HIV-1 Protease Inhibitor Induced Oxidative Stress Suppresses Glucose Stimulated Insulin Release: Protection with Thymoquinone

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The highly active anti-retroviral therapy (HAART) regimen has considerably reduced the mortality rate in HIV-1 positive patients. However, long-term exposure to HAART is associated with a metabolic syndrome manifesting cardiovascular dysfunction, lipodystrophy, and insulin resistance syndrome (IRS). The inclusion of HIV-1 protease inhibitors (PIs) in HAART has been linked to the induction of IRS. Although several molecular mechanisms of PI-induced effects on insulin action have been postulated, the deleterious effects of PIs on insulin production by pancreatic β-cells have not been fully investigated and therapeutic strategies to ameliorate insulin dysregulation at this level have not been targeted. The present study showed that exposure to several different PIs, nelfinavir (5–10 μM), saquinavir (5–10 μM) and atazanavir (8–20 μM), decreases glucose stimulated insulin secretion from rat pancreatic β-cells (INS-1). Nelfinavir significantly increased reactive oxygen species (ROS) generation and suppressed cytosolic, but not mitochondrial superoxide dismutase (SOD) levels. Nelfinvair also decreased both glutathione and ATP and increased UCP2 levels in these cells. Simultaneous treatment with thymoquinone (TQ) (2.5 μM), an active ingredient of black seed oil, significantly inhibited the effect of nelfinavir on augmented ROS production and suppressed SOD levels. Both TQ and black seed oil exposure increased glucose stimulated insulin secretion and ameliorated the suppressive effect of nelfinavir. The present findings imply a direct role of ROS in PI induced deleterious effects on pancreatic β-cells. Our findings also suggest that TQ may be used as a potential therapeutic agent to normalize the dysregulated insulin production observed in HAART treated patients. Exp Biol Med 234:442–452, 2009

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Introduction

The advancement of HIV-1 anti-retroviral therapy from a monotherapy to the combination therapy known as Highly Active Antiretroviral Therapy (HAART) has helped reduce mortality rate in AIDS patients. Since the inclusion of protease inhibitors (PIs) in the HAART regimen, the prognosis of HIV-1 patients has significantly improved but has also been associated with several metabolic disorders including lipodystrophy, insulin resistance syndrome (IRS) and cardiovascular diseases (1–7). Although similar abnormalities have been observed in AIDS patients receiving only reverse transcriptase inhibitors or no therapy (8), the frequency and severity is significantly higher in patients undergoing treatment that includes PIs (9). These clinical observations have spurred an interest in delineation of the mechanisms involved in PI-induced IRS. The mechanism of PI-induced IRS had previously been linked to suppressed insulin action on the skeletal muscle (10), adipose tissue (11) or the hepatic cells (12), but the effect of PIs on insulin secretion by the β-cells of the pancreas has not been thoroughly investigated (13). Indeed, clinical data suggests that PI-containing regimens impair glucose tolerance in HIV-1 infected patients by induction of peripheral IRS in skeletal muscle and adipose tissue and by impairment of the β-cells to compensate for the insulin resistance (14).

Glucose is a major regulator of insulin secretion from pancreatic β-cells, and insulin also regulates its own secretion via a feedback mechanism. Therefore, any dysregulation in glucose metabolism would affect insulin secretion. To implicate a direct role of PIs in insulin secretion rather than a secondary effect of hyperglycemia, several *in vitro* studies have been done. Long-term as well as acute treatment with the PIs, ritonavir, nelfinavir, indinavir and saquinavir were found to significantly inhibit

insulin secretion in rat insulinoma cell line, INS-1 (13) and mouse insulinoma cell line (MIN6) (15); however, the molecular mechanism manifesting these β-cells impairments is not well understood.

Oxidative stress has been implicated in the pathophysiology of diabetes and has been associated with its etiology (16). Previous findings from our laboratory have shown that in human aortic endothelial cells (HAECs), PIs alone and as part of HAART regimen increase generation of reactive oxygen species (ROS) (17). Nelfinavir induced oxidative stress can also lead to necrosis of adipocytes (18). Since pancreatic β-cells have a relatively low expression of antioxidant enzymes, such as catalase and glutathione peroxidases, they are more sensitive to oxidative stress (19). Acute exposure of isolated mouse islets and INS-1 cells to oxidative stress inducers like arsenite and methylglyoxal were found to inhibit glucose induced insulin secretion (20).

Oxidative phosphorylation in the mitochondria plays a critical role in the glucose stimulated insulin secretion from the pancreatic β-cells. Superoxide production in pancreas can lead to the activation of uncoupling protein 2 (UCP2), a protein associated with the mitochondria (21). Uncoupling proteins cause partial depolarization of the mitochondrial membrane potential due to the dissociation between electron transport chain and ATP synthesis (22). Up-regulation of UCP2 in the pancreas has been associated with decreased production of ATP, closure of ATP sensitive K^+ channels and impairment of insulin secretion (23, 24).

Several clinical trials with antioxidant supplements like vitamin E and/or C have failed to show any significant improvement in markers of oxidative stress in type 2 diabetic patients (25). Hence, plant based antioxidants are gaining increasing importance as they can be used as dietary supplements and have minimal side effects. Thymoquinone (TQ), an active ingredient of the black seed oil (BSO) from the plant *Nigella sativa,* has been shown to possess potent antioxidant properties (26). The antioxidant property of TQ may also serve an important role as a protective agent against chemically induced hepatic damage (27). The compound TQ shares structural homology with coenzyme Q, which is an important antioxidant in the electron transport chain, and both BSO as well as TQ have been shown to inhibit lipid peroxidation (28) and alleviate nitric oxide induced hypertension in rats (29).

In this study, we have investigated the role of PI induced oxidative stress in suppressing glucose stimulated insulin secretion in rat pancreatic β-cell line (INS-1) and possible pharmacological intervention by TQ and BSO, to investigate a possible therapeutic strategy to ameliorate the PI-induced deleterious effects on INS-1 cells.

Materials and Methods

Reagents. The HIV-1 PIs (nelfinavir, saquinavir and atazanavir) were purchased from Tulane Pharmacy and thymoquinone (TQ) from Sigma (St. Louis, MO). Nelfinavir (Viracept®) was dissolved in ethanol to a stock concentration of 10 mg/ml (15 mM), saquinavir (Invirase®) in water to a concentration of 2 mg/ml (2.6 mM) and atazanavir (Reyataz®) in water to a stock concentration of 4 mg/ml (6.23 mM). BSO and TQ were dissolved in DMSO to a stock concentration of 12 mg/ml and 25 mM, respectively. All stocks were aliquoted and stored at −20°C and each aliquot was thawed and used once. The fluorescent dyes H_2DCFDA (intracellular ROS) and MitoSox red (mitochondrial ROS) were purchased from Invitrogen (Carlsbad, CA). High range rat insulin ELISA kit was obtained from Alpco Diagnostics (Salem, NH), glutathione assay kit from Northwest Life Science Specialties (Vancouver, WA), ATP assay kit from Biovision (Mountain View, CA) and SOD assay kit was obtained from Cayman Chemicals (Ann Arbor, MI). The primary antibody to Cu/Zn SOD was purchased from Calbiochem (La Jolla, CA), Mn SOD from Abcam (Cambridge, MA), rabbit anti-actin antibody from Sigma (St. Louis, MO) and UCP2 antibody from Alpha Diagnostics (San Antonio, TX).

Cell Culture and Treatment. The rat pancreatic insulinoma cell line, INS-1 was a kind gift from Dr. Donald Scott (University of Pittsburgh, PA). Cells were grown in RPMI-1640 medium containing 11.1 mM glucose supplemented with 10% FBS, 10% penicillin-streptomycin and 50 μM of β-mercaptoethanol and sub-cultured at a ratio of 1:4 and used for experiments within first 40 passages. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

The following treatment protocol was used in each experiment. Prior to treatment with the drugs, INS-1 cells were grown to 70% confluence in complete media and thereafter starved overnight in RPMI medium containing 3.3 mM glucose. The cells were treated with nelfinavir (1–10 μM), atazanavir (4–20 μM), or saquinavir (2–10 μM) in the presence or absence of TQ (1–2.5 μM) for 24 hr. Parallel cultures were exposed to proper vehicle controls, consisting of the drug diluents only. In specified experiments, cells were stimulated with PBS containing 22 mM glucose to monitor ROS production and insulin secretion.

Insulin ELISA. INS-1 cells were plated in 6-well plates at a density of 5×10^5 cells/well and allowed to grow to 70% confluence and treatment with PIs and TQ were carried out. After the treatments, cells were washed once with warm Krebs Ringer Bicarbonate Buffer (KRBB-140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 2 mM $NaHCO_3$, pH 7.4) with 0.1% BSA for 5 min, followed by stimulation with 0 or 22 mM glucose in KRBB with 0.1% BSA for 30 min at 37°C. Supernatant was collected, centrifuged at 12,000 *g*/10 min at 4°C and the cells were lysed to determine protein content by BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL). Ten μl of standard and 10 μl of supernatant from each sample were added to the primary antibody coated wells of the ELISA

plate. Fifty µl of enzyme conjugate was then added to each well and the plate incubated on a shaker at 800 rpm for 2 hr at room temperature. Following 4–6 washes with wash buffer, 200 µl of substrate was added to each well and the plate further incubated in dark for 15 min. The reaction was stopped by adding 50 µl of stop solution to each well, mixed well and absorbance was determined immediately using the microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA) at 450 nm. The values obtained are expressed as ng of insulin secreted per mg of total cellular protein.

ROS Assay. Dichlorodihydrofluorescein diacetate (H_2DCFDA) is a cell-permeant indicator for ROS. This dye is retained within the cells following removal of the acetate groups by intracellular esterases and is activated to a fluorophor via oxidation occurring inside the cytosol. Similarly, the MitoSox red dye (Invitrogen) is rapidly targeted to mitochondria where it gets oxidized by ROS and exhibits fluorescence. For these experiments, cells were cultured in 24-well Krystal black plates (Labnet International, Woodbridge, NJ). Following treatment with PIs and/or TQ, cells were washed once with warm PBS, loaded with 10 µM H_2DCFDA or 5 µM MitoSox in serum free RPMI media for 20 min at 37°C and subsequently washed with warm PBS. After the last wash, cells were stimulated with PBS containing 22 mM glucose and fluorescence was read at excitation and emission wavelengths of 485 and 520 nM for H_2DCFDA and 520 and 590 nm for MitoSox dye, respectively. The fluorescence was detected by using a Bio-Tek fluorometer, *FL_x800* (Winooski, VT), by taking readings after 30 min post incubation with the dyes. In each experiment, no dye controls were used to measure background fluorescence, which was subtracted from the experimental groups. After the assay, cells were lysed and total protein contents were determined by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL). The fluorescence values were normalized to protein levels in each sample and results are expressed as percent change in fluorescence per mg of protein and compared to the vehicle controls.

Glutathione Assay. The glutathione assay kit from Northwest Life Science Specialties (Vancouver, WA) was used for determinations of glutathione (GSH) levels. After treatment of the cells, approximately 10^6 cells/ml were taken and disrupted by sonication, centrifuged and supernatants were used for GSH determination. Approximately 50 µl of calibrator, diluted sample and diluted control, were added to designated wells of a 96-well microtiter plate. To this, 50 µl of thiol reagent (DTNB) and 50 µl of glutathione reductase (GR) were added and plates were incubated for 3–5 min at room temperature. Following this, 50 µl of reconstituted NADPH was added to each well and absorbance recorded using the microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA) at 405 nm at 15–20 sec intervals up to 3 min. Total protein contents were determined by the BCA protein assay kit. GSH values (µM) were normalized to protein (mg) and results are percent change compared to vehicle control.

Cu/Zn SOD Assay. The kit from Cayman Chemicals (Ann Arbor, MI) was employed to measure SOD activity in the INS-1 cells treated with nelfinavir. For the assay, the cells were scraped using cold 20 mM HEPES buffer, pH 7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose and further homogenized on ice. The cells were collected after centrifuging at 1500 *g* for 5 min at 4°C and supernatant was used for the assay. Two hundred µl of the radical detector was added to the wells of a 96-well plate followed by 10 µl of the sample or the standards. The reaction was initiated by adding 20 µl of xanthine oxidase to each well and readings were taken 20 min after incubation at room temperature using the microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA) at 450 nm. The concentration in the sample was calculated from the standard curve and values were expressed in units of enzyme normalized to cellular protein in mg.

ATP Assay. ATP levels in the INS-1 cells were determined using the Aposensor^{TM} ADP/ATP Ratio Assay Kit (Biovision, CA). After the nelfinavir treatments, cells were stimulated with 22 mM glucose. For these assays, cells (10^3–10^4) were resuspended in 10 µl of medium after trypsinization and treated with 100 µl of the nuclear releasing reagent, for 5 min at room temperature with gentle shaking. One µl of the ATP monitoring enzyme was then added and the samples were read within 1 min using an Optocomp I luminometer (MGM Instruments, Inc., Hamden, CT) to measure the ATP levels. The values were represented in percent change as compared to control treatments.

Western Immunodetection. Western Blot analysis was carried out to monitor the protein levels of Cu/Zn SOD, Mn SOD, UCP2 and β-actin from cell lysates. The cells were lysed using the lysis buffer (Cell Signaling Technology, Boston, MA), protein estimated by BCA method and approximately 50 µg of protein was electrophoresed in 7.5% or 18% SDS-PAGE gels (Bio-Rad) at 90 V. Separated proteins were electro-transferred to nitrocellulose membrane for 1 hr and non-specific binding was blocked by using 5% non-fat dry milk prepared in Tris buffered saline containing 0.1% Tween-20. Hybridization with the primary antibodies was performed by incubation at 4°C for overnight. The membranes were developed using horseradish peroxidase-linked secondary antibody and a chemiluminescent detection system (LumiGLO, KPL, Gaithersburg, MD). Levels of immunoreactive proteins were determined by using densitometry (Quantity One; Version 4.6.1). Densitometric ratios were calculated as compared to β-actin levels in each sample and fold changes were reported as compared to the untreated control samples.

Statistical Analysis. All statistical analyses were performed with GraphPad Prism version 4.03 (San Diego, CA). Experiments were performed at least 3–5 times and the values obtained from 2–4 replicate samples were averaged

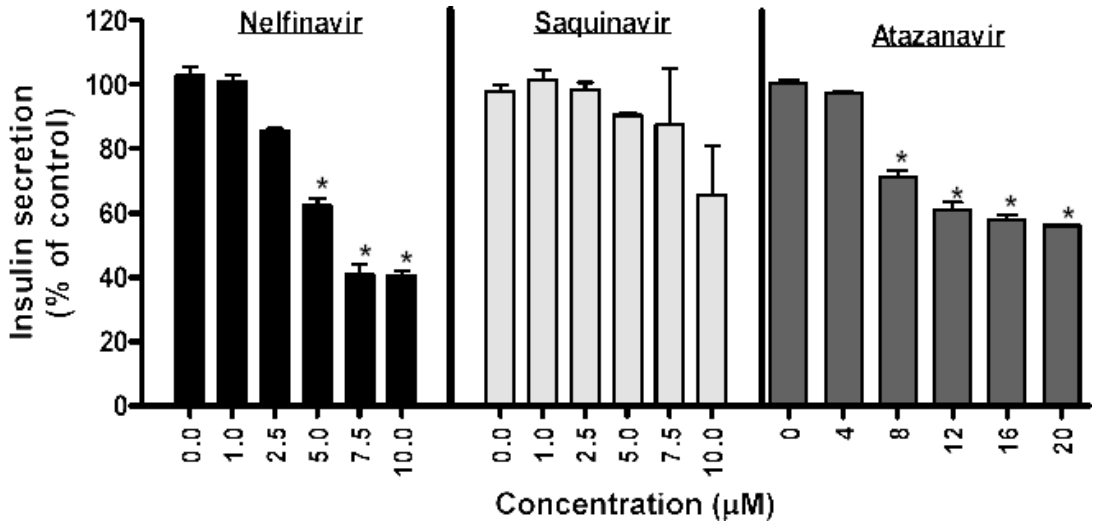

Figure 1. Effect of HIV-1 PIs on glucose stimulated insulin secretion in INS-1 cells. INS-1 cells were treated for 24 hr with different concentrations of the HIV-1 PIs, nelfinavir (0–10 μM), saquinavir (0–10 μM) and atazanavir (0–20 μM). Cells were monitored for glucose (22 mM) stimulated insulin secretion at 30 min. Glucose stimulated insulin secretion in culture supernatants were monitored by ELISA and insulin levels (ng/ml) are expressed as percent change as compared to control. Data from five independent experiments ($n = 5$) carried out in triplicates were analyzed. The error bars represent ±SEM of values and significant change from controls is represented as * $P < 0.05$.

in each experiment. The results were expressed as standard error of means (± SEM). The significance of changes from control values was determined by using a two-tailed Student's *t* test and comparisons between three or more groups were carried out by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. The *P* values of $<$ 0.05 were considered to be significant.

Results

Exposure to the PIs, Nelfinavir, Saquinavir or Atazanavir Showed Differential Suppression of Glucose Stimulated Insulin Secretion by the INS-1 Cells. In INS-1 cells treated with the PIs, nelfinavir (1–10 μM), saquinavir (2–10 μM) or atazanavir (4–20 μM) for 24 hr, we measured both basal (3 mM) and hyperglycemic (22 mM) levels of glucose stimulation for 30 min on insulin secretion, by using an insulin specific ELISA assay (Fig. 1). Basal levels of insulin secretion were not affected by PI exposure (data not shown); however, glucose stimulated insulin secretion showed a concentration dependent decrease following treatment with nelfinavir (60 ± 2%), saquinavir (25 ± 6%) and atazanavir (40 ± 2%). Since nelfinavir exposure showed the most significant suppressive effect on glucose stimulated insulin secretion, we monitored the molecular mechanism linked to nelfinavir mediated effects on INS-1 cells.

Nelfinavir Exposure Increases Oxidative Stress and Decreases Antioxidant Levels in INS-1 Cells. Previous studies from our lab have shown that ROS generation is increased with PIs and PI-based HAART regimen in human aortic endothelial cells (17). Since oxidative stress is commonly implicated in the pathophysiology of diabetes (16), we wanted to determine whether PIs induce oxidative stress in INS-1 cells which might account for the decrease in insulin secretion. INS-1 cells were treated for 24 hr with different concentrations of nelfinavir (0–10 μM), saquinavir (0–10 μM) or atazanavir (0–20 μM), and intracellular ROS production was assessed by using the fluorescent dye H_2DCFDA (Fig. 2). Change in ROS levels was normalized to total cellular protein content and represented as fluorescent units/mg of protein. It was observed that, as compared to the vehicle controls, cells exposed to nelfinavir showed significantly increased ROS levels and a 50 ± 5% increase was seen at 10 μM concentration of nelfinavir (Fig. 2A). However, exposure to neither atazanavir nor saquinavir showed a significant enhancement in ROS levels. Furthermore, our results showed 6 hr treatment with nelfinavir (10 μM) did not increase oxidative stress; however, a significant increase in ROS generation was evident after 24 hr of exposure to nelfinavir (Fig. 2B).

In the subsequent studies, increased oxidative stress in INS-1 cells was correlated with a simultaneous decrease in the levels of antioxidants. We monitored the activities of essential endogenous antioxidants, glutathione (GSH) and superoxide dismutase (SOD). We observed that exposure to nelfinavir (10 μM) significantly decreased GSH levels (Fig. 3A) and intracellular SOD (Cu/Zn SOD) activity (Fig. 3B) in INS-1 cells. ATP levels are also dysregulated during mitochondrial oxidative stress. ATP levels increase in response to glucose stimulation in β-cells. We observed that 24 hr nelfinavir (10 μM) treatment of INS-1 cells significantly decreased (40 ± 3%) glucose stimulated ATP production (Fig. 3C). The above findings suggested that antioxidant defense mechanism is affected with nelfinavir treatment in INS-1 cells and the ROS generation may be

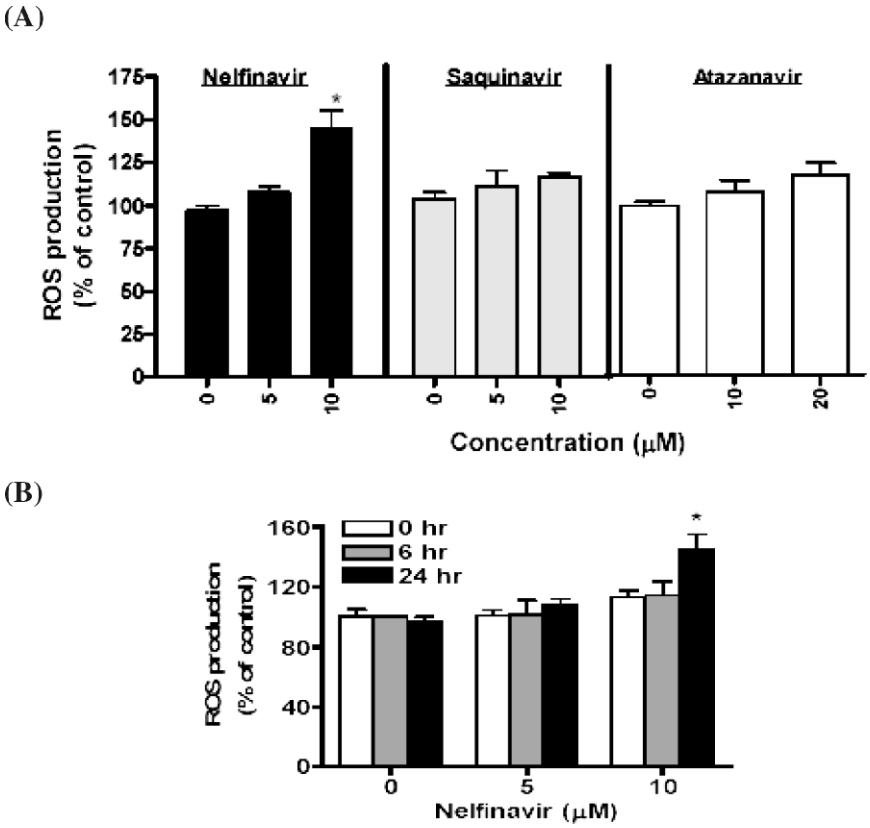

Figure 2. Effect of HIV-1 PIs on ROS production by INS-1 cells. In INS-1 cells treated with the HIV-1 PIs (nelfinavir, atazanavir and saquinavir), accumulation of cytosolic ROS was measured by fluorometric analysis using H_2DCFDA. (A) Following exposure to different concentrations of the PIs for 24 hr, cytosolic ROS levels were measured. (B) INS-1 cells were treated for 6 and 24 hr with nelfinavir (10 μM) and cells were monitored for ROS accumulation. Values from three independent experiments ($n = 3$) and significant change from controls is represented as * $P < 0.05$.

mediated via dysfunctioning of the mitochondrial electron transport chain.

Nelfinavir Treatment of INS-1 Cells Reduced SOD Protein Levels. Since we observed a decrease in the SOD enzyme activity following nelfinavir treatment, we determined whether the cytosolic SOD (Cu/Zn SOD) and mitochondrial SOD (Mn SOD) protein levels are altered in INS-1 cells. Western blot analysis illustrated that the expression of Cu/Zn SOD protein is significantly reduced following exposure to nelfinavir (Fig. 4A); however, the Mn SOD levels were not critically affected (Fig. 4B). The bar graph data (Fig. 4C) represents the average of three independent experiments, depicting almost a 2-fold decrease in Cu/Zn SOD protein levels.

Thymoquinone Decreases Nelfinavir Induced Oxidative Stress and UCP2 Expression and Increases Cu/Zn SOD Levels in INS-1 Cells. Antioxidant effects of plant based antioxidant TQ have been shown by several investigators (27–29). To investigate whether antioxidant therapy using TQ may be an approach to mitigate the nelfinavir induced ROS production, INS-1 cells were simultaneously treated with nelfinavir (0–10 μM) and TQ (1 μM and 2.5 μM) for 24 hr. We observed that TQ co-exposure at 2.5 μM level significantly reduced the oxidative stress induced by nelfinavir (Fig. 5A). Exposure to 1 μM TQ was not as effective in suppressing oxidative stress (Fig. 5A), and TQ at concentrations higher than 2.5 μM showed cytotoxicity in nelfinavir treated cells (data not shown). The decrease in ATP production via nelfinavir (Fig. 3C) suggested a direct involvement of the mitochondrial pathway. The mitochondrial UCP2 protein gets up-regulated during oxidative stress and directly affects ATP levels in cells. We observed that nelfinavir (10 μM) treatment significantly increased (50%) UCP2 protein levels, and simultaneous treatment with TQ suppressed both basal and nelfinavir induced UCP2 levels (Fig. 5B). This further suggested that nelfinavir induced oxidative stress may be partially mediated via disruption of a mitochondrial pathway and TQ which has structural similarities with Co-Q, may be acting as a mitochondrial antioxidant, as well. Although the levels of Mn SOD were not affected by nelfinavir, Cu/Zn SOD levels were significantly decreased (Fig. 4A). Our experiments clearly show that TQ co-treatment can restore the decreased levels of Cu/Zn SOD protein in nelfinavir treated INS-1 cells (Fig. 5C) and significant (2-fold) reversal

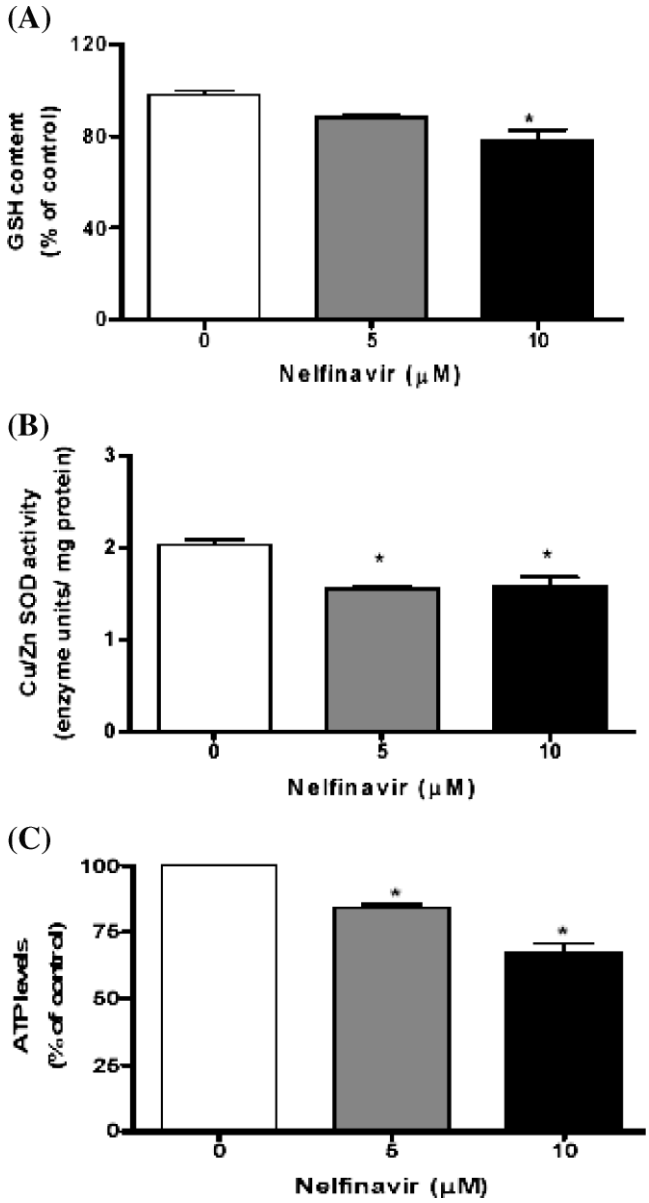

Figure 3. Effect of nelfinavir on GSH content, Cu/Zn SOD activity and ATP levels. INS-1 cells were treated for 24 hr with nelfinavir (10 μM) and (A) GSH levels were monitored by using an assay kit. Data are expressed as percent change of glutathione compared to vehicle control. (B) Cu/Zn SOD enzyme activity was monitored by using an assay kit and data are expressed as units of enzyme activity normalized to total cellular protein content in mg. (C) Glucose (22 mM) stimulated ATP levels in INS-1 cells exposed to nelfinavir (10 μM) for 24 hr were monitored using an assay kit. The ATP levels are expressed in percent change compared to vehicle control. Data from 3–4 independent experiments were analyzed. Error bars represent ±SEM of values and significant change from controls is represented as * $P < 0.05$.

of Cu/Zn SOD with TQ is observed at 10 μM of nelfinavir treatment (Fig. 5D).

Both Black Seed Oil and Its Active Component, TQ Can Restore the Nelfinavir-Mediated Decrease in Glucose Stimulated Insulin Secretion in INS-1 Cells. Previous studies have reported the hypoglycemic action of TQ in diabetic rats and have associated the suppression of nitric oxide production as a possible mechanism (30, 31). In this study, we investigated whether the antioxidant effects of TQ (2.5 μM) can suppress the deleterious effects of nelfinavir on glucose stimulated insulin secretion in INS-1 cells (Fig. 6A). Similarly, co-exposure of INS-1 cells to black seed oil (BSO) was also able to ameliorate the deleterious effects of nelfinavir treatment in these cells (Fig. 6B). The concentration of BSO used was equivalent to the concentration of pure TQ used in other experiments and was calculated assuming the concentration of TQ as 20% in the oil (32, 33). Cells were simultaneously exposed with nelfinavir (10 μM) in combination with either TQ (2.5 μM) or BSO (8 μg/ml) for 24 hr and insulin secretion was monitored by ELISA. We observed that TQ (2.5 μM) co-treatment significantly (35 ± 4%) increases insulin secretion in nelfinavir (5 and 10 μM) exposed cells. In addition, BSO co-treatment also showed increase in insulin secretion and significant (30 ± 2%) protective effect was seen in cells exposed to the lower concentration (5 μM) of nelfinavir. Our findings implicate a therapeutic value of BSO and/or thymoquinone supplementation to ameliorate the deleterious effects of nelfinavir on oxidative stress, antioxidant levels and insulin production by the pancreatic β-cells.

Discussion

Long-term treatment with HIV-1 PI containing HAART regimen has been associated with clear manifestations of the metabolic syndrome in HIV-1 positive patients, including cardiovascular dysfunction, lipodystrophy and insulin resistance (1, 4, 5, 34). IRS may be manifested by both a decreased response of insulin at the peripheral tissues and concomitant attenuation or dysregulation of glucose stimulated insulin production by the pancreatic β-cells. The deleterious effects of HIV-1 PIs have been associated with oxidative stress in endothelial cells (17, 35, 36), adipocytes (11, 18) and macrophages (37). In addition, several PIs have been shown to impair glucose stimulated insulin release in INS-1 cells (13). These studies had suggested several mechanism of action, either via inhibition of voltage dependent K+ currents (38) or decrease in intracellular calcium concentrations (39). Our study, in concert with a number of previous publications (13, 39), has shown that the PIs, nelfinavir, atazanavir and saquinavir, significantly reduce glucose stimulated insulin secretion in rat pancreatic insulinoma cell line, INS-1. This implicates a direct inhibitory effect of PIs on insulin production by pancreatic β-cells. However, the molecular pathways ultimately leading to pancreatic β-cell dysfunction were not analyzed. We wanted to determine whether the PI-mediated decrease in insulin secretion from the pancreatic β-cells is also associated with oxidative stress. We observed that within 24 hr of exposure, but not within 6 hr, nelfinavir increased intracellular ROS levels in INS-1 cells. However,

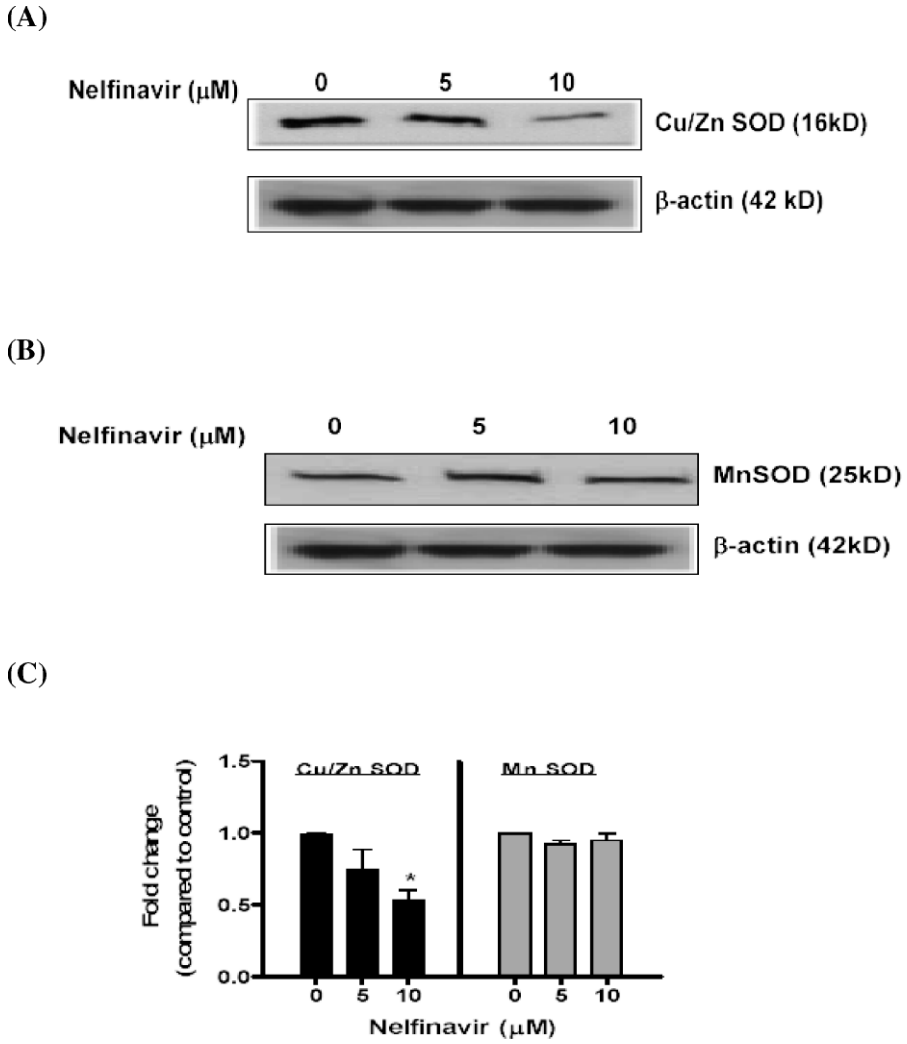

Figure 4. Effect of nelfinavir on SOD protein levels in INS-1 cells. A representative gel picture of Western blot analysis of Cu/Zn SOD (A) and Mn SOD (B) protein expression is shown in INS-1 cells treated with nelfinavir (10 μM) for 24 hr. (C) Densitometric ratios for Cu/Zn SOD and Mn SOD protein expression were calculated after normalizing to β-actin levels. Data is expressed as fold change compared to controls. Bar graph shows the Cu/Zn SOD and Mn SOD levels from 3 independent experiments. Error bars represent ±SEM of values and significant change from controls is represented as * $P < 0.05$.

neither atazanavir nor saquinavir exposure showed a significant increase in oxidative stress, but a trend towards higher ROS levels was seen with both of these later PIs.

Although the concentrations of PIs used in our current study are 2–3-fold higher as compared to free plasma concentrations observed in patients, since plasma protein binding is high for most of the PIs, the concentrations used in our *in vitro* study may correspond to plasma concentrations achievable. In addition, these concentrations have also been used in several other published studies (10, 12, 15, 40). Our findings clearly indicated a drug-specific instead of a class-specific effect on the INS-1 cells. Only nelfinavir was seen to suppress glucose stimulated insulin secretion and increase oxidative stress to a significant level. These findings may be due to the short-term exposure (24 hr) experiments carried out in these studies. A chronic and long-term exposure that occurs in patients may further exacerbate these deleterious effects of nelfinavir and may enhance the effects of other PIs as well, which may not be apparent in our *in vitro* experiments. Indeed, a number of previous studies have shown that nelfinavir besides indinavir is one of the PIs with the most side effects (11, 13, 18) and the newer PI, atazanavir, is associated with much lower cardiovascular events (41, 42). However, since nelfinavir showed the most potent effect, further mechanistic studies were performed by treating the INS-1 cells with nelfinavir.

The postprandial regulation of insulin levels is a critical parameter in regulating hyperinsulinemia and hyperglycemia (43, 44). Insulin levels may also play a direct role in the development of both PI-induced manifestations and in the investigations of therapeutic approaches to suppress their side effects. It has been suggested by a number of different investigators that both dysregulation of insulin action and insulin production need to be normalized in patients on

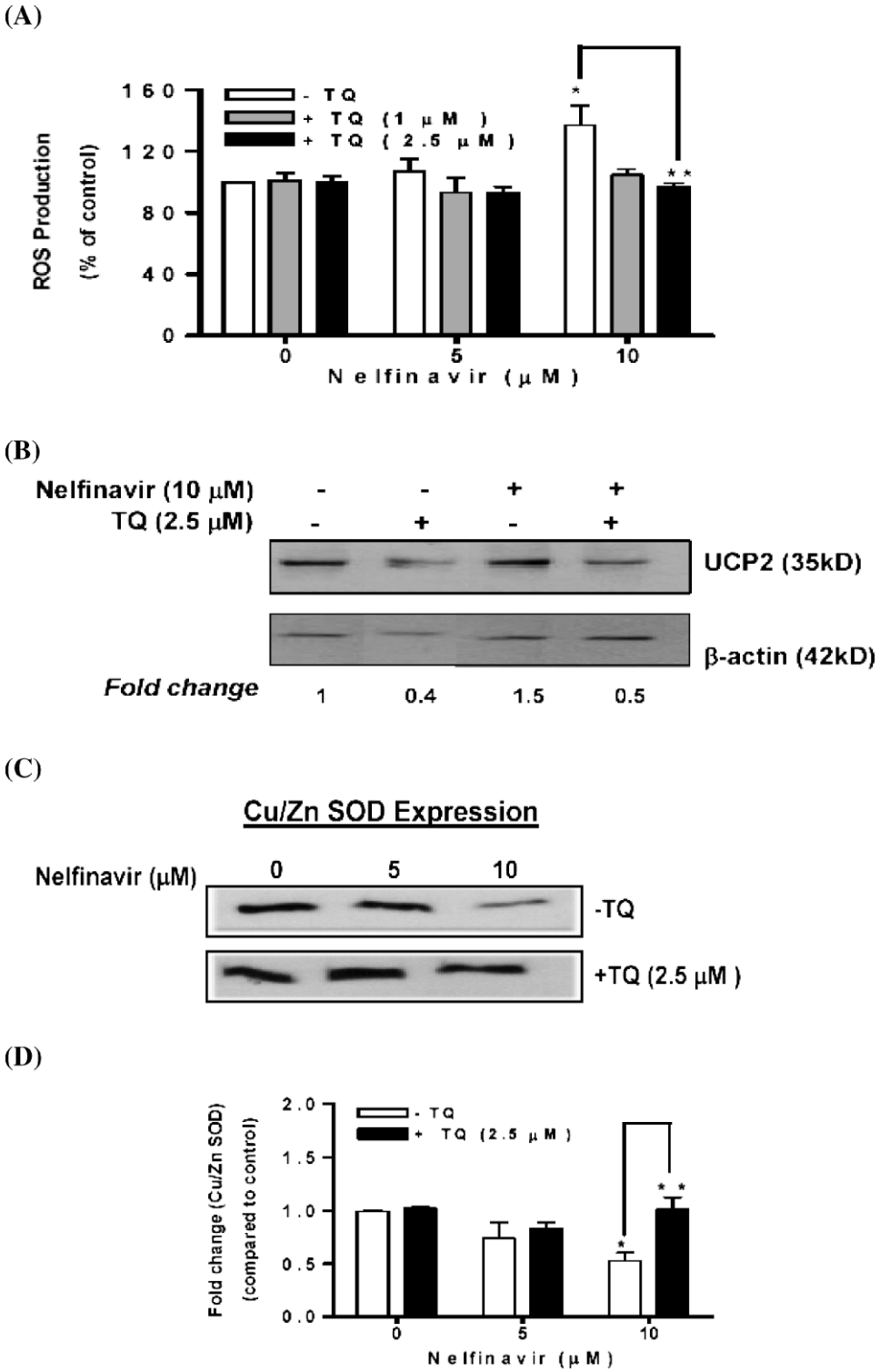

Figure 5. Effect of TQ on cytosolic ROS levels in nelfinavir treated INS-1 cells. (A) INS-1 cells were simultaneously treated with nelfinavir (10 μM) and TQ (1 and 2.5 μM) for 24 hr and ROS levels were monitored. Data are expressed as percent change of fluorescence units after normalizing to the cellular protein content. Significant changes are represented as * $P < 0.05$ in nelfinavir treatment vs. vehicle control and ** $P < 0.05$ in nelfinavir + TQ vs. nelfinavir alone group. (B) UCP2 protein levels were monitored using Western blot technique. Effects of nelfinavir and/or TQ on UCP2 levels in INS-1 cells are shown. The densitometric values for UCP2 protein were normalized to β-actin and data represents the fold change as compared to control, which is presented at the bottom of the gel pictures. (C) Cu/Zn SOD protein levels were monitored using Western blot technique. Effects of nelfinavir and/or TQ on Cu/Zn SOD expression in nelfinavir treated INS-1 cells. (D) The fold changes in Cu/Zn SOD protein levels as compared to controls were analyzed and normalized to the respective levels of β-actin. Data from three independent experiments is presented in the bar graphs. Error bars represent ±SEM of values and significant changes from controls are represented as * $P < 0.05$ in nelfinavir treatment vs. vehicle control and ** $P < 0.05$ in nelfinavir + TQ vs. nelfinavir alone.

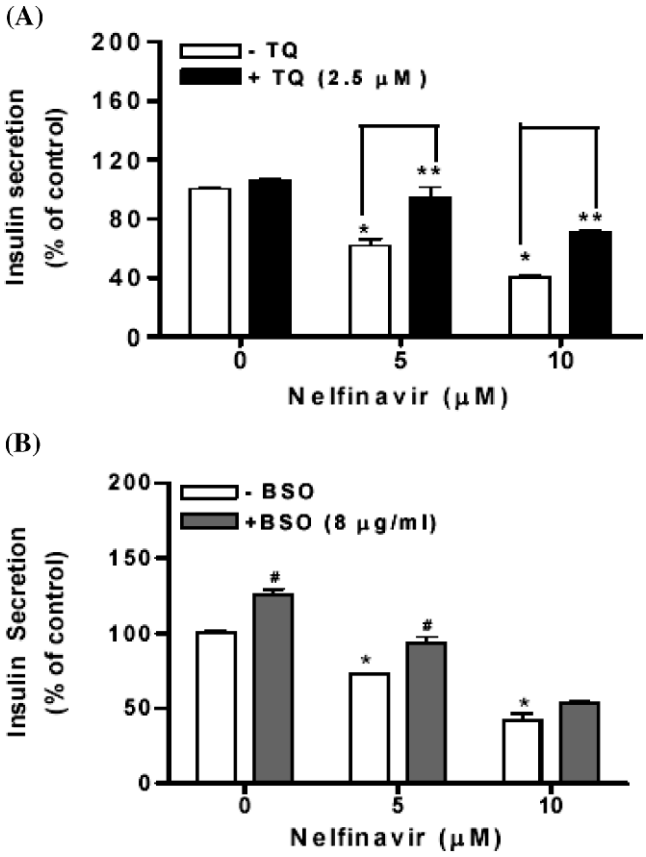

Figure 6. Effect of exposure to TQ or BSO on glucose stimulated insulin secretion in nelfinavir treated INS-1 cells. INS-1 cells were treated with nelfinavir (10 μM) alone or in combination with either (A) TQ (2.5 μM) or (B) BSO (8 μg/ml) for 24 hr. Glucose (22 mM) stimulated insulin secretion was monitored by ELISA. Data are represented as percent fold change compared to controls. Error bars represent ±SEM of values and significant changes from controls are represented as * $P < 0.05$ in nelfinavir treatment vs. vehicle control and ** $P < 0.05$ in nelfinavir + TQ vs. nelfinavir alone groups.

long-term HAART (36, 45, 46). Indeed, several therapeutic strategies are being investigated to suppress the insulin resistance and the PI-associated abnormalities in HIV-1 positive patients (45, 47, 48). Both insulin sensitizers, such as thiazolidinediones (49) and metformin (50), as well as several statins (51), are in clinical trial to suppress the cardiovascular dysfunction (CVD) and IRS. In addition, several lipid lowering agents such as omega-3 fatty acids are being tested to decrease the incidence of lipodystrophy in HIV-1 positive patients (52). Recent evidences implicate that adjunct therapy with antioxidants may ameliorate these deleterious effects of PI containing HAART regimen (11). Several known antioxidants have been tested in pre-clinical and clinical trials (53). One of the most common and potent antioxidants, N-acetylcysteine, has limited use due to the toxicity associated with its overdose as a result of poor absorption (54). In addition, vitamin based antioxidants such as Vitamin E and Vitamin C have been used, but have not proven to be very effective in suppression of insulin resistance in patients (25). Ethnobotanical studies to isolate plant antioxidants are being carried out and are being monitored for active compounds that work potently as antioxidants, both *in vitro* as well as *in vivo* (55, 56). In this study, we explored the antioxidant properties of TQ, the active component of BSO from the plant *Nigella sativa*. A number of previous studies using TQ have shown antioxidant properties of this compound (27–29) and its use has exhibited hypoglycemic effects *in vivo* (31).

Our data showed that simultaneous treatment of INS-1 cells with TQ suppressed nelfinavir induced oxidative stress levels and protected SOD expression in nelfinavir treated cells, thus suggesting the potential role of TQ as an oxygen radical scavenger. Nelfinavir induced oxidative stress was associated with lowered protein levels as well as enzyme activity of Cu/Zn SOD and concomitant decrease in the intracellular levels of GSH. Mitochondrial UCP2 protein, commonly associated with the dissipation of oxidative stress and decrease in ATP production, was monitored to correlate the effect of nelfinavir on insulin secretion from pancreatic β-cells (23, 24). The results from the present study show that UCP2 levels were increased with nelfinavir treatment and concomitantly a significant decrease in ATP production was also observed, which may account for the decrease in glucose stimulated insulin secretion with nelfinavir treatment.

The hypoglycemic effects of *Nigella sativa* oil have also been shown in diabetic rats (32). We postulate that nelfinavir induced oxidative stress is primarily responsible for the deleterious effects of this PI on pancreatic β-cells. Exposure to TQ suppressed UCP2 protein levels. Since TQ shares a structural homology with ubiquinone (mitochondrial component), it is likely that TQ may act as a mitochondrial antioxidant. Some investigators (29, 57) suggested that TQ treatment improves oxidative stress by increasing the glutathione level; however, the exact mechanism for its action still needs to be investigated. Similar to TQ, exposure to black seed oil restored nelfinavir induced decrease in insulin secretion to normal levels. This clearly suggests a potential for the use of black seed oil or TQ as protective agents for diabetes. It is likely that administration of a single antioxidant may not be sufficient to counteract the oxidative stress in patient population even though our *in vitro* studies show a beneficial effect. A naturally occurring antioxidant, alpha-lipoic acid has been shown to significantly regulate blood glucose and mitigate neuropathy in diabetes mellitus in addition to its role in reducing oxidative stress (58). It may be certainly interesting to investigate the effects of a combination therapy of TQ with other antioxidants such as alpha-lipoic acid, which may prove more efficient in controlling diabetic conditions in the HAART treated patients.

In summary, we have shown that exposure to HIV-1 PIs, especially nelfinavir, suppresses glucose mediated insulin secretion in pancreatic β-cells. This effect is mediated through increased oxidative stress and decreased antioxidant mechanisms. Furthermore, our studies showed

that the use of either black seed oil or its active component TQ might ameliorate the deleterious effect of nelfinavir on INS-1 cells.

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