N-acetyl cysteine attenuates oxidative stress and glutathione-dependent redox imbalance caused by high glucose/high palmitic acid treatment in pancreatic Rin-5F cells

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Abstract

Elevated levels of glucose and fatty acids are the main characteristics of diabetes, obesity and other metabolic disorders, associated with increased oxidative stress, mitochondrial dysfunction and inflammation. Once the primary pathogenesis of diabetes is established, which is potentially linked to both genetic and environmental factors, hyperglycemia and hyperlipidemia exert further destructive and/or toxic effects on β-cells. The concept of glucolipotoxicity has arisen from the combination of deleterious effects of chronic elevation of glucose and fatty acid levels on pancreatic β-cell function and/or survival. Though numerous studies have been conducted in this field, the exact molecular mechanisms and causative factors still need to be established. The aim of the present work was to elucidate the molecular mechanisms of oxidative stress, and inflammatory/antioxidant responses in the presence of high concentrations of glucose/fatty acids on pancreatic β-cell line (Rin-5F) and to study the effects of the antioxidant, N-acetyl cysteine (NAC) on β-cell toxicity. In our study, we investigated the molecular mechanism of cytotoxicity in the presence of high glucose (up to 25 mM) and high palmitic acid (up to 0.3 mM) on Rin-5F cells. Our results suggest that the cellular and molecular mechanisms underlying β-cell toxicity are mediated by increased oxidative stress, imbalance of redox homeostasis, glutathione (GSH) metabolism and alterations in inflammatory responses. Pretreatment with NAC attenuated oxidative stress and alterations in GSH metabolism associated with β-cells cytotoxicity.

Introduction

Glucose and fatty acids are the main sources of energy production and cell survival. However, overload of these nutrients, has been implicated in diabetes and obesity-induced metabolic reprogramming and complications as also in cardiovascular disorders and cancer [1–5].

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However, the specific pathogenesis of these diseases remains unclear. Glucotoxicity and lipo-
toxic caused by chronic hyperglycemia/dyslipidemia have been proposed to play a critical
role in disease development [2, 4, 6, 7, 8]. Persistent hyperglycemia reduces β-cell function and
insulin action by attenuation of insulin-mediated glucose transport and impairment of glu-
cose-induced insulin secretion, which subsequently leads to deterioration of β-cell function. In
addition, excessive exposure to high levels of fatty acids causes β-cell dysfunction, inhibits glu-
cose-induced insulin secretion, and induces β-cell death by apoptosis [9]. The combination of
glucolipotoxicity exacerbates the deleterious effects of chronic elevation of glucose and fatty
acids on pancreatic β-cell function and/or survival [10,11]. Studies have shown that elevated
glucose levels augment the effect of free fatty acid (FFA)-induced cell death, because high glu-
cose concentration inhibits fat oxidation, and consequently lipid detoxification [12]. We have
recently demonstrated that HepG2 cells treated with a high (25 mM) glucose concentration
induces glucotoxicity and metabolic stress, which is further augmented by the treatment of satu-
rated fatty acids [13].

Though numerous studies have been carried out in this field, the exact molecular mecha-
nisms and causative factors involved in glucolipotoxicity is not clearly understood. This is due
to the fact that under in vivo conditions, several physiological, physical, endocrine, dietary and
environmental factors work in tandem. Therefore, our aim in the present study was to eluci-
date the molecular and cellular mechanisms underlying pancreatic β-cell toxicity in the pres-
ence of high glucose/ palmitic acid using an in vitro model of insulin-secreting pancreatic cells,
Rin-5F. The main focus in this study was to investigate the oxidative stress induced, changes in
redox homeostasis, GSH metabolism and inflammatory responses in pancreatic β-cells after
treatment with high levels of glucose and, palmitic acid. Furthermore, we also investigated the
effects of N-acetyl cysteine (NAC), a reactive oxygen species (ROS) scavenger, on the modula-
tion of oxidative stress and inflammation in glucolipotoxicity-induced cells. Our results indi-
cate that NAC pre-treatment selectively restores redox homeostasis, while exerting a marginal
effect on the inflammation induced alterations in these cells.

Materials and methods

Materials

Reduced and oxidized glutathione (GSH/GSSG), 1-chloro 2, 4-dinitrobenzene (CDNB),
cumene hydroperoxide, glutathione reductase, 3-((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-
zolium bromide (MTT), NADH, NADPH, LookOut mycoplasma PCR detection kit, fatty
acid-free bovine serum albumin (BSA), palmitic acid and N-acetyl cysteine (NAC) were pur-
chased from Sigma (St Louis, MO, USA), while 2',7'-dichlorofluorescein diacetate (DCFDA)
was procured from Molecular Probes (Eugene, OR, USA). Kits for nitric oxide (NO) were pur-
chased from R & D Systems (MN, USA) and that for lipid peroxidation (LPO) from Oxis Int,
Inc. (Portland, OR, USA). Kits for GSH/GSSG assays were procured from Promega Corp.
(Madison, WI, USA). Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) kits were
purchased from BD Pharmingen (BD Biosciences, San Jose, USA) and kits for prostaglandin
E2 (PGE2) were purchased from Abcam (Cambridge, UK). Kits for catalase were purchased
from Cayman (MI, USA) while those for superoxide dismutase (SOD) were purchased from
Trevigen (Gaithersburg, MD, USA). Rin-5F cells were obtained from the American Type Cul-
ture Collection (Manassas, VA, USA). The Rin-5F cell line, a commonly used in vitro model
for insulin secreting cells, is a clone derived from the Rin-m rat pancreatic islet cell line. Poly-
clonal antibodies against NF-kBp65 and actin were purchased from Santa Cruz Biotechnology
Inc. (Santa Cruz, CA, USA). Reagents for cell culture, SDS-PAGE and Western blot analyses
were purchased from Gibco BRL (Grand Island, NY, USA) and Bio Rad Laboratories (Richmond, CA, USA).

Cell culture and treatment
Rin-5F cells were cultured in RPMI 1640 with glutaMAX medium supplemented with 10% heat inactivated FBS and 1% non-essential amino acids in a humidified incubator in the presence of 5% CO₂-95% air at 37°C. The cell line was tested for mycoplasma contamination using the LookOut mycoplasma PCR detection kit (Sigma, St Louis, MO, USA) and tested negative (S1 Fig). Palmitic acid was dissolved in 100% ethanol and heated to 40°C for 10 min to make a stock solution of 100 mM and then conjugated to 1% fatty acid-free BSA in a molar ratio of 6:1, according to the method described in previously published reports [14, 15]. The palmitate/BSA conjugate was added to cultured cells in RPMI supplemented with 1% FBS to generate a final concentration of 0.06 mM and 0.3 mM palmitate. For high glucose concentration, similar treatment was carried out in the presence of high glucose (25 mM) media (to mimic the in vivo diabetic condition). To normalize the effect of BSA/ethanol used in palmitic acid treatment, control cells in both normal and high glucose media, were treated with medium containing equivalent amounts of vehicle (BSA/ethanol), in the absence of palmitic acid. For NAC treatment, cells were treated with 10 mM NAC 2 h prior to the palmitic acid treatment. Concentrations of glucose and palmitic acid and time points used in the study, were based on cytotoxicity tests and numerous previously published reports [14,15]. These concentrations were used to mimic the human hyperglycemia/hyperlipidemia under experimental in vitro conditions [14–15]. After the required treatments, harvested cells were washed twice with cold phosphate buffered saline (PBS, pH 7.4) and homogenized in H-medium buffer (70 mM sucrose, 220 mM mannitol, 2.5 mM HEPES, 2 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, pH 7.4) at 4°C, to prepare total cell lysates. Cellular fractionation to prepare nuclear and cytosolic extracts were performed by centrifugation and the purity of isolated fractions for cross contamination was checked as described previously [16]. Bradford method was used to determine protein concentrations of the lysates [17].

MTT cell viability assay
The mitochondria dehydrogenase activities were determined by MTT assay. Briefly, cells were treated with different concentrations of palmitic acid (0.02–0.5 mM) for different time intervals (2–48 h) under normal and high glucose conditions. The cell viability was tested after treatment and assessed by the reduction of MTT dye to form insoluble purple formazan crystals. The crystals were dissolved in acidified alcohol, and the viable cells were quantitated using an ELISA reader (TECAN Infinite M 200 PRO, Austria) at 550 nm after subtracting the appropriate control values.

Measurement of ROS, NO and LPO
Intracellular production of peroxides were measured fluorometrically using the DCFDA-dependent fluorescence method. This compound by itself is not fluorescent, it and is converted by the intracellular esterases to 2,7’-dichlorodihydrofluorescin which is subsequently oxidized by hydrogen peroxide to the highly fluorescent 2,7’-dichlorodihydrofluorescein (DCF) and ROS production was then measured microscopically and fluorometrically as described previously [18]. The measurement of ROS production was also done using FACS analysis. For this, cells were incubated with 5 μM DCFDA for 30 min at 37°C, the cells were then washed with PBS, trypsinized, resuspended in PBS and fluorescence analyzed immediately by flow cytometry as described previously [19]. For the analysis of the flow cytometry data, the untreated cells
were used as the negative control, and the statistical tool in the software was used to calculate the percentage fluorescence in the experimental cells.

For the NO assay, NO production was determined based on nitric oxide synthase (NOS) activity by measuring the concentration of total nitrite in culture supernatants using Griess reagent (R & D Systems Inc.).

The LPO in cell extracts was measured using the LPO-586 kit according to the manufacturer’s recommended protocol and the concentration of malondialdehyde (MDA) calculated from the standard curve using MDA as standard [20].

**Measurements of SOD and catalase activity**

Measurement of catalase was based on its peroxidatic activity, which depends on its ability to catalyze the oxidation of alcohol by hydrogen peroxide. The formaldehyde produced was measured colorimetrically with a chromogen, Purpald, which on oxidation, changes from colorless to purple, and the absorbance read at 540 nm.

The SOD assay was based on the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase. Superoxide ions reduced the nitro blue tetrazolium (NBT, yellow water soluble) to NBT-diformazan (dark-blue water insoluble). SOD activity was measured as the percent inhibition in NBT-diformazan formation, according to the vendor’s protocol (R &D System, MN, USA).

**Measurements of GSH and GSH metabolism**

The GSH/GGSG ratio was measured using the GSH/GGSG-Glo kit as per the vendor’s protocol. The activity of glutathione S-transferase (GST), using CDNB as substrate, was measured as described by Habig et al. [21]. The activity of GSH reductase, using oxidized glutathione (GSSG) as substrate, was measured by a standard protocol by Smith et al. [22]. Glutathione peroxidase (GSH-Px) activity was measured indirectly by a coupled reaction with glutathione reductase using cumene hydroperoxide as a substrate as described previously[23].

**Measurement of cytokines (TNF-α, IL-6 and PGE2)**

TNF-α, IL-6 and PGE2 were measured in culture supernatants using specific ELISA kits. These kits were procured from BD Pharmingen (BD Biosciences, San Jose, USA) for TNF-α and IL-6 assays and from Abcam (Cambridge, UK) for the PGE2 assay. The cytokines were measured as described in the vendor’s protocols [19].

**Measurement of protein expression**

Proteins from cell extracts (30 μg) were resolved by 7.5% SDS-PAGE and electrophoretically transferred on to nitrocellulose membranes by Western Blotting, as described [18,24,25]. The blots were then developed using an ECL Plus Western Blotting Luminal Reagent kit and the bands visualized using the Typhoon FLA 9500 system (GE Healthcare, Uppsala, Sweden). Densitometric analysis was performed using Image J software and expressed as relative ratio normalized against the loading control, β-actin.

**Statistical analysis**

Values shown are expressed as the mean ± S.E.M. of three individual experiments. Statistical significance of the data was assessed using SPSS software (version 23) by analysis of variance (ANOVA) followed by least significant difference (LSD) post-hoc analysis. P values ≤ 0.05 were considered statistically significant.
Results

Effect of high glucose/high palmitic acid treatment on Rin-5F cell viability

Fig 1A shows the inhibition of cell survival in the presence of high glucose/high palmitic acid. Maximum inhibition was observed in cells treated with 0.3–0.5 mM palmitic acid (~ 56% and 74%, respectively). Negligible toxicity was observed using palmitic acid at concentrations below 0.06 mM. However, cells treated with 0.08 mM or 0.1 mM palmitic acid exhibited an inhibition of cell survival by 30% and 36%, respectively. Fig 1B shows the time-dependent inhibition of cell survival in the presence of normal and high glucose. Maximum inhibition of cell viability was observed with 0.3–0.5 mM palmitic acid at 24–48 h. For further studies, we selected 0.06 mM and 0.3 mM palmitic acid concentrations, since 0.5 mM was found to be extremely toxic with low yield of cells.

Effect of high glucose/high palmitic acid treatment on oxidative stress

Treatment with palmitic acid showed a concentration-dependent increase in intracellular ROS production under normal and high glucose conditions. Reactive oxygen species was measured and captured microscopically using DCFDA. Maximum fluorescence was observed with high concentration of palmitic acid (0.3 mM) under both normal as well as high glucose conditions (Fig 2A). Intracellular ROS production was also measured spectrofluorometrically (Fig 2B) and by flow cytometry using the BD FACSDiva software (Fig 2C). The higher concentration of palmitic acid (0.3 mM) showed a marked increase in ROS production (5-fold and 1.5-fold compared with the control of normal and high glucose conditions, respectively).

Nitric oxide production was significantly increased (~ 50%) in Rin-5F cells treated with 0.06 mM palmitic acid under normal and high glucose conditions (Fig 3A). In contrast, 0.3 mM palmitic acid showed a slight reduction in NO production.

In parallel with ROS production, LPO was significantly increased in a concentration-dependent manner after treatment with palmitic acid under normal and high glucose conditions (Fig 3B). Treatment with 0.3 mM palmitic acid under high glucose conditions caused a marked increase (~2-fold) in MDA production.

Effect of high glucose/high palmitic acid treatment on SOD and catalase activity

Superoxide dismutase activity was significantly increased in the presence of high amounts of glucose, which further increased with palmitic acid treatment (Fig 4A). Under normal glucose conditions, 0.06 mM and 0.3 mM palmitic acid caused ~ 45% and 68% increase in SOD activity, respectively. Comparatively, under high glucose conditions, a ~15%-25% increase in SOD activity was observed after palmitic acid treatment. The increase in SOD activity could be due to the increase in superoxide production, which in turn contributed to the increase in ROS production.

Fig 4B shows a concentration-dependent increase in catalase activity under normal and high glucose conditions in the presence of palmitic acid. Furthermore, high glucose significantly increased the catalase activity compared to normal glucose. The increase in catalase activity could clear excess hydrogen peroxide produced by the increase in SOD activity.

Effect of high glucose/high palmitic acid treatment on GSH metabolism

Fig 5A shows the inhibitory effect of palmitic acid on the GSH/GSSG ratio, in a concentration-dependent manner, in the presence of normal and high glucose. The inhibitory effect of palmitic acid was more pronounced in the presence of high glucose.
In parallel, the activity of the GSH reductase was significantly decreased with high concentrations of palmitic acid in the presence of high glucose (Fig 5B), suggesting a reduction in the recycling mechanism, thus causing an increase in oxidized glutathione. Palmitic acid seemed to have no appreciable effects on GSH-reductase activity in normal glucose-treated cells.

As observed in Fig 5C, palmitic acid evidently reduced the activity of GSH-Px under normal and high glucose conditions. The activity of GSH-conjugating enzyme, GST, slightly decreased with high concentrations of palmitic acid in the presence of normal glucose (Fig 5D). These results may suggest the reduced availability of GSH and a compromised detoxification mechanism in Rin-5F cells treated with high glucose/high palmitic acid.
Effects of high glucose/high palmitic acid treatment on the release of inflammatory cytokines in Rin-5F cells

Fig 6 shows the effect of palmitic acid treatment, under normal and high glucose conditions, on TNF-α and IL-6 levels. A significant increase in the levels of TNF-α and IL-6 were observed with 0.06 mM palmitic acid compared with the 0.3 mM treatment (Fig 6A and 6B). A pronounced increase (approximately 3-fold) in TNF-α levels was observed with 0.06 mM palmitic acid compared with 0.3 mM palmitic acid. However, in the presence of high glucose, the levels of TNF-α increased with both concentrations of palmitic acid. Similar effects were observed with IL-6 levels after palmitic acid treatment. A significant increase was observed with 0.06 mM palmitic acid under normal as well as high glucose conditions. On the other hand, 0.3 mM palmitic acid caused a significant increase only in the presence of high glucose.

Palmitic acid treatment also caused a concentration-dependent increase in prostaglandin E2 (PGE2) levels under both normal and high glucose conditions (Fig 6C).
Effect of high glucose/high palmitic acid on NF-kB expression

As shown in Fig 7, a significant reduction in the expression of cytosolic NF-kB was observed in palmitic acid treated cells suggesting an increased translocation of NF-kB from the cytosol in glucolipotoxicity.

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Effect of NAC pre-treatment on oxidative stress in high glucose/high palmitic acid-treated cells

Fig 8 shows the effect of high glucose/high palmitic acid treatment with/without NAC, (10 mM for 2 h), on ROS production in Rin-5F cells. As observed previously, immunofluorescent microscopic, fluorometric and flow cytometric studies showed a significant increase in ROS production with increasing concentrations of palmitic acid, which was significantly reduced (15–25%) in the presence of NAC (Fig 8A, 8B and 8C).
As shown in Fig 9A, NAC pre-treatment caused a 20%-25% reduction in NO production with both 0.06 mM and 0.3 mM palmitic acid in the presence of high glucose. Similarly, NAC treatment also caused a significant reduction in LPO, caused by palmitic acid treatment in the presence of high glucose (Fig 9B).

Effect of NAC pre-treatment on SOD and catalase enzyme activities in high glucose/high palmitic acid-treated cells

Pre-treatment with NAC significantly reduced (> 50%) SOD activity, which was increased with 0.3 mM palmitic acid treatment in the presence of high glucose. However, little effect was observed with 0.06 mM palmitic acid (Fig 10A).

Pre-treatment with NAC also caused a mild decrease in catalase activity, which increased with palmitic acid in the presence of high glucose (Fig 10B). These results suggest that the cells were under reduced oxidative stress in the presence of NAC, and a reduction in the concentration of ROS metabolizing enzymes was observed due to reduced ROS production in NAC-treated cells.

Effect of NAC pre-treatment on GSH levels in high glucose/high palmitic acid-treated cells

As shown in Fig 11, a significant reduction in GSH/GSSG ratio was observed after high glucose/high palmitic acid treatment. Pre-treatment with NAC markedly enhanced the GSH/GSSG ratio by 20% and 50% after 0.06 mM and 0.3 mM palmitic treatment, respectively.
Effect of NAC pre-treatment on TNF-α release in high glucose/high palmitic acid-treated cells

As shown in Fig 12, TNF-α significantly increased after treatment with 0.06 mM palmitic acid in the presence of high glucose. However, no significant alterations in TNF-α production were observed after NAC pre-treatment.

Discussion

The concept of glucolipotoxicity has arisen from the combination of deleterious effects of chronic elevation of glucose and free fatty acid levels on pancreatic β-cell function and/or survival [10,11]. Studies have also shown that elevated glucose levels augment the effect of FFA-induced cell death [12]. The precise mechanism of glucolipotoxicity in pancreatic β-cells is still not clear owing to numerous inconsistent reports. Moreover, it is not clearly understood how pancreatic cells adapt to excess fuel and the subsequent fate of cells under such conditions. Nutrient overload induces inflammatory responses causing β-cell mass destruction affecting pancreatic β-cell neogenesis, proliferation and function. Therefore, our aim was to elucidate the cytotoxic mechanisms of high glucose/high palmitic acid treatment in insulin-secreting Rin-5F cells with the main focus on oxidative stress, redox metabolism and inflammatory...
Furthermore, we also investigated the cytoprotective effects of NAC, a glutathione precursor antioxidant with known ROS scavenging properties, on toxicity induced by high glucose/high palmitic acid in Rin-5F cells.

Pancreatic Rin-5F cells were treated with high glucose (25 mM) and high saturated fatty acid, palmitic acid (up to 0.3 mM). These concentrations were based on previous literature and our preliminary assays on cell viability/survival. The human physiological range for plasma glucose is 5.5–6 mmol/L with a maximum of ~ 9 mmol/L postprandially [26,27]. Similarly, the physiological range of plasma fatty acids is 0.3 mmol/L to 4 mmol/L [28]. Palmitic acid was selected since it is the most abundant saturated fatty acid [29] and a precursor of several other fatty acids in vivo, and has been reported to exert more effects in dyslipidemia when compared to other saturated fatty acids. Moreover, it has been demonstrated clinically, that the level of palmitate in plasma increases 1.5- and 3-fold in type-2 diabetic patients during nocturnal and postprandial states, respectively, compared with a healthy patient [30].

Our results, consistent with other studies [13,31,32], showed that exposure of Rin-5F cells to palmitic acid increased oxidative stress in a concentration-dependent manner under high glucose conditions. NAC attenuates glucolipotoxicity in β-cells.

**Fig 7. High glucose/high palmitic acid treatment-induced alterations in the expression of NF-κB.** Cytosolic extracts (30 μg protein) from treated cells were separated on 7.5% SDS-PAGE and transferred on to nitrocellulose paper by Western blotting. NF-κBp65 protein was detected using a specific polyclonal antibody. The quantitation of the protein is expressed as relative ratios normalized against the loading control, actin. The figures are representative of three experiments. Asterisks indicate significant difference (*p ≤ 0.05, **p ≤ 0.01) relative to untreated control cells under normal glucose conditions, (Δp ≤ 0.05, ΔΔp ≤ 0.01) relative to untreated control cells under high glucose conditions.

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This finding is confirmed by an increase in ROS/reactive nitrogen species (RNS) production, increase in lipid peroxidation, inhibition in GSH/GSSG ratio and alterations in GSH metabolism, redox homeostasis and inflammatory response.

The decrease in the activities of GSH-reductase and GSH-Px in the presence of high palmitic acid with normal/high glucose could have triggered the activation of antioxidant enzymes, SOD and catalase. This could indicate the adaptive response of cells to glucose/palmitic acid toxicity. The competency of cells to resist oxidative damage is determined by the effect of a battery of physiological antioxidants, among which GSH is the most abundant. Antioxidant deficiencies could develop as a consequence of either decreased synthesis or increased utilization [33]. Both cases can be explained by a decline in the reduced-to-oxidized GSH ratio. In our study, we observed a significant inhibition of both the recycling enzyme, GSH-reductase, as well as the detoxifying and scavenging enzyme, GSH-Px. It has been well established experimentally and clinically that GSH level is decreased in diabetes. Moreover, GSH deficiency has substantial implications in the pathogenesis of diabetic complications [34–36].

A clinical study has also shown that dietary supplementation of GSH precursors (glycine and...
glutamine) can restore GSH synthesis and lower the oxidative stress in uncontrolled diabetic patients [34]. Similarly, we observed a significant increase in the GSH/GSSG ratio in high glucose/high palmitic acid-treated Rin-5F cells that have been pre-treated with 10 mM NAC. This resulted in a reduction of oxidative stress in the cells, as observed by a decrease in ROS/RNS production, decreased LPO, and a decrease in antioxidant activity (SOD). This may explain...
the consequential equilibrium of the redox status provided by NAC that does not require the effects of multiple antioxidant enzymes.

Oxidative stress and inflammation act as cooperative and synergistic partners in the pathophysiology of numerous diseases such as diabetes, obesity, cardiovascular diseases, neurological disorders and cancer. Studies have shown that palmitic acid triggers the production of proinflammatory cytokines and oxidants, leading to cellular hypertrophy and apoptosis [37,38]. Fatty acids can directly activate inflammatory pathways themselves, to potentiate inflammatory toxicity. In our study, we observed a decreased expression of cytosolic NF-kB, with

Fig 10. Effect of NAC pre-treatment on SOD and catalase activities in high glucose/high palmitic acid-treated Rin-5F cells. Rin-5F cells were grown to about 80% confluence and treated with different concentrations of palmitic acid with/without NAC. SOD (A) was measured as the percentage conversion of NBT to NBT-diformazan according to the vendor’s protocol. Catalase measurement (B) was dependent on its ability to catalyze the oxidation of alcohol by hydrogen peroxide, and the produced formaldehyde was measured colorimetrically. Results are expressed as the mean +/- S.E.M. of three experiments. Asterisks indicate significant difference (\*p \leq 0.05) relative to untreated control cells under normal glucose conditions, (\*\*p \leq 0.01) relative to untreated control cells under high glucose condition, (\(\triangle\)p \leq 0.05, \(\triangle\triangle\)p \leq 0.01) relative to 0.3 mM palmitic acid in the presence of high glucose.

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Fig 11. Effect of NAC pre-treatment on GSH levels in high glucose/high palmitic acid-treated Rin-5F cells. GSH/GSSG ratio was measured using a GSH/GSSG-Glo kit as described in Materials and Methods. Results are expressed as the mean +/- S.E.M. of three experiments. Asterisks indicate significant differences (*p ≤ 0.05) relative to untreated control cells under normal glucose conditions, (ΔΔp ≤ 0.01, ΔΔΔp ≤ 0.001) relative to untreated control cells under high glucose conditions, (p ≤ 0.05) relative to 0.06 mM palmitic acid in the presence of high glucose, (○○p ≤ 0.01) relative to 0.3 mM palmitic acid in the presence of high glucose.

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Fig 12. Effect of NAC pre-treatment on the release of TNF-α in high glucose/high palmitic acid-treated Rin-5F cells. TNF-α was measured using standard ELISA kit as described earlier in Materials and Methods. Results are expressed as the mean +/- S.E.M. of three experiments. Triangles indicate significant difference (ΔΔΔp ≤ 0.001) relative to untreated control cells under high glucose conditions.

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increasing palmitic acid concentrations, suggesting an increased translocation into the nucleus, which in turn, triggered the release of pro-inflammatory cytokines such as TNF-α, IL-6 and PGE2.

Additionally, there has been a growing interest in studying the therapeutic effects of N-acetyl cysteine (NAC) in the prevention of diseases characterized by increased oxidative stress, such as diabetes [39]. Therefore, we extended our study to elucidate the effects of NAC pre-treatment under high glucose/high palmitic acid conditions. Our results showed that NAC attenuated the high glucose/high palmitic acid-induced ROS/RNS production and lipid peroxidation and decreased the activities of SOD and catalase. The GSH levels were significantly recovered with NAC pre-treatment. However, NAC pre-treatment did not restore the inflammatory response in pancreatic cells under glucolipotoxicity conditions.

In summary, our results suggest that high glucose/palmitic acid toxicity is partly mediated by increased ROS/RNS production, oxidative stress and inflammatory responses accompanied by alteration of GSH metabolism and an imbalance of redox homeostasis. The NAC treatment, however, has restored most of the redox homeostasis in these cells induced by glucolipotoxicity.

Supporting information

S1 Fig. Checking for mycoplasma. Contamination in the cell-line used was performed using the LookOut mycoplasma detection kit which utilizes the polymerase chain reaction, as described in the vendor’s protocol.

(PDF)

S2 Fig. Original Western blot images. Proteins from cell extracts (30 μg) were resolved by 7.5% SDS-PAGE and electrophoretically transferred on to nitrocellulose membranes by Western Blotting. The blots were then developed using an ECL Plus Western Blotting Luminol Reagent kit and the bands visualized using the Typhoon FLA 9500 system (GE Healthcare, Uppsala, Sweden). Actin was used as the loading control. The original blot images after blotting with NF-kB and actin are shown.

(PDF)

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