Original Paper

Cytoprotective Effects of N-Acetylcysteine on Streptozotocin- Induced Oxidative Stress and Apoptosis in RIN-5F Pancreatic β -Cells

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Key Words

Streptozotocin • Pancreatic Rin-5F cells • Oxidative stress • Apoptosis • N-acetylcysteine

Abstract

Background/Aims: Numerous studies have reported overproduction of reactive oxygen species (ROS) and alterations in mitochondrial energy metabolism in the development of diabetes and its complications. The potential protective effects of N-acetylcysteine (NAC) in diabetes have been reported in many therapeutic studies. NAC has been shown to reduce oxidative stress and enhance redox potential in tissues protecting them against oxidative stress associated complications in diabetes. In the current study, we aimed to investigate the molecular mechanisms of the protective action of NAC on STZ-induced toxicity in insulin secreting Rin-5F pancreatic β -cells. **Methods:** Rin-5F cells were grown to 80% confluence and then treated with 10mM STZ for 24h in the presence or absence of 10mM NAC. After sub-cellular fractionation, oxidative stress, GSH-dependent metabolism and mitochondrial respiratory functions were studied using spectrophotometric, flow cytometric and Western blotting techniques. **Results:** Our results showed that STZ-induced oxidative stress and apoptosis caused inhibition in insulin secretion while NAC treatment restored the redox homeostasis, enhanced insulin secretion in control cells and prevented apoptosis in STZ-treated cells. Moreover, NAC attenuated the inhibition of mitochondrial functions induced by STZ through partial recovery of the mitochondrial enzymes and restoration of membrane potential. STZ-induced DNA damage and expression of apoptotic proteins were significantly inhibited in NAC-treated cells. **Conclusion:** Our results suggest that the cytoprotective action of NAC is mediated via suppression of oxidative stress and apoptosis and restoration of GSH homeostasis and mitochondrial bioenergetics. This study may, thus, help in better understanding the cellular defense mechanisms of pancreatic β -cells against STZ-induced cytotoxicity.

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Introduction

Diabetes mellitus (DM) is a complex metabolic disorder characterized by elevated levels of blood glucose (hyperglycemia), impairment of insulin secretion and insulin resistance [1]. The WHO projects that diabetes will be the seventh leading cause of death in 2030 [2]. In 2015, 415 million cases of diabetes were reported worldwide, one in 11 adults had diabetes and one in two adults with diabetes went undiagnosed [3]. In the same year, 5 million deaths were reported; one person died every 6 seconds from diabetes. The fact that the number of diabetic patients has doubled over the past decades has made this disease a global challenge.

Hyperglycemia, the hall mark of diabetes, induces oxidative stress as a result of over production of reactive oxygen species (ROS) and alterations in the mitochondrial energy metabolism play a key role in the pathogenesis of β -cell dysfunction and development of diabetic complications [4, 5]. The different sources responsible for ROS overproduction and oxidative stress in diabetes are activation of NADPH oxidase, uncoupling of NO synthases, stimulation of xanthine oxidase and glycated proteins [6–8]. Several studies, including our own, have suggested that pancreatic beta cells are extremely sensitive to oxidative stress due to relatively low levels of antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase [9–11]. Studies have shown also that prolonged exposure to elevated levels of glucose down-regulates the rate limiting enzyme for glutathione synthesis, γ -glutamylcysteine synthetase (γ -GCS) [12]. Therefore, enhancing the antioxidant mechanisms could help in attenuating the oxidative stress induced by hyperglycemia. Studies have shown that overexpression of superoxide dismutase in transgenic mice diminished the oxidative stress induced by diabetes in β -cells [13]. Also, catalase overexpression in transgenic mouse have shown to produce remarkable protection against hydrogen peroxide produced by oxidative stress and significantly reduce the diabetogenic effect of streptozocin [14]. Additionally, studies have shown a strong correlation between the depletion of GSH and development of diabetic complications [15]. GSH is the most important intracellular antioxidant. The changes in GSH level influence the redox status in cells and induce cellular damage, as a consequence of oxidant/pro-oxidant imbalance.

N-Acetylcysteine (NAC) is an acetylated cysteine residue and a precursor of reduced glutathione (GSH). It is a sulfhydryl donor which regenerates GSH, stimulates the activity of glutathione-S-transferase and acts as a scavenger through its direct interaction with ROS [16]. NAC is a membrane permeable cysteine precursor that does not require active transport and once inside the cell, it is rapidly hydrolyzed to release cysteine residue [17]. The synthesis of GSH is limited by the availability of cysteine [18]. Clinically, NAC is used as a mucolytic agent in patients with cystic fibrosis [19], as an antidote in the treatment of acetaminophen overdose [20] and also used in the prevention of non-alcoholic steatohepatitis (NASH), characterized by decreased GSH and increased oxidative stress [21]. NAC is considered a better source of glutathione than glutathione itself, due to the low oral absorption of the latter. Several studies have reported the potential protective effects of NAC against diabetes, in reducing hyperglycemia and inhibiting ROS production. Experimental studies have reported that NAC reduced hyperglycemia and inhibited alloxan induced NF- κ B activation in mice [22]. Studies have shown also that NAC prevented the decrease in insulin content and mRNA expression [23, 24]. NAC also has a beneficial role on diabetic complications. Studies have shown that NAC inhibited the development of the peripheral neuropathy in streptozotocin-induced diabetic rats [25] and protects from diabetic cardiomyopathy, through inhibiting the ROS production and fibrosis [26].

We have previously investigated the cytotoxicity mechanisms of the STZ, a known pancreatic β -cell specific cytotoxin, in Rin-5F pancreatic cells [27]. Our results showed that STZ increased the ROS/RNS production, lipid peroxidation, expression of oxidative stress marker proteins and inhibited the GSH level and caused alterations in GSH metabolism. Moreover, STZ treatment caused a significant inhibition in the activities of the mitochondrial respiratory enzymes and ATP production. We have further extended this study to elucidate

the protective mechanisms of a well-known GSH precursor anti-oxidant and ROS scavenger, NAC, on the STZ-induced toxicity in these cells.

Materials and Methods

Materials

Streptozotocin (STZ), NAC, reduced and oxidized glutathione (GSH/GSSG), cumene hydroperoxide, glutathione reductase, 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT), NADH, NADPH, reduced cytochrome c, coenzyme Q2, antimycin A, sodium dodecyl maltoside and kits for hexokinase were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-Dichlorofluorescein diacetate (DCFDA) was procured from Molecular Probes (Eugene, OR, USA). Kits for nitric oxide and mitochondrial membrane potential were purchased from R & D System (Minneapolis, MN, USA) and that for lipid peroxidation (LPO) and aconitase from Oxis Int, Inc. (Burlingame, CA, USA). Kits for GSH/GSSG assay were procured from Promega Corp. (Madison, WI, U.S.A). Apoptosis detection kits for flow cytometry were purchased from BD Biosciences (San Jose, CA, USA). Kits for glutamate dehydrogenase was purchased from Abcam (Cambridge Science Park, Cambridge, UK). Kits for insulin was purchased from Cisbio (Parc Marcel Boiteux, Codolet, France). Rin-5F cells were obtained from American Type Culture Collection (Manassas, VA, USA). Polyclonal antibodies against beta-actin, caspase-3, caspase-9, PARP, iNOS, Bcl-2 and Bax 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 cell line is an insulin-secreting pancreatic cell line clone derived from the Rin-m rat islet cells, and has been widely used for toxicological and pharmacological studies. It has been identified as a continuous cell line, which retains many of the features of pancreatic beta-cells, including insulin secretion and sensitivity to oxidative stress. Rin-5F cells were grown in poly-L-lysine coated 75 cm² flasks (~2.0–2.5 × 10⁶ cells/mL) in RPMI1640 medium supplemented with 1% nonessential amino acids, 2mM glutamine, 10% heat inactivated fetal bovine serum in a humidified incubator in the presence of 5% CO₂-95% air at 37°C. Cells were cultured to 80% confluence and then treated with 10mM STZ for 24h in the presence or absence of 10 mM NAC, 2h prior to STZ treatment. Control cells were treated with vehicle (media containing citrate buffer) alone. Cells were harvested after treatment, washed with PBS (pH 7.4) and homogenized in H-medium buffer (70mM sucrose, 220mM mannitol, 2.5mM HEPES, 2mM EDTA, and 0.1mM phenylmethylsulfonylfluoride, pH 7.4) at 4 °C. Mitochondrial and post-mitochondrial fractions were then isolated by differential centrifugation and used for further analysis [28]. Protein concentration was determined by Bradford method [29].

MTT Cell Viability Test

The MTT cell viability test was assessed after reaction of cells with STZ (24h) and/or NAC (2h) based on the metabolic activity of mitochondrial dehydrogenases, which reduces tetrazolium dye MTT to form insoluble purple formazan crystals. The viable cells were quantitated using an ELISA reader (TECAN Infinite M 200 PRO, Austria) at 570 nm.

Measurement of apoptosis

The apoptosis assay using flow cytometry was performed according to the vendor's protocol (BD Biosciences, San Jose, CA, USA) as described before [30]. Briefly, control untreated and STZ-treated cells were trypsinized, washed in PBS and resuspended (1 × 10⁶ cells/ml) in binding buffer (10mM HEPES, pH 7.4, 140mM NaCl, 2.5mM CaCl₂). A fraction (100 µl/1 × 10⁵ cells) of the cell suspension was incubated with 5µl Annexin V conjugated to FITC and 5µl propidium iodide (PI) for 15min at 25°C in the dark. Binding buffer (400µl) was added to the suspension and apoptosis was measured immediately using a Becton Dickinson FACSscan analyzer. The apoptotic cells were estimated by the percentage of cells that stained positive for Annexin V-FITC while remaining impermeable to PI (AV+/PI-). This method was also able to distinguish viable cells (AV-/PI-) and cells undergoing necrosis (AV+/PI+).

Measurement of Reactive Oxygen Species (ROS), NO and LPO

Cells were cultured in 6 wells plate ($\sim 2 \times 10^5$ cells/well) and treated with STZ and/ or NAC for 24h. The intracellular production of ROS was measured by FACS analysis using DCFDA. For this, cells were incubated with 5 μ M DCFDA for 30min at 37°C. Cells were washed twice with PBS, trypsinized, resuspended in 3ml PBS and fluorescence immediately analyzed by flow cytometry as described before [31].

For NO assay, NO production was determined by measuring the concentration of total nitrite in the culture supernatants using Griess reagent (R & D Systems Inc.) as described before [32].

LPO in the cell extracts of treated and control cells were measured using the LPO-586 kit according to the manufacturer's recommended protocol and the concentration of MDA calculated from the standard curve as described before [33].

Measurement of GSH Metabolism

GSH/GSSG ratios and activities of GSH-Px, and GSH reductase were measured in treated and untreated control cell extracts. GSH/GSSG ratios were measured using the GSH/GSSG-Glo kit as per the vendor's protocol. Briefly, treated and untreated control cells were lysed with either total or oxidized glutathione reagent. For the oxidized glutathione measurement, the total GSH was blocked using NEM reagent and the oxidized glutathione was reduced. The total reduced glutathione then converts a specific probe, Luciferin-NT to luciferin in the presence of a GST enzyme coupled to firefly luciferase. The luciferin formed gives a luminescent signal, which is proportional to the amount of GSH. The total and oxidized glutathione are then measured from the standard curve and the GSH/GSSG ratios calculated. GSH-Px activity using cumene hydroperoxide [34] and GSH-reductase using oxidized glutathione (GSSG) [35] as substrates were measured by standard protocols as described before [31, 33, 36, 37].

Measurement of Mitochondrial Bioenergetics

Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$). The mitochondrial membrane potential ($\Delta\psi_m$) was measured by flow cytometry using a fluorescent cationic dye according to the vendor's protocol (DePsipher™, R & D System Inc.) as described before [30]. DePsipher has the property of aggregating upon membrane polarization forming an orange-red fluorescent (absorption/emission 585/590nm) compound. If the membrane potential is reduced, the dye cannot access the transmembrane space and remains in its green fluorescent (510/527nm) monomeric form.

Measurement of Mitochondrial enzyme activities. Measurement of mitochondrial respiratory enzyme complexes was done by suspension of the cell extracts (5 μ g protein), from STZ and/or NAC treated and untreated control Rin-5F cells in 1.0ml of 20mM KPi buffer, pH 7.4, in the presence of the detergent, lauryl maltoside (0.2%). NADH ubiquinone oxidoreductase (Complex I) and cytochrome c oxidase (Complex IV) were measured using the substrates coenzyme Q2 and reduced cytochrome c, respectively by the methods of Birch-Machin and Turnbull [38] as described before [37].

Mitochondrial aconitase activity was measured by the NADPH coupled conversion of citrate to isocitrate in the presence of isocitrate dehydrogenase using the Bioxytech Aconitase-340 assay kit. Aconitase activity was expressed as the rate of formation of NADPH determined at 340 nm.

Measurement of glutamate dehydrogenase and hexokinase activities

Glutamate dehydrogenase (GDH) activity was measured using the GDH kit (Abcam, Cambridge Science Park, Cambridge, UK) as per the vendor's protocol. Briefly, it is a coupled enzyme assay in which mitochondria from treated cells were reacted with buffer containing glutamate as a specific substrate, resulting in the generation of NADH, which reacts with a probe generating a colorimetric (450nm) product proportional to the GDH activity. One unit of GDH is the amount of enzyme that will generate 1.0 μ mole of NADH per minute at pH 7.6 at 37°C.

Hexokinase activity was measured using the HK assay kit (Sigma-Aldrich, St.Louis MO, USA) as per the manufacturer's instructions. Briefly, it is a coupled enzyme assay in which glucose is converted to glucose-6-phosphate by HK, which in turn is oxidized by glucose-6-phosphate dehydrogenase to form NADH. The resulting NADH reduces a colorless probe to a colored product which is read at 450 nm.

Measurement of insulin level

Insulin levels were measured using insulin assay kit (Cisbio, Codolet, France) as per the vendor's instructions. Briefly, it involves Fluorescence Resonance Energy Transfer (FRET) using two different anti-insulin antibodies, one labelled with europium cryptate, and the other with XL665. The FRET phenomenon occurs when a sandwich is formed when insulin molecule and the two antibodies come into close proximity and is proportional to the concentration of insulin.

SDS-PAGE and Western blot analysis

Proteins from cell extracts (30µg) from control and treated cells were separated electrophoretically on 12% SDS-PAGE [39] and transferred on to nitrocellulose paper by Western blotting [40]. Transferred proteins were probed with primary antibodies against iNOS, caspase-3, caspase-9, PARP, Bax and Bcl-2. Immunoreactive bands were visualized using the appropriate conjugated secondary antibodies. Equal loading of protein was confirmed using beta-actin as the loading control. After development of the blots, the bands were visualized and further densitometric analysis performed using the Typhoon FLA 9500 system (GE Healthcare, Uppsala, Sweden) and expressed as relative ratios normalized against actin or other proteins as appropriate.

Statistical analysis

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

Results

Effect of NAC and STZ on Rin-5F cell viability

Cell survival measured by the mitochondrial enzyme based MTT assay, showed that STZ-treatment caused about 40% reduction in cell viability (Fig. 1). NAC pretreatment caused a significant recovery in cell survival in STZ-treated cells.

Effect of NAC on STZ-induced apoptosis

Rin-5F cells were treated with STZ with/without NAC and apoptosis was measured by FACS analysis. As shown in Fig. 2, a significant increase in the percentage of cells undergoing early/late apoptosis ($\sim$ 25%) was observed with 10mM STZ treatment for 24h compared to control untreated cells ($\sim$ 3-5%). NAC pretreatment resulted in a remarkable reduction in apoptotic cell death in STZ-treated cells suggesting the protection of cell death by NAC.

Effect of NAC on STZ-induced oxidative stress

Rin-5F cells were treated with STZ and/or NAC and assays were performed to measure the oxidative stress. A significant increase in intracellular ROS production (Fig. 3A), NO production (Fig. 3B) and lipid peroxidation as measured by malondialdehyde (MDA) levels (Fig. 3C) was observed after STZ treatment. NAC pretreatment, on the other hand, caused a remarkable reduction in ROS production and lipid peroxidation. However, NO production was only marginally affected after NAC treatment.

Fig. 1. Cell viability assay by MTT. Rin-5F cells ($\sim 2 \times 10^4$) were grown in 96-well plates for 24h and treated with STZ with/without NAC. The formazan crystals formed, following the reduction of MTT by metabolically active (viable) cells, were solubilized in acidified isopropanol and quantitated using the ELISA reader at 570 nm. Results are expressed as mean $\pm$ SEM for three experiments. Asterisks indicate significant difference (**p $\leq$ 0.005) relative to the untreated control cells and triangle (Δ p $\leq$ 0.05) indicate significant difference from STZ-treated cells.

Fig. 2. Effect of NAC on apoptosis. Apoptosis was measured in Rin-5F cells treated with STZ with/without NAC as described before in the Materials and Methods by flow cytometry using FACSDiva software. Representative dot plots are shown and % age of apoptotic cells is represented as a histogram. Results are expressed as mean $\pm$ SEM of at least three experiments. Asterisks indicate significant difference (**p $\leq$ 0.005) from control untreated cells and triangle ($\Delta\Delta$ p $\leq$ 0.005) indicate significant difference from STZ-treated cells.

Effect of NAC on GSH metabolism in STZ treated cells

Since NAC is a glutathione precursor, we investigated the effects of STZ/NAC on GSH level by measuring the ratio of reduced to oxidized GSH, as well as on the enzymes involved in regulating the GSH-metabolism. A significant reduction in GSH/GSSG ratio (70%) was observed after STZ treatment in Rin-5F cells (Fig. 4A). NAC pretreatment caused a moderate recovery in the GSH levels. The activity of GSH-Px was significantly increased in STZ-treated cells (Fig. 4B). This could be due to the increased oxidative stress and increase in peroxide levels. NAC pretreatment, however, caused a significant reduction in the enzyme activity. On the other hand, the activity of GSH-reductase, which catalyzes the reduction of the oxidized glutathione, was significantly reduced in STZ-treated cells, explaining the low ratio of GSH/GSSG (Fig. 4C). However, pretreatment with NAC showed a significant recovery of the activity of the enzyme.

Fig. 3. Effect of NAC on ROS, NO and LPO. Rin-5F cells were cultured to 80% confluence and treated with STZ with/without NAC as described in the Materials and Methods. ROS production was measured in cell lysate using DCFDA and fluorescence was measured by flow cytometry using FACSDiva software as described before [30]. Histogram represents percent change in ROS production (3A). NO production was determined by measuring the concentration of total nitrite in the culture supernatants (3B) with Griess reagent (R & D Systems Inc.). Lipid peroxidation (LPO) was measured as total amount of malondialdehyde (3C) as per the vendor's protocol (Oxis Research, Inc.) Results are expressed as mean $\pm$ SEM of at least three experiments. Asterisks indicate significant difference (** $p \leq 0.005$) from control untreated cells and triangle ($\Delta\Delta p \leq 0.005$) indicate significant difference from STZ-treated cells.

Effect of NAC on mitochondrial bioenergetics

Effect of NAC on mitochondrial membrane potential (MMP). The role of MMP is very important in determining the fate of cells undergoing oxidative stress and mitochondrial dysfunction. Disturbances in the membrane potential results in the opening of the mitochondrial pores, thus releasing cytochrome C into the cytosol, which subsequently triggers the downstream apoptotic cascade. Our results showed a significant loss in the

Fig. 4. Effect of NAC on GSH metabolism. GSH/GSSG ratio (4A), GSH-Px (4B) and GSH reductase (4C) were measured as described before in the Materials and Methods. Results are expressed as mean $\pm$ SEM of three experiments. Asterisks indicate significant difference (** $p \leq 0.005$) from control untreated cells and triangle ($\Delta p \leq 0.05$, $\Delta\Delta p \leq 0.005$) indicate significant difference from STZ-treated cells.

Fig. 5. Effect of NAC on mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\psi_m$) was measured by flow cytometry using a fluorescent cationic dye according to the vendor's protocol (DePisiphe™, R & D System Inc.). A typical histogram representing the % age loss of mitochondrial membrane potential is shown. Results are expressed as mean $\pm$ SEM of three experiments. Asterisks indicate significant difference (* $p \leq 0.05$, ** $p \leq 0.005$) from control untreated cells and triangle ($\Delta\Delta p \leq 0.005$) indicate significant difference from STZ-treated cells.

Fig. 6. Effect of NAC on mitochondrial enzyme activity. Activities of mitochondrial respiratory enzyme complexes, (Complex I (6A), Complex IV (6B)) and the matrix enzyme, aconitase (6C) were measured using their respective substrates as described in the Materials and Methods. Results are expressed as mean $\pm$ SEM of three experiments. Asterisks indicate significant difference (** $p \leq 0.005$) from control untreated cells and triangle ($\Delta\Delta p \leq 0.005$) indicate significant difference from STZ-treated cells.

Fig. 7. Effects of NAC on glutamate dehydrogenase and hexokinase activities. Glutamate dehydrogenase (7A) and hexokinase (7B) enzyme activities were measured by a coupled enzyme assay method as mentioned in the Materials and Methods. Results are expressed as mean $\pm$ SEM of three experiments. Asterisks indicate significant difference (** $p \leq 0.005$) from control untreated cells.

membrane potential after treatment with STZ in Rin-5F cells (Fig. 5). NAC pretreatment however, showed a significant recovery of the MMP in STZ treated cells.

Effect of NAC on mitochondrial enzyme activity. The activities of the mitochondrial respiratory complexes as well as the Krebs' cycle enzyme, aconitase are sensitive indicators of mitochondrial bioenergetics and oxidative stress. As shown in Fig. 6 (A, B), STZ treatment caused a significant reduction in the activities of Complex I and Complex IV. NAC pretreatment, however, showed only a marginal recovery in the activity of Complex IV but not in the activity of Complex I. NAC appeared to exhibit some inhibitory effect on the Complex

I activity presumably affecting the substrate (e.g. NADH) availability or the glutathionylation of this enzyme resulting in the alteration of the enzyme activity. Similarly, a marked inhibition (90%) was also observed in the activity of aconitase, a ROS sensitive mitochondrial matrix enzyme, after STZ treatment (Fig. 6C). NAC pretreatment caused a significant recovery in the activity. Also, NAC treatment alone showed a significant activation of this enzyme. This may be due to the scavenging of endogenous ROS production by NAC.

Effect of NAC on glutamate dehydrogenase and hexokinase activities

Glutamate dehydrogenase is a mitochondrial matrix enzyme, which plays a key role in nitrogen and glutamate metabolism and energy homeostasis. Fig. 7A shows a significant inhibition in the activity of glutamate dehydrogenase after STZ treatment. However, pretreatment with NAC showed only a marginal recovery. Similarly, the activity of another key enzyme, hexokinase, required for glucose metabolism and hormonal regulation, was also significantly inhibited (~60%) after STZ treatment (Fig. 7B). NAC treatment, however, didn't help in recovering the activity back to normal control level.

Effect of NAC treatment on insulin level

Assessment of insulin level and its secretion has been a useful guide to determine the therapeutic efficacy and management in diabetic patients. In our study, we observed a significant decrease in the level of insulin after STZ treatment (Fig. 8). NAC pretreatment however did not restore the insulin level in STZ treated cells while a moderate increase in insulin level was observed in control cells treated with NAC alone.

Effect of NAC on the expression of oxidative stress and apoptotic protein markers

In cells, the increased oxidative stress also triggers a cascade of signaling events, including cleavage of caspases leading to apoptosis. As shown in Fig. 9A, STZ treatment increased the cleavage of molecular oxidative stress and apoptotic marker proteins such as PARP, iNOS, caspase-3 and caspase-9. NAC pretreatment showed a moderate to significant reduction in the cleavage of PARP, caspase-3 and caspase-9 demonstrating protection against the oxidative stress induced by STZ. On the other hand, no significant effect on increased iNOS expression was observed after NAC treatment. This also confirmed the effect of NAC on the NO level measured in STZ-treated cells (Fig. 3B). Treatment with STZ also showed increase in the expression of the pro-apoptotic protein, Bax, and decrease in the expression of anti-apoptotic protein, Bcl-2, (Fig. 9B). NAC pretreatment again significantly decreased the expression ratio of the Bax/Bcl-2 reconfirming its protective effects against STZ-induced toxicity.

Fig. 8. Effect of NAC on insulin level. Insulin levels were measured using the Fluorescence Resonance Energy Transfer (FRET) method by using two different anti-insulin antibodies as mentioned in the Materials and Methods. Results are expressed as mean $\pm$ SEM of three experiments. Asterisks indicate significant difference (** $p \leq 0.005$) from control untreated cells.

Fig. 9. Expression of apoptotic protein markers. Total extracts (30 μ g protein) from control and STZ-treated cells were separated on 12% SDS-PAGE and transferred on to nitrocellulose paper by Western blotting. iNOS, PARP, caspase-3, caspase-9 (9A) Bax, and Bcl-2 (9B), proteins were detected using specific antibodies against these proteins. Beta-actin was used as loading control. The quantitation of the protein bands are expressed as relative ratios normalized against actin or other specific proteins as appropriate. The figures are representative of three experiments. Asterisks indicate significant difference (** $p \leq 0.005$) from control untreated cells and triangle ($\Delta p \leq 0.05$, $\Delta\Delta p \leq 0.005$) indicate significant difference from STZ-treated cells.

Discussion

Experimental and clinical studies suggest the involvement of oxidative stress and mitochondrial dysfunction in the development and progression of diabetes and its complications [4, 41–43]. Pancreatic β -cells are extremely sensitive to oxidative stress due to excessive production of ROS/RNS and relatively low level of antioxidant enzymes, particularly, associated with GSH metabolism. The therapeutic potential of anti-oxidants in preventing diabetes and its complications have been suggested in numerous studies, including our own.

GSH is a master regulator of redox homeostasis, therefore, there is a growing interest in studying the therapeutic effects of N-acetylcysteine (NAC), a pro-drug and a GSH precursor, in the prevention of diseases characterized by increased oxidative stress, such as diabetes [44].

In the present study, our aim was to elucidate the cytoprotective effects of NAC on STZ-induced toxicity in Rin-5F cells. STZ is a known pancreatic beta-cell specific cytotoxin and is therefore being widely used for its diabetogenic properties [45, 46]. We have previously demonstrated that diabetes (both *in vivo* and *in vitro* models) induces oxidative stress and mitochondrial respiratory dysfunction [10, 28, 47]. In the present study we intended to investigate the cytoprotective mechanisms of NAC on oxidative stress, mitochondrial bioenergetics, GSH metabolism and apoptosis. The selection of the STZ dose and time point treatment were based on our previous study on Rin-5F cells [27] and HepG2 [36] where lower dose (10mM) for short time period (24h) had minimal effect on the cell viability.

A significant recovery of altered GSH metabolism by NAC treatment was observed in STZ-treated cells suggesting the potential mechanism of NAC action in protecting cells from oxidative stress. NAC has also been shown to exert its antioxidant and anticancer effects by modulating the glutathione metabolism and the biotransformation of carcinogenic substances *in vivo* [48].

Studies have shown that glutamate dehydrogenase plays a key role in regulating redox homeostasis through the activation of GSH-Px [49]. In our study we found that STZ treatment significantly decreased the activity of GDH and increased ROS production, which might have activated the ROS scavenger enzyme, GSH-Px, to regulate the redox homeostasis. NAC treatment significantly decreased the ROS levels, subsequently reducing the activity of GSH-Px.

Endogenous ROS production plays an important role in the regulation of glycolytic activity and expression of glycolytic enzymes, including HK, which could be reversed by NAC pre-treatment [50]. Our study also indicated a similar decrease in HK activity after NAC pre-treatment, which could be related to its antioxidant effects.

The key parameter for evaluating mitochondrial function is mitochondrial membrane potential, which drives the synthesis of ATP [51]. A significant loss of MMP was observed in Rin-5F cells after treatment with STZ accompanied by a decrease in the activities of the respiratory complexes, Complex I and Complex IV as well as that of the mitochondrial matrix enzyme, aconitase. NAC treatment had significantly recovered the MMP and the activity of ROS-sensitive aconitase enzyme. However, NAC treatment inhibited the activity of Complex I but not Complex IV. This could be because NAC acts as a redox sensor by inhibiting the Complex I activity and limiting the oxygen and NADH utilization, thus reducing oxidative stress [52]. Complex I may also be inhibited by excess GSH through glutathionylation [53] since NAC treatment results in an increase in GSH levels. Moreover, the suppression of Complex I activity could also be a result of S-nitrosation of Complex I by NO, which is an effective mechanism to prevent ROS formation, thus protecting the cells from oxidative stress [54, 55]. We have previously shown that NAC treatment partially protected the oxidative modification of mitochondrial complexes and helped in preserving the mitochondrial function in HepG2 cells [56]. Studies have also shown membrane permeabilization to be related to apoptosis [57]. Our present study has also demonstrated activation of caspase-3, caspase-9 and increased ratio of Bax/Bcl-2 expression, which confirmed the increased oxidative stress and thus the increased apoptosis after STZ treatment, which recovered significantly after NAC pre-treatment.

Another important finding of our study was the marked reduction in insulin secretion, which, however was not normalized by NAC pre-treatment. This could be due to NAC scavenging the ROS, physiologically required for insulin secretion. However, a moderate increase in insulin secretion was observed in cells treated with NAC alone. This could be due to the improvement of pancreatic physiological and mitochondrial functions, which control the glucose-stimulated insulin secretion. This is supported by previous studies suggesting the improvement of insulin secretion by NAC treatment associated with improvement of GLUT

Fig. 10. Schematic model representing the cytoprotective mechanisms of NAC in STZ treated Rin-5F cells. As demonstrated in our previous study [27], STZ induces cytotoxicity and apoptosis by increased ROS/NOS production and LPO and decreased GSH/GSSG ratio and mitochondrial dysfunction. The schematic model shows the restoration of redox homeostasis, GSH metabolism and mitochondrial bioenergetics after NAC treatment in STZ-treated Rin-5F cells. Upward arrows (↑) indicate increase and downward arrows (↓) indicate decrease.

2 expression and insulin synthesis [23, 58]. However, in the long-run, the regeneration of beta cells and preservation of the physiology and mitochondrial bioenergetics in pancreatic cells, along with the scavenging of ROS and stimulation of antioxidant defense mechanisms by NAC may improve insulin synthesis and secretion in the damaged beta cells. GDH also plays a key role in insulin secretion [59]. Studies have shown that overexpression of GDH enhances insulin secretion whereas deletion of GDH significantly reduced insulin secretion in pancreatic cells [60, 61]. In the present study we found that NAC pre-treatment didn't significantly recover the GDH activity which could be the reason for the decreased insulin secretion observed in these cells.

Conclusion

In summary, our results demonstrate that NAC treatment protects the Rin-5F cells from STZ-induced toxicity via modulation of energy and redox homeostasis and suppression of oxidative stress. The activation of antioxidant defense mechanisms and regeneration of cellular GSH followed by the preservation of key mitochondrial bioenergetics functions, at least in part, may be involved. We have presented a schematic model to highlight the protective effects of NAC on STZ-induced cytotoxicity in Rin-5F cells (Fig. 10). However, the exact therapeutic role of NAC in diabetes needs to be further elucidated in human subjects. The persistent hyperglycemia and inflammatory conditions associated with both type 1 and type 2 diabetes results in increased oxidative stress and associated complications in diabetes. Therefore, the present study may contribute to a better understanding of the oxidative stress induced cytotoxicity and the cellular defense mechanisms of insulin secreting pancreatic β -cells. Moreover, these results may have implications in understanding the aetiology and pathophysiology of diabetes and therapeutic responses.

Abbreviations

STZ (streptozotocin); NAC (N-acetylcysteine); MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide); ROS (reactive oxygen species); NO (nitric oxide); LPO (lipid peroxidation); GSH (glutathione); GSH-Px (glutathione peroxidase); DCFDA (2',7'-dichlorofluorescein diacetate); SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

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Disclosure Statement

The authors declare no conflict of interest.

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