA cycle intermediates protects against high glucose/palmitate-induced INS-1 beta cell death

Sung-E Choi¹,¹, Youn-Jung Lee¹,¹, Geum-Sook Hwang¹,b,¹, Joo Hee Chung¹, Soo-Jin Leea, Ji-Hyun Lee¹, Seung Jin Hanc, Hae Jin Kimm, Kwan-Woo Lee c, Youngsook Kimd, Hee-Sook June, Yup Kanga,⁎

⁠¹ Institute for Medical Sciences, Ajou University School of Medicine, Suwon, Republic of Korea
b Korea Basic Science Institute, Seoul, Republic of Korea
c Department of Endocrinology and Metabolism, Ajou University School of Medicine, Suwon, Republic of Korea
d Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Republic of Korea
e Lee Gil-yo Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon, Republic of Korea

ARTICLE INFO

Article history:
Received 25 August 2010
and in revised form 12 October 2010
Available online 18 October 2010

Keywords:
Anaplerosis
Beta cell
Glucolipotoxicity
Mitochondria
TCA cycle

ABSTRACT

The aim of this study is to investigate the effect of mitochondrial metabolism on high glucose/palmitate (HG/PA)-induced INS-1 beta cell death. Long-term treatment of INS-1 cells with HG/PA impaired energy-producing metabolism accompanying with depletion of TCA cycle intermediates. Whereas an inhibitor of carnitine palmitoyl transferase 1 augmented HG/PA-induced INS-1 cell death, stimulators of fatty acid oxidation protected the cells against the HG/PA-induced death. Furthermore, whereas mitochondrial pyruvate carboxylase inhibitor phenylacetic acid augmented HG/PA-induced INS-1 cell death, supplementation of TCA cycle metabolites including leucine/glutamine, methyl succinate/α-ketoisocapric acid, dimethyl malate, and valeric acid or treatment with a glutamate dehydrogenase activator, aminobicycloheptane-2-carboxylic acid (BCH), significantly protected the cells against the HG/PA-induced death. In particular, the mitochondrial tricarboxylate carrier inhibitor, benzene tricarboxylate (BTA), also showed a strong protective effect on the HG/PA-induced INS-1 cell death. Knockdown of glutamate dehydrogenase or tricarboxylate carrier augmented or reduced the HG/PA-induced INS-1 cell death, respectively. Both BCH and BTA restored HG/PA-induced reduction of energy metabolism as well as depletion of TCA intermediates. These data suggest that depletion of the TCA cycle intermediate pool and impaired energy-producing metabolism may play a role in HG/PA-induced cytotoxicity to beta cells and thus, HG/PA-induced beta cell glucolipotoxicity can be protected by nutritional or pharmacological maneuver enhancing anaplerosis or reducing cataplerosis.

© 2010 Elsevier Inc. All rights reserved.

Introduction

Insulin deficiency caused by a loss of pancreatic beta cells and a subsequently impaired compensation for insulin resistance contributes toward the development of type 2 diabetes [1]. Increased levels of free fatty acids (FFAs)² are believed to induce a beta cell loss in type 2 diabetic patients, and this was termed ‘lipotoxicity’. Since FFAs in conjunction with hyperglycemia potentiates the lipotoxicity, the high glucose/FFA-induced toxicity was an augmented form of lipotoxicity and termed ‘glucolipotoxicity’ [2]. In fact, long-term exposure to FFA induced beta cell death in culture and in isolated islets [3], and elevated levels of glucose augmented this FFA-induced cell death [4]. This in vitro beta cell death by elevated FFA and high concentration of glucose is considered to represent in vivo glucolipotoxicity. Cell death was mainly apoptotic with cytochrome c release, caspase 3 activation, and DNA fragmentation [3]. Saturated fatty acids such as palmitic and stearic acids are generally cytotoxic to beta cells, while unsaturated fatty acids like linoleic, oleic, and palmitoleic acids, are not, and actually protect cells from saturated FFA-induced death [5].

The molecular and cellular mechanisms involved in FFA-induced beta cell death as well as the potentiation of FFA-induced death by glucose are not fully understood. Ceramide and the ceramide-induced up-regulation of nitric oxide (NO) have been suggested to be important mediators of FFA-induced beta cell death.

Abbreviations used:
CPT-1, carnitine palmitoyl transferase-1; ER, endoplasmic reticulum; FFA, free fatty acid; FAO, fatty acid oxidation; GDH, glutamate dehydrogenase; LC-CoA, long-chain acyl-coenzyme A; PPAR-α, peroxisome proliferator-activated receptors-alpha; ROS, reactive oxygen species; PARP, poly-(ADP-ribose) polymerase; PC, pyruvate carboxylase; TC, tricarboxylate carrier; TCA, tricarboxylate acid; TG, triacylglycerol.

⁎ Corresponding author. Address: Institute for Medical Science, Ajou University School of Medicine, Wonchon-dong san 5, Yongtong-gu, Suwon, Gyeonggi-do 442-749, Republic of Korea. Fax: +82 31 219 4482.
E-mail address: kangy@ajou.ac.kr (Y. Kang).
¹ These authors contributed equally to this work.
² Abbreviations used: CPT-1, carnitine palmitoyl transferase-1; ER, endoplasmic reticulum; FFA, free fatty acid; FAO, fatty acid oxidation; GDH, glutamate dehydrogenase; LC-CoA, long-chain acyl-coenzyme A; PPAR-α, peroxisome proliferator-activated receptors-alpha; ROS, reactive oxygen species; PARP, poly-(ADP-ribose) polymerase; PC, pyruvate carboxylase; TC, tricarboxylate carrier; TCA, tricarboxylate acid; TG, triacylglycerol.
induced insulin resistance suggest that mitochondrial dysfunction, through depletion of TCA cycle intermediates contributed to FFA-elimination to a useless energy exhaustion metabolism [30]. Collectively, TG/FFA cycling was postulated to divert toxic glucose/FFA metabolism away from glucolipotoxicity [29]. Enhanced activity with increased lipolysis, was suggested to act as the mechanism through over-expression of stearoyl-CoA desaturase 1 (SCD1) was found to provide survival signals to FFA-treated beta cells by sequestering fatty acids as phosphatidic acid, lysophosphatidic acid, diacylglycerol (DAG), and ceramide were suggested to play a critical role in FFA-induced toxicity to beta cells [23,32,33].

The present study was initiated to determine whether metabolic impairment in mitochondria was involved in high glucose/FFA-induced glucolipotoxicity to beta cells and whether the maintenance of TCA cycle intermediate pool could protect against the toxicity. We initially tested whether mitochondrial energy-producing metabolism was impaired in high glucose/palmitate (HG/PA)-treated INS-1 beta cells. We measured the level of intracellular ATP and the oxidation rate of glucose and palmitate and determined the levels of TCA cycle intermediates in the HG/PA-treated cells. We next investigated the role of glucose/FFA metabolism in HG/PA-induced cytotoxicity using modulators of anaplerosis and cataplerosis as well as modulators of fatty acid oxidation. The effect of reduced anaplerosis and cataplerosis on HG/PA-induced INS-1 cell death was examined through gene knockdown of glutamate dehydrogenase (GDH) and mitochondrial tricarboxylate carrier (TC), respectively. We finally investigated whether GDH activator and TC inhibitor would be able to restore the HG/PA-induced reduction of oxidation metabolism and depletion of TCA cycle intermediates.

Materials and methods

Reagents

All chemicals, including glucose, palmitate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), bezafibrate, etomoxir, phenylpyruvate, leucine, and methyl pyruvate, leucine, glutamine, monomethyl succinate, α-ketoisocaproate, dimethyl malate, valeric acid, 2-aminobicyclo[2.2.1]heptan-2-carboxylic acid (BCH), 2,3-benzenetricarboxylate (BTA), and ATP were purchased from either Merck Bioscience (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO). The chemicals were dissolved in either appropriate media solution or dimethyl sulfoxide (DMSO) and then treated at the required working dilution. All chemicals were handled in accordance with the supplier’s recommendations. Anti-caspase 3 and PARP antibodies were purchased from Cell Signalling Technology (Beverly, MA).

Preparation of palmitate

Preparation of palmitate was slightly modified from Listenberg’s protocol [34]. Palmitate/BSA (bovine serum albumin) conjugates were prepared through soaking palmitate with sodium hydroxide and mixing with BSA. Briefly, a 20 mM solution of palmitate in 0.01 M NaOH was incubated at 70 °C for 30 min and the fatty acid soaps were then complexed with 5% fatty acid-free BSA in phosphate-buffered saline (PBS) in 1:3 volume ratio. The complexed fatty acids consisted of 5 mM palmitate and 3.75% BSA. The palmitate/BSA conjugates were diluted in 10% FBS culture medium (approximately 0.4% BSA) and administered to cultured cells. Molar ratio of palmitate to BSA in 0.4 mM palmitate is 3.5:1 and the concentration of BSA is approximately 0.7%.

Cells

INS-1 rat insulinoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad,
CA), 100 U/mL penicillin, 100 g/mL streptomycin, and 10 mM HEPES at 37 °C in a humidified atmosphere containing 95% air and 5% CO2.

Viability assay

Briefly, the cells were treated with MTT (0.5 mg/ml) at 37 °C for 2 h. Supernatants were discarded and acidic isopropanol (0.04 N HCl) was then added. After incubating at room temperature for 30 min, absorbance was measured at 570 nm using a microplate reader (BIO-RAD, Hercules, CA).

Quantitation of cell death

Cell death was determined by measuring cytoplasmic histone-associated DNA fragments using the Cell Death Detection ELISA kit (Roche Applied Science, Mannheim, Germany). Briefly, the cells that were treated with palmitate were lysed by adding incubation buffer (supplied from kit). After centrifugation (20,000g, 10 min), the supernatant was pipetted onto an anti-histone-coated microplate. After incubation for 90 min at 25 °C, the wells were rinsed with washing solution (supplied from kit). Anti-DNA PODs were then bound to the nucleosome complex by adding conjugation solution (supplied from kit) and incubating at 25 °C for 90 min. After washing the wells with washing solution, the colour was developed by adding ABTS (2,2′-azino-di-[3-ethylbenzothiazoline sulphonate]) substrate solution (1 mg/ml) and by incubating with shaking at 250 rpm for 10–20 min. The amount of peroxidase retained in the nucleosome complex was determined by measuring the absorbency at 405 nm.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression levels of GDH and mitochondrial TC mRNAs were determined using the semi-quantitative RT-PCR technique. Semi-quantitative RT-PCR was performed with the Takara RNA PCR kit Version 3.0 (Takara, Shiga, Japan). Briefly, INS-1 cell cDNAs were synthesised with avian myeloblastosis virus (AMV) reverse transcriptase and random 9-mers, and underwent PCR amplification with primer sets of GDH (forward primer: 5′-CTCAGGTACCCGTCT- GATCTTCTGTGCTCCCG, backward primer: 5′-CTCACTCGAGGCGAGG TGAAGAGGGTGGTAAAG) and TC (forward primer: 5′-GCTGAGGCT ATCGAAATCTGCT, backward primer: 5′-TAGCTGAGCCCTTGG TATGTC). GDH DNAs were amplified under the following conditions: denaturation at 95 °C for 5 min followed by 26 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. On the other hand, TC DNA was amplified by 29 cycles under the same conditions. The reaction was terminated by extension at 72 °C for 10 min. The amplified DNA was analysed by 1% agarose gel electrophoresis, and the relative DNA quantities were measured by densitometric analysis on the basis of amplified cyclophilin DNA.

Immunoblotting

The cells were suspended in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris–Cl, (pH 7.5), and protease inhibitor cocktail (Roche Applied Science, Mannheim Germany)] and then incubated on ice for 30 min. Whole proteins were extracted by differential centrifugation (10,000g, 10 min) and protein concentrations in lysates were determined using protein assay kits (Bio-Rad, Hercules, CA). An equal volume of 2 × sodium dodecyl sulphate (SDS) sample buffer [125 mM Tris–Cl, (pH 6.8), 4% SDS, 4% 2-mercaptoethanol, 20% glycerol] was added to cell lysates, and equivalent amounts of protein (30 μg) were loaded onto 10–15% polyacrylamide gels, electrophoresed, and then electrophotographically transferred onto Polyvinylidene Fluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking these membranes with 5% skimmed milk for 30 min, target antigens were reacted with primary antibodies and subsequently secondary antibodies (horseradish peroxidase-conjugated mouse IgG or anti-rabbit IgG antibodies). Immunoreactive bands were then developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Height, IL). Band intensity was determined by densitometric analysis using a one-dimensional Quantity One® 1D image analysis system. Maximum intensity of the band was converted to 100%, and relative intensities were calculated on the basis of maximum.

Measurement of oxaloacetate, alpha-ketoglutarate and citrate

To determine the level of TCA cycle intermediates, metabolism assay kits for oxaloacetate, alpha-ketoglutarate, and citrate (BioVision Inc., Mountain View, CA) were used. Briefly, cells were scraped, washed with PBS, and homogenised in assay buffer provided by supplier. Insoluble particles were removed by differential centrifugation (15,000g, 10 min). Protein concentrations in extracts were determined using protein assay kits (Bio-Rad, Hercules, CA). Residual enzymes in extracts were removed by perchloric acid/KOH precipitation protocol (BioVision Inc., Cat. # K808-200). The resulting supernatant was used for measurement of metabolites. The supernatants were mixed with each assay buffer containing enzyme mix, developer, and probe. After incubation at room temperature for 30 min, absorbance was measured at 570 nm. The quantity of each metabolite was determined by normalisation to intracellular protein content.

Measurement of oxidation rate of glucose and palmitate

Cells were incubated in 0.2% BSA/KRB (0.2 mM glucose, 24 mM NaHCO3, 1.2 mM MgCl2, 1 mM HEPES, 129 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2) containing 2 mM glucose and 0.1 μCi/ml [U-14C] glucose for 1 h or in 0.2% BSA/KRB containing 2 mM glucose, 1 mM carnitine, 0.2 mM palmitate, 0.5 μCi/ml [1-14C] palmitate for 2 h, respectively. The supernatant containing labelled CO2 was transferred into a new tube and then the reaction was stopped by adding 1/6 volume of cold 40% perchloric acid. The CO2 was absorbed in 1 M KOH solution by sealing the bottle containing the reaction tube and KOH tube for 16 h. The amount of labelled CO2 was measured by counting the radioactivity of absorbed CO2 in KOH solution with liquid scintillation. Normalisation relative to intracellular protein content in live cells was performed. The relative amount of labelled CO2 compared to CO2 produced from non-treated INS-1 cells represented the relative oxidation rate of glucose or palmitate in HG/PA-treated cells.

Measurement of ATP

Quantitation of intracellular ATP levels was determined using a CellTiter-Glo™ luminescence kit (Promega Life Science, WI). INS-1 cells were mixed with the same volume of CellTiter-Glo reagent and lysed by vortexing. After incubating at room temperature for 10 min, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The quantity of ATP was calculated using an ATP standard curve. The relative level of ATP compared to that from non-treated INS-1 cells represented the relative percent of ATP in HG/PA-treated cells.

Small interfering RNAs and transfection

The 21-nucleotide small interfering RNA duplexes were designed and synthesised from Samchully Pharm Co. (Seoul, Korea). The sequences are as follows: GFP, 5′-GUUCAGCGUGUCCGCGAGTT; rat
GDH (GenBank: X144233.1), 5'-GAUCAAUCCCAAGAACUAU; rat TC (GenBank: NM_017307), 5'-GUGUCAUGUGACUGUCAU. INS-1 cells were transfected with siRNA duplex using a pipet type electroporator (Digital biotechnology, Seoul, Korea) according to manufacturer’s instruction. Briefly, 2.5 µg each siRNA duplex with 3 µg pCDNA in 100 µl R buffer (Digitalbiotechnology) were transfected into 5 × 10^6 INS-1 cells under conditions of 1650 V and 10 ms width. After transfection, 1.5 × 10^6 cells were seeded into each well of a 6-well plate and incubated for 36 h.

Statistics

Data are represented as means ± SE from at least three independent experiments. Differences between the groups were determined by one way analysis of variance (ANOVA) using the SPSS statistical analysis program version 7.5 (SPSS, Gray, NC, USA). Duncan’s multiple range test was performed for evaluation of differences between the groups. A value of p < 0.05 was considered to indicate a significant difference between groups.

Results

Augmentation of palmitate-induced INS-1 cell death by high concentration of glucose

Since saturated fatty acid was reported to be cytotoxic to beta cells [3], we re-examined the palmitate-induced cytotoxicity to INS-1 beta cells. INS-1 cells were exposed to different concentrations of palmitate in the presence of 11 mM glucose for 18 h. As shown in Fig. 1A, a low dose of palmitate (0.1 mM) slightly increased viability of INS-1 cells but 0.3 mM and 0.4 mM of palmitate (PA) significantly reduced the cell viability. Palmitate at a concentration of 0.4 mM reduced cell viability to 64%. Since FFA-induced lipotoxicity was reported to be synergized with high concentration of glucose [4], we examined the PA-induced viability reduction in response to varying concentrations of glucose. As shown in Fig. 1B, glucose dose-dependently potentiated the palmitate-induced reduction of INS-1 cell viability. Incubation of INS-1 cells with 0.4 mM palmitate in the presence of 5 mM glucose for 18 h reduced cell viability by around 13%. Incubation of the cells in the presence of 25 mM glucose (high glucose: HG), however, reduced viability by around 56%. HG/PA-induced viability reduction of INS-1 cells was time-dependent (Fig. 1C). Viability began to decline at 12 h after HG/PA treatment although that at 6 h was slightly increased. The Cell Death Detection assay demonstrated that fragmented DNAs were detected by palmitate treatment in the presence of 5 mM glucose and that the palmitate-induced DNA fragmentation was augmented by 25 mM glucose (Fig. 1D). The DNA fragmentation by HG/PA treatment was also time-dependent (Fig. 1E). In particular, DNA fragmentation at 12 h was not significantly increased although cell viability at the same time was significantly reduced (Fig. 1D and E), suggesting that viability reduction by HG/PA was followed by HG/PA-induced DNA fragmentation. The level of the cleaved form of caspase 3 and poly (ADP-ribose) polymerase (PARP), demonstrating caspase 3 activation, was gradually increased in palmitate-treated cells in proportion to increasing concentrations of glucose (Fig. 1E). These data demonstrated that saturated fatty acid-induced apoptotic cell death in INS-1 beta cells and the fatty acid-induced apoptotic death was augmented by high concentration of glucose.

Reduction of ATP level, oxidation rate of glucose and palmitate, and TCA cycle intermediates in HG/PA-treated INS-1 cells

Since mitochondrial dysfunction was suggested to be a mediator of HG/FFA-induced glucolipotoxicity, we examined the intracellular level of ATP in HG/PA-treated INS-1 cells. As shown in Fig. 2A, the ATP level was slightly increased at 6 h after HG/PA treatment, but the level was significantly reduced by around 35% at 12 h, the time when viability reduction began to be observed. Since ATP level was lower, oxidation rate of glucose and palmitate was next investigated in HG/PA-treated cells. As shown in Fig. 2B and C, pre-treatment with HG/PA for 12 h significantly reduced oxidation rate of glucose and palmitate. On the other hand, the oxidation rate of glucose and palmitate was slightly increased at 6 h after HG/PA treatment. These data also demonstrated that energy-producing capability in mitochondria was impaired with similar kinetics with viability reduction in HG/PA-treated INS-1 cells. Whereas bezafibrate as a peroxisome proliferator-activated receptors-alpha (PPAR-α) agonist increased the oxidation rate of palmitate, etomoxir as an inhibitor of CPT-1 reduced the oxidation rate of palmitate (Fig. 2C). Since impaired glucose metabolism and subsequent reduction of TCA cycle intermediate pool was suggest to be a characteristic of lipid-induced mitochondrial stress [31], we next examined the level of TCA cycle intermediates including oxaloacetate, citrate, and α-ketoglutarate using a metabolism assay kit. As shown in Fig. 2D, the oxalacetate level began to decrease at 3 h after HG/PA treatment and then continuously declined. On the other hand, the level of citrate and α-ketoglutarate in HG/PA-treated INS-1 cells peaked at 6 h after HG/PA treatment and then began to decrease at 9 h. These data demonstrated that levels of all three TCA cycle intermediates in HG/PA-treated INS-1 cells were significantly lower than those in untreated cells at the time when the viability reduction was detected, but when HG/PA-induced DNA fragmentation was not observed. Reduction kinetics of TCA cycle intermediates was in accord with that of intracellular ATP and oxidation rate of glucose and palmitate (Fig. 2A–D). These results suggest that long-term treatment of INS-1 cells with HG/PA impaired the oxidative metabolism of glucose and FFA, partly through depletion of TCA cycle intermediates.

Role of fatty acid oxidation in HG/PA-induced INS-1 cell death

Enhanced fatty acid oxidation (FAO) metabolism was supposed to protects against palmitate-induced cell death since inhibition of FAO promotes the palmitate-induced beta cell death [4]. We re-examined the effect of FAO on HG/PA-induced INS-1 cell death. FAO was stimulated by treatment of the cells with the PPAR-α agonist bezafibrate or the AMP-activated protein kinase (AMPK) activator 5-aminooimidazole-4-carboxamide ribonucleoside (AICAR) [35]. These two agents dose-dependently and significantly protected against HG/PA-induced DNA fragmentation (Fig. 3A and B). The effective concentrations of AICAR or bezafibrate did not increase DNA fragmentation of INS-1 cell in the absence of palmitate. In contrast, etomoxir, an inhibitor of carnitine palmitoyltransferase 1 (CPT-1) that catalyzes the rate-limiting step of the oxidation of FFAs [4], dose-dependently and significantly augmented HG/PA-induced DNA fragmentation (Fig. 3C). These data demonstrated that stimulation of FAO protected against HG/PA-induced INS-1 cell death, but inhibition of FAO promoted the cell death.

Supplement of TCA cycle intermediates protects against HG/PA-induced INS-1 cell death

Pyruvate produced through glycolysis is converted to oxaloacetate through pyruvate carboxylase (PC), resulting in replenishment of tricarboxylic acid (TCA) cycle intermediates (anaplerosis) and finally providing cytosolic citrate (cataplerosis) for fatty acid/lipid synthesis (Supplemental Fig. 1). To determine whether depletion of TCA cycle intermediates was involved in HG/PA-induced INS-1 cell death, the activity of PC was blocked by treatment with PC inhibitor phenylacetic acid (PAA) and the effect of PAA on
HG/PA-induced death was then examined. PAA dose-dependently augmented HG/PA-induced DNA fragmentation at the concentrations that did not influence the death of INS-1 cells in the absence of palmitate (Fig. 4A). This data strongly suggest that depletion of...
TCA cycle intermediates through reduced anaplerosis is involved in HG/PA-induced INS-1 cell death. Since TCA cycle intermediates are replenished through treatment with various metabolic fuels, the effect of the metabolic fuels on HG/PA-induced INS-1 cell death was tested (Supplemental Fig. 1). While treatment of the cells with methyl pyruvate, a membrane transferable form of pyruvate, did not influence HG/PA-induced DNA fragmentation (Fig. 4B), leucine and glutamine, which replenish α-ketoglutarate, as well as mono-methyl succinate and α-ketoisocaproic acid, which replenish succinic acid and acetyl-CoA, significantly and dose-dependently reduced HG/PA-induced DNA fragmentation (Fig. 4C and D). Dimethyl malate, a membrane transferable TCA cycle intermediate malate, also significantly reduced HG/PA-induced DNA fragmentation (Fig. 4E). In addition, valeric acid, a five carbon-chain FFA that replenishes succinyl-CoA through propionyl-CoA significantly and dose-dependently reduced HG/PA-induced DNA fragmentation (Fig. 4F). In particular, BCH, an activator of glutamate dehydrogenase (GDH) that can replenish α-ketoglutarate in the TCA cycle from glutamate, very effectively reduced HG/PA-induced DNA fragmentation and the protective effect was dose-dependent (Fig. 4G). Since the TCA cycle intermediate pool could be expanded by blocking transportation of mitochondrial citrate to the cytosol, the effect of BTA as an inhibitor of mitochondrial tricarboxylate carrier (TC) on HG/PA-induced cytotoxicity was examined (Supplemental Fig. 1). BTA also dose-dependently and significantly reduced the HG/PA-induced DNA fragmentation (Fig. 3H). Since activator of GDH or inhibitor of TC reduced HG/PA-induced DNA fragmentation, we confirmed the protective effect of BCH or BTA on HG/PA-induced INS-1 cell death.
induced cell death through reduced cleavage of caspase 3 and PARP. As shown in Fig. 5, exposure of INS-1 cell to HG/PA for 15 h increased the level of cleaved caspase 3 in conjunction with cleaved PARP. However, treatment with BCH or BTA significantly attenuated HG/PA-induced cleavage of PARP and caspase 3. FAO stimulator bezafibrate also showed similar attenuating effect on HG/PA-induced caspase 3 cleavage. Collectively, all our attempts for enhancing TCA cycle intermediate pool through enhanced anaplerosis or reduced cataplerosis showed evident protective effect on HG/PA-induced INS-1 cell death.

Augmentation of HG/PA-induced INS-1 cell death by knockdown of glutamate dehydrogenase (GDH) and attenuation of the death by knockdown of mitochondrial tricarboxylate carrier (TC)

Since pharmacological agents enhancing anaplerosis or reducing cataplerosis showed a protective effect on HG/PA-induced INS-1 cell death, we examined whether gene knockdown of GDH or TC would demonstrate similar augmenting or attenuating effects on HG/PA-induced INS-1 cell death, respectively. While knockdown of GDH is thought to inhibit supply of TCA cycle intermediate pool to the mitochondria and subsequent impaired energy-producing metabolism could be rescued by supplementation of TCA cycle intermediates through enhanced anaplerosis or reduced cataplerosis.

Restoration of HG/PA-induced reduction of TCA cycle intermediates, glucose/palmitate oxidation rate, and ATP level by BCH or BTA treatment

Since the attempts to enhance TCA cycle intermediate pool protected INS-1 cells against HG/PA-induced cell death, depletion in the TCA cycle intermediates and subsequent reduction of energy-producing metabolism was postulated to be involved in HG/PA-induced cytotoxicity. We tested whether treatment of the cells with BCH or BTA would increase the level of TCA cycle intermediates and restore ATP levels with enhanced oxidation rate in HG/PA-treated INS-1 cells. Treatment of the INS-1 cells with BCH or BTA without palmitate tended to increase the level of TCA cycle intermediates including oxaloacetate, citrate and α-ketoglutarate (Fig. 7A–C). In particular, both BCH and BTA significantly prevented HG/PA-induced reduction of these intermediates (Fig. 7A–C). FAO stimulator bezafibrate also showed similar preventive effect on HG/PA-induced reduction of the intermediates. In accordance with an increase of TCA cycle intermediates, both BCH and BTA significantly prevented HG/PA-induced reduction of glucose or palmitate oxidation (Fig. 7D and E). On the other hand, bezafibrate significantly increased the oxidation rate of palmitate (Fig. 7E), but decreased glucose oxidation in HG-treated INS-1 cells (Fig. 7D). Bezafibrate also prevented HG/PA-induced reduction of oxidation rate of glucose and palmitate in INS-1 cells (Fig. 7D and E). In accordance with a restoration of oxidation rate, treatment with bezafibrate, BCH or BTA significantly restored the reduced ATP level in HG/PA-treated INS-1 cells (Fig. 7F). These data suggested that HG/PA-induced inhibition of energy-producing metabolism could be rescued by supplementation of TCA cycle intermediates through enhanced anaplerosis or reduced cataplerosis.

Discussion

High concentration of glucose amplified palmitate-induced INS-1 cell death. Bezafibrate and AICAR, which is known to increase FAO, protected against this HG/PA-induced cytotoxicity. However, etomoxir, which inhibits FAO by blocking acyl-CoA transport into mitochondria, potentiated this cytotoxicity. On the other hand, most attempt to expand the TCA cycle intermediate pool, including treatment of cells with leucine/glutamine, monomethyl succinate/α-ketoisocaproic acid, dimethyl malate, valeric acid, BCH and BTA, significantly protected against HG/PA-induced cytotoxicity. In fact, the level of TCA cycle intermediates in HG/PA-treated INS-1 cells was significantly low at the time when HG/PA-induced viability reduction began to be observed. The oxidation rate of glucose and palmitate was declined and the level of ATP was also reduced in HG/PA-treated cells. The reductions of oxidation rate and ATP level in HG/PA-treated cells were restored after treatment of the cells with bezafibrate, BCH or BTA. These results suggest that an insufficient supply of TCA cycle intermediates or fuel substrates into mitochondria and subsequent impaired energy-producing metabolism may play a role in HG/PA-induced beta cell cytotoxicity.

![Fig. 5](image_url). Protective effect of FAO stimulator, anaplerosis enhancer, and cataplerosis inhibitor on HG/PA-induced caspase 3 activation. INS-1 cells were treated with 25 mM glucose (HG) and 0.4 mM palmitate (PA) in the presence of 0.5 mM bezafibrate (Bez), 10 mM BCH or 2.5 mM BTA for 15 h. Cleaved caspase 3 and cleaved PARP were detected by immunoblotting (IB) with anti-cleaved caspase 3 and anti-PARP antibodies, respectively (A). P-Cas3, C-Cas3, P-PARP, and C-PARP, represent pro-caspase 3, cleaved caspase 3, uncleaved PARP, and cleaved PARP, respectively. **p < 0.01 vs. cleaved caspase 3 from HG/PA-treated INS-1 cells. ##p < 0.01 vs. cleaved PARP from HG/PA-treated cells.
Initially it was suggested that the accumulation of toxic lipid intermediates such as ceramide, LC-CoA, DAG, or phospholipids and subsequent activation of the lipid-mediated signals was linked in beta cell glucolipotoxicity [19]. Reactive oxygen species (ROS) produced during augmented lipogenesis was suggested to contribute to beta cell glucolipotoxicity [11,13]. However, several reports opposed these suggestions. Ceramide was not believed to play a role in FFA-induced lipotoxicity since a ceramide synthase inhibitor did not block FFA-induced cytotoxicity [26,33,36]. We also observed that fumonisin B1 and myriocin, inhibitors of sphingosine N-acetyl transferase and serine palmitoyltransferase, respectively, afforded little protection against HG/PA-induced cytotoxicity (Supplemental Fig. 2A). Although inhibiting PKC was weakly protective (Supplemental Fig. 2B), Welters et al. reported that PKCδ was not required for palmitate-induced cytotoxicity to beta cells [37]. The data that treatment of the INS-1 cells with antioxidants such as NAC and GSH did not protect against HG/PA-induced cytotoxicity [26,33,36]. We also observed that fumonisin B1 and myriocin, inhibitors of sphingosine N-acetyl transferase and serine palmitoyltransferase, respectively, afforded little protection against HG/PA-induced cytotoxicity [26,33,36]. Welters et al. reported that PKCδ was not required for palmitate-induced cytotoxicity to beta cells [37].

Of particular note was that T0901317, an liver X receptor (LXR) agonist that stimulated FFA/lipid synthesis, strongly protected against HG/PA-induced cytotoxicity, weakening support for a potential role of glucose-induced lipogenesis in HG/PA-induced cytotoxicity (Supplemental Fig. 2D). Collectively, recent reports as well as our data do not support the hypothesis that accumulation of a specific lipid intermediate and subsequent activation of the lipid-mediated signal may be the direct mediators in HG/PA-induced beta cell glucolipotoxicity.

Stimulation or inhibition of FAO protected or augmented HG/PA-induced beta cell death, respectively. These data are consistent with a study published by El-Assaad et al., which reported that FAO stimulator AICAR protected beta cells against HG/FFA-induced glucolipotoxicity while FAO inhibitor etomoxir amplified the toxicity (Supplemental Fig. 2D). This data including ours suggested that FAO might be impaired in HG/PA-treated cells. When beta cells are simultaneously exposed to high levels of glucose and FFA, a transient increase of malonyl-CoA is believed to block acyl-CoA transport into the mitochondria and ultimately block FAO since malonyl-CoA inhibits CPT-1 [19,21]. A report showing that over-expression of CPT-1 counteracted beta cell glucolipotoxicity also suggests that transportation of acyl-CoA to mitochondria may be impaired in HG/PA-treated cells [38]. In addition to impaired transportation, oxida-
tion of FFAs in mitochondria may be incomplete in beta cells since incomplete FAO was reported to be a contributor to FFA-induced insulin resistance [31]. On the other hand, Xu et al. reported that high level palmitate as well as high concentration of glucose up-regulated expression of pyruvate dehydrogenase kinase in beta cells [39], which reduced pyruvate dehydrogenase activity and subsequently inhibited supply of pyruvate to the TCA cycle. This result suggests that the supply of both pyruvate and FFA as energy substrates is not sufficient in mitochondria, and, subsequently, energy-producing metabolism to compensate the enhanced lipogenic pathway can be impaired in HG/PA-treated cells. In fact, our experimental data showing that the oxidation rate of glucose as well as palmitate declined and that intracellular ATP level was significantly low in HG/PA-treated INS-1 cells support that impaired energy-producing metabolism may be due to an insufficient supply of pyruvate and FFA into mitochondria. Reports showing that the flux of the TCA cycle was reduced in type 2 diabetes and that an enhanced mitochondrial metabolism accounted for adaptation of beta cells to high-fat diet-induced insulin resistance [40,41] suggested that insufficient supply of the TCA cycle intermediates can also be a critical mediator for HG/PA-induced toxicity. In fact, most attempts to increase the TCA cycle intermediates protected the INS-1 cells against HG/PA-induced cytotoxicity. A knockdown study of GDH or TC augmenting or attenuating HG/PA-induced INS-1 cell death, respectively, supports that maintenance of TCA cycle intermediate pool through enhanced anaplerosis and reduced cataplerosis is critical for maintaining cell viability in HG/PA-treated INS-1 cells. In fact, our preliminary experiment that treatment with both PAA and etomoxir, which may impair supply of TCA cycle metabolites, induced similar mode of cytotoxicity induced by HG/PA. Treatment of INS-1 cells with PAA and etomoxir induced apoptotic death and the PAA/etomoxir-induced death was prevented by treatment with FAO stimulators or GDH activator (Data not shown). Since FFA-induced insulin resistance was able to be caused by lack of TCA intermediates, we measured cellular level of TCA cycle intermediates, levels of oxaloacetate, citrate, and α-ketoglutarate in HG/PA-treated INS-1 cells. Oxidation rates of glucose or palmitate were determined by measurement of labelled CO2 produced from [U-14C] glucose (D) or [1-14C] palmitate (E). *p < 0.05 vs. HG/PA-treated INS-1 cells. **p < 0.01 vs. oxaloacetate, citrate, and α-ketoglutarate in HG/PA-treated INS-1 cells. Oxidation rates of glucose or palmitate were determined by measurement of labelled CO2 produced from [U-14C] glucose (D) or [1-14C] palmitate (E). *p < 0.05 vs. oxidation rate in HG-treated INS-1 cells. **p < 0.01 vs. oxidation rate in HG/PA-treated INS-1 cells. ATP level was measured using the CellTiter-Glo™ luminescence kit. *p < 0.05; **p < 0.01 vs. ATP level in HG/PA-treated INS-1 cells.

Fig. 7. Restoration of the HG/PA-induced reduction of mitochondrial metabolism by BCH and BTA. INS-1 cell were treated with HG/PA for 12 h in the absence or presence of 0.5 mM bezafibrate (Bez), 10 mM BCH, or 2.5 mM BTA. The levels of oxaloacetate (A), citrate (B), and α-ketoglutarate (C) in cellular extracts were then determined using a metabolism assay kit (Biovision). Data are represented as means ± SEM from three independent experiments. *p < 0.05; **p < 0.01 vs. oxaloacetate, citrate, and α-ketoglutarate in HG/PA-treated INS-1 cells. Oxidation rates of glucose or palmitate were determined by measurement of labelled CO2 produced from [U-14C] glucose (D) or [1-14C] palmitate (E). *p < 0.05 vs. oxidation rate in HG-treated INS-1 cells. **p < 0.01 vs. oxidation rate in HG/PA-treated INS-1 cells. ATP level was measured using the CellTiter-Glo™ luminescence kit. *p < 0.05; **p < 0.01 vs. ATP level in HG/PA-treated INS-1 cells.
may play a role in HG/PA-induced beta cell cytotoxicity. Our data showing that methyl pyruvate did not afford protection against HG/PA-induced cell cytotoxicity suggested the anaplerotic pathway through PC is impaired in HG/PA-treated INS-1 cells. Recent report stating that an LXR agonist could augment anaplerotic pathway through enhanced activation of PC may explain our result that T0901317 had strong protective effect on HG/PA-induced death (Supplemental Fig. 2D), even though its enhanced lipogenic activity [47]. Furthermore, a report that over-expression of PPAR up-regulated expression of anaplerotic pyruvate carboxylase [48] also suggests that FAO stimulation can also up-regulate the anaplerotic process, suggesting that FAO stimulators protect against HG/PA-induced glucolipotoxicity through a sufficient supply of TCA cycle intermediates. In particular, our experiments showing that enhanced anaplerosis by BCH and reduced cataplerosis by BTA restored HG/PA-induced reduction of oxidation rate and ATP level suggest that the impaired energy metabolism in HG/PA-treated cells is partly due to insufficient supply of TCA cycle intermediates. Interestingly, imbalanced mitochondrial energy production in HG/PA-treated cells may explain beta cell dysfunctions observed in type 2 diabetes, such as impaired glucose-stimulated insulin secretion and impaired expression of insulin gene [49–51].

Although metabolic failure through depletion of TCA cycle intermediates was suggested to be a cause of HG/PA-induced glucolipotoxicity, the intracellular mechanism how failure of energy metabolism activates toxic signals in INS-1 beta cells is not clearly defined. Earlier reports suggest that oxidative stress, ER stress, Ca^2+ signal, and impaired insulin signal were intracellular mediators for HG/PA-induced cytotoxicity. In fact, these cytotoxic mediators can be linked to mitochondrial dysfunction. Enhanced production of ROS and subsequent activation of oxidative stress signal was a typical toxic mediator produced from mitochondrial dysfunction and therefore, could be a possible mediator for HG/PA-induced cytoxicity to INS-1 cells [23]. However, since antioxidants such as NAC and GSH did not protect against HG/PA-induced cytotoxicity, ROS and oxidative stress does not seem to be a mediator for HG/PA-induced toxicity. Since coupling mitochondrial dysfunction to ER stress responses was recently reported in hepatic cells [52], metabolic failure in mitochondrion through depletion of TCA cycle intermediates may induce HG/PA-induced cytotoxicity through ER stress induction. It will be further studied whether the ER stress can be a critical linker between mitochondrial metabolic failure and HG/PA-induced INS-1 cell death. Another mediator linking mitochondrial dysfunction to glucolipotoxicity can be elevation of intracellular Ca^{2+}. Since it was reported that defective mitochondrial function caused the cells to be vulnerable to Ca^{2+}-mediated toxic signals [53], metabolic failure in beta cell mitochondria may induce HG/PA-induced cytotoxicity through activation of Ca^{2+} signals. A recent report that mitochondrial dysfunction inhibited insulin signalling pathway [54] strongly suggests that mitochondrial dysfunction may induce beta cell glucolipotoxicity through inhibition of insulin signalling pathway.

To test whether HG/PA treatment induced cytotoxicity in primary beta cells and whether the HG/PA-induced toxicity was reduced by BCH and BTA, we initially investigated the glucose-induced augmentation of PA-induced viability reduction of primary islet cells. High concentration of glucose did not potentiate PA-induced viability reduction of primary islet cells isolated from non-diabetic rats, but potentiated the viability reduction of primary islet cells isolated from diabetic animals, such as Otsuka Long Evans Tokushima Fatty (OLETF) and Zucker Diabetic Fatty (ZDF) (Supplemental Fig. 3). Protective effect through BCH and BTA on HG/PA-induced cytotoxicity was also observed in primary islet cells isolated from these diabetic rats (Supplemental Fig. 3). These results suggest that metabolic failure in mitochondria can also be a mediator for glucolipotoxicity to primary beta cells. Although our data demonstrate that supplement of TCA cycle intermediates protects against HG/PA-induced toxicity to primary beta cells, further research is needed to establish whether elevated FFA and high concentration of glucose induce beta cell loss in diabetic subjects through metabolic failure in mitochondria. The concentrations of glucose and palmitate utilized in this study are higher than the concentrations observed in normal human plasma. However, the concentrations would be similar to the concentrations experienced in diabetic patients. Even in extreme cases, the postprandial blood glucose concentration can easily be over 25 mM [55] and the level of saturated fatty acid can also be over 0.4 mM in severely diabetic patients [56]. At least, the concentrations we used can be relevant concentrations for explaining beta cell loss in severely diabetic patients.

In conclusion, metabolic failure in mitochondrion is thought to play a critical role in HG/PA-induced cytotoxicity to beta cells. The HG/PA-induced cytotoxicity could be protected by supplement of the TCA cycle intermediates as well as stimulation of FAO. Thus, insufficient capacity of mitochondrial energy metabolism in beta cells may determine the shift from glucolipoadaptation to glucolipotoxicity in diabetic subjects. Nutritional or pharmacological approaches enhancing TCA cycle intermediate pool in mitochondria can be novel targets to protect against beta cell loss characterised by type 2 diabetes.

Acknowledgments

This study was supported by grants from Korea Research Foundation (KRF 2009-0072584) and Innovative Research Institute for Cell Therapy (A062260).

Appendix A. Supplementary data


References
