The mitochondria-targeted antioxidant MitoQ modulates mitochondrial function and endoplasmic reticulum stress in pancreatic β cells exposed to hyperglycaemia

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Running title: MitoQ alleviates endoplasmic reticulum stress under hyperglycaemia

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Abstract

Background/Aims: Mitochondria-targeted antioxidants such as mitoquinone (MitoQ) have demonstrated protective effects against oxidative damage in several diseases. The increase in reactive oxygen species (ROS) production during glucose metabolism in β cells can be exacerbated under hyperglycaemic conditions such as type 2 diabetes (T2D), becoming a key contributor to β cell function impairment. In the present work, we aimed to evaluate the effect of MitoQ on oxidative stress, endoplasmic reticulum (ER) stress and nuclear factor kappa B (NFκB) signalling in a pancreatic β cell line under normoglycaemic (NG, 11.1 mM glucose) and hyperglycaemic (HG, 25 mM glucose) conditions.

Methods: We employed the pancreatic β cell line INS-1E incubated with or without MitoQ (0.5μM) under NG or HG conditions and assessed: O_2 consumption with a Clark-type electrode; mitochondrial function, oxidative stress parameters and calcium levels by fluorescence microscopy; ER stress markers and NFκB-p65 protein levels by western blotting.

Results: Our results showed that MitoQ prevented the enhanced ROS production and O_2 consumption and decreased GSH levels found under HG. MitoQ was also able to reduce protein levels of the ER stress markers (GRP78 and P-eif2α) and the proinflammatory nuclear transcription factor NFκB-p65, both of which increased under HG.

Conclusion: Our findings suggest that MitoQ treatment modulates mitochondrial function and thereby ameliorates endoplasmic reticulum stress and NFκB activation and thus has potential benefits as a treatment for insulin resistance-related diseases such as T2D.

Non standard abbreviations: ΔΨm, mitochondrial membrane potential; CMFDA, 5-chloromethylfluorescein diacetate; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; ER, endoplasmic reticulum; FCS, fetal calf serum; Fluo-4 AM, (acetyloxy)me-thyl ester; GRP78, glucose-regulated protein 78 chaperone; GSH, gluthatione; HG, hyperglycaemia; INS-1E, pancreatic β cells isolated from insulinoma; MitoQ, mitoquinone; NFκB, nuclear factor kappa B; NG, normoglycaemia; P-eif2α, phosphorylated eukaryotic translation initiation factor 2 alpha; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methylester; T2D, type 2 diabetes; TPP, triphenylphosphonium; WB, western blotting.

Introduction

During the onset of type 2 diabetes (T2D), glucose homeostasis is altered due to an impairment of the compensatory response of β cells [1]. In addition to the destruction of pancreatic β cells [2,3], T2D is also associated with oxidative stress and mitochondrial dysfunction [4,5,6], with mitochondria representing the main source of reactive oxygen species (ROS) in mammalian cells. Therefore, the increase in ROS levels that takes place in pancreatic β cells under hyperlipidaemia and hyperglycaemia, is one of the key events leading to the impairment of β cell function in T2D [7], although the exact underlying mechanisms are yet to be elucidated.

Under normal conditions, pancreatic β cells metabolize glucose via glycolysis and the tricarboxylic acid cycle in order to generate ATP. During this process, ROS are released and counteracted by the cell antioxidant defences. Moreover, β cells modulate blood glucose concentration by releasing insulin, which occurs when the enhanced metabolism of glucose in β cells increases the ATP/ADP ratio and leads to a rapid influx of Ca^{2+}, which in turn triggers the release of insulin. Hyperglycaemic conditions (HG) such as those found in T2D patients lead to increased ROS production [8,9]. This situation can be exacerbated under hyperlipidaemia, during which oxidation of free fatty acids-derived acetyl CoA by the TCA cycle and β-oxidation is enhanced, leading again to increased ROS production [10]. As pancreatic β cells are highly susceptible to oxidative damage [11], antioxidant therapies that improve or avoid oxidative stress may help ameliorate damage.

Oxidative stress has also been related to endoplasmic reticulum (ER) stress under insulin resistance conditions [12]. In fact, ER stress plays an important role in β cell impairment under hyperlipidaemic and hyperglycaemic conditions [13,14], and constitutes a key mediator of β cell apoptosis [15]. Furthermore, it has been reported

that ER stress signalling might be triggered by oxidized LDL through the induction of P-eif2α, ATF6 and IRE1α in vascular cells [16]. In addition, oxidative stress can activate NFκB, leading to changes in proinflammatory gene expression, eventually inducing cardiovascular impairment [17].

Therefore, therapies that decrease mitochondrial impairment, oxidative stress and, consequently, ER stress may be of use in the treatment of T2D and its deleterious effects [4]. For this reason, mitochondria-targeted antioxidants are emerging as potential protective therapies against oxidative damage in cardiovascular diseases, T2D and other important diseases [18,19]. The most widely used mitochondria-targeted antioxidant is mitoquinone (MitoQ), an ubiquinone derivate that specifically targets mitochondria by covalent attachment to a lipophilic triphenylphosphonium (TPP) cation [20,21]. Due to the considerable mitochondrial membrane potential (ΔΨm), this cation is accumulated within mitochondria inside cells.

Therefore, the aim of the current study was to evaluate the effect of the mitochondria-targeted antioxidant MitoQ on oxidative stress, ER stress and NFκB in the pancreatic β cell line INS-1E, as a widely used beta-cell surrogate, under normoglycaemic (NG) and HG conditions.

Materials and methods

Cell culture and treatments

The insulinoma cell line INS-1E pancreatic β cell was cultured in a humidified chamber with 5% CO_2 in RPMI-1640 medium (Biowest, Nuaillé, France) at 11.1 mM glucose supplemented with 5% (vol./vol.), heat-inactivated foetal calf serum (FCS), 10 mM HEPES, 2mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol, as previously described [22]. All

experiments were performed on cells from passages 40-50. After 4 days of pre-culture in T75 flask, cells were maintained a further 2 days at 11.1 mM glucose (NG) or were exposed to a high glucose concentration (25 mM, HG) [23]. Cells were then transferred to T25 flasks or 48-well plates and, upon 85% confluency, were treated for 3 hours with 0.5 μM MitoQ or decyl-TPP, in order to evaluate the non-specific effects of mitoQ. Decyl-TPP is similar in hydrophobicity to MitoQ but without the ubiquinol antioxidant moiety [24]. Treatments did not affect viability and morphology of these cells.

Measurement of O_2 consumption

INS-1E β cells were detached by trypsinization, counted with a Scepter 2.0 cell counter (Millipore Iberica, Madrid, Spain), resuspended (5×10^6 cells/mL) in Hanks' balanced salt solution (Sigma Aldrich, Missouri, US) and placed in a gas-tight chamber. Mitochondrial O_2 consumption was then measured with a Clark-type O_2 electrode (Rank Brothers, Bottisham, U.K.) [25]. An inhibitor of the electron transport chain, sodium cyanide (10^{-3} mol/L), was used to confirm that O_2 consumption was mainly mitochondrial (95-99%).

Membrane potential (ΔΨm), ROS production, glutathione (GSH) and calcium levels measurement

Fluorescence probes tetramethylrhodamine methylester (TMRM; 5×10^{-6} mol/l), MitoSOX (5×10^{-6} mol/l), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 5×10^{-6} mol/l), 5-chloromethylfluorescein diacetate (CMFDA; 1×10^{-6} mol/l), and (acetyloxy)methyl ester (Fluo-4 AM; 1×10^{-6} mol/l) were employed to estimate ΔΨm, mitochondrial ROS, total ROS, GSH content and calcium levels, respectively. For these measurements, cells were seeded in 48-well plates and incubated for 30 min with the

respective fluorescent probe. Fluorescence was then measured by fluorometry using a fluorescence microscope (IX81; Olympus) coupled to the static cytometry software "ScanR" (Olympus). Nuclei were visualized with Hoechst 33342. Measures of fluorescence are expressed in arbitrary units. Experiments were performed in triplicate and 16 images per well were recorded and analysed. All fluorochromes were purchased from Thermo Fisher Scientific, Waltham, US.

Western blotting (WB)

Total protein extracts from INS-1E β cells were obtained following the previously described treatments by lysing on ice for 15 min with an extraction buffer (400 mM NaCl, 20 mM HEPES pH 7.5, 20% Glycerol, 0.1 mM EDTA, 10 μM Na_2MoO_4 and 0.5%, and Nonidet P-40), containing protease inhibitor mixture (10 mM NaF, 1 mM Na_3VO_4, 10 mM PNP, and 10 mM β-glycerolphosphate) and dithiothreitol 1mM, and performing centrifugation at 4°C for 15 min. Concentration of the isolated supernatant containing protein extract was determined using the BCA protein assay kit (Thermo Fisher Scientific, IL, US). Twenty-five micrograms of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with the following primary antibodies: anti-GRP78 rabbit polyclonal antibody (Abcam, Cambridge, MA), anti-P-eif2α-pS52 rabbit polyclonal antibody (Life Technologies, California, US), anti-NFκB-p65 rabbit polyclonal antibody (Abcam, Cambridge, MA) and anti-actin rabbit polyclonal antibody (Sigma Aldrich, Missouri, US). Blots were incubated with the secondary antibody HRP goat anti-rabbit (Millipore Iberica, Madrid, Spain) and developed for 2 min with ECL plus reagent (GE Healthcare, LC, UK) or Supersignal West Femto (Thermo Fisher Scientific, IL, US). The protein signal was detected by chemiluminescence and visualized in a Fusion FX5 acquisition

system (Vilbert Lourmat, Marne La Valle´e, France). Images were analysed and quantified by densitometry using Bio1D software (Vilbert Lourmat, Marne La Valle´e, France) and protein bands were normalized to the expression of actin in the same sample.

Statistical analysis

Data analysis was performed with SPSS 17.0. Bar graphs show mean ± SEM. Data were compared with a one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test. Significant differences were considered when $p<0.05$.

Results

Mitochondrial Function

An O_2 electrode was used to monitor the rate of O_2 consumption in INS-1E β cells with and without the mitochondria targeted antioxidant MitoQ. The O_2 consumption by the cells was mainly mitochondrial, since addition of sodium cyanide resulted in almost complete (95-99%) inhibition of O_2 consumption (not shown). Rates of O_2 consumption in β cells treated and not treated with the mitochondrial targeted antioxidant MitoQ remained constant under NG. Interestingly, although HG enhanced O_2 consumption rates ($p<0.05$), MitoQ-treated β cells showed a downward trend under these conditions (Figure 1A, B). No changes were observed with respect in $\Delta\Psi m$, measured as TMRM fluorescence, in any of the conditions studied (Figure 1C).

INS-1E β cells exhibited higher levels of MitoSOX oxidation under HG than under NG conditions, which is consistent with an increase in mitochondrial ROS

production; however, MitoQ decreased MitoSOX oxidation specifically in the cells cultured under HG (Figure 1D, $p<0.01$).

None of these oxidative stress parameters were affected by treatment with the control compound decyl-TPP, which has the same mitochondrial targeting moiety as MitoQ but lacks the antioxidant effect.

Oxidative stress parameters

Total ROS fluorescence, evaluated by DCFH-DA fluorescence, was significantly higher in untreated cells under HG when compared with NG ($p<0.05$). Interestingly, MitoQ treatment decreased total ROS ($p<0.05$) to values similar to those observed under NG conditions, thus revealing a decrease in oxidative stress under HG. MitoQ did not alter total ROS in INS-1E β cells under NG (Figure 2A).

On the other hand, CMFDA fluorescence, which is proportional to the content of the free thiol form of GSH in INS-1E β cells, decreased under HG ($p<0.05$) and was restored in the presence of MitoQ ($p<0.05$), suggesting a protective role. MitoQ did not alter the levels of GSH in NG conditions (Figure 2B).

These results highlight that HG conditions induce oxidative stress in INS-1E β cells and that MitoQ prevents it by reducing levels of ROS and preserving antioxidant content. None of the oxidative stress parameters were affected by treatment with the control compound decyl-TPP.

ER stress

As oxidative stress is closely related to ER stress, we investigated whether UPR activation occurred in INS-1E β-cells by assessing protein levels of the ER stress markers GRP78 and P-eif2α.

INS-1E β cells cultured under HG displayed enhanced protein levels of the glucose-regulated protein 78 (GRP78) chaperone (Figure 3A) and phosphorylated eukaryotic translation initiation factor 2 alpha (P-eif2α, Figure 3B) with respect to control cells ($p<0.01$), which pointed to activation of the UPR in response to HG. These effects were reversed in HG conditions by the addition of MitoQ ($p<0.01$, Figure 3A and 3B). MitoQ did not modify protein levels in NG conditions.

Fluo4-AM fluorescence, employed to measure calcium content, showed a similar pattern as that of ER stress proteins. Whereas calcium levels remained constant in INS-1E β cells with or without MitoQ under NG conditions, there was an increase in intracellular calcium levels in the HG condition that was reverted in the presence of MitoQ, suggesting a preventive effect (Figure 3C). Decyl-TPP treatment did not affect any of these parameters.

Levels of NFκβ-p65

INS-1E β cells showed an increase in NFκB-p65 levels (Figure 4, $p<0.01$) under HG conditions, but MitoQ treatment returned NFκB-p65 protein levels to those observed under NG conditions (Figure 4, $p<0.01$), thus suggesting an anti-inflammatory effect. MitoQ did not modify NFκB-p65 levels under NG conditions. Decyl-TPP treatment did not affect NFκB-p65 protein expression.

Discussion

In the present study we demonstrate that HG induces an increase of mitochondrial oxygen consumption, mitochondrial ROS production, ER stress markers GRP78 and P-eif2α, calcium levels and NFκB-p65 protein expression, while, on the contrary, HG decreases GSH levels in pancreatic β cells. All of these effects were restored by treatment with the antioxidant MitoQ.

INS-1E is a cell line that displays stable glucose responsiveness and faithfully mimics the function of normal pancreatic islets. In addition INS-1E are responsive to known potentiators of glucose signalling, which makes them of valuable for studying the mechanisms involved in the regulation of insulin secretion. It is important to highlight their suitability for testing potential therapeutic agents for diabetes treatment, such as mitochondrial targeted antioxidants [26].

HG is closely related to oxidative stress and mitochondrial dysfunction [27,28]. In this sense, we have previously demonstrated that leukocytes from T2D patients presented oxidative stress, mitochondrial dysfunction and ER stress [27]. Furthermore, different studies have shown beneficial effects of mitochondrial antioxidant enzymes by protecting against oxidative stress under HG [29]. For these reasons, we considered whether mitochondria-targeted antioxidants could exert beneficial effects under HG, as we have previously demonstrated in leukocytes from T2D patients [30]. In fact, MitoQ has been shown to be an effective therapeutic strategy for diabetic nephropathy in the $Ins2^{+/-AkitaJ}$ mouse model [18], and promotes the survival and function of pancreatic β cells when subjected to glucotoxicity and glucolipotoxicity by improving insulin secretion [31]. In addition to the above, we selected MitoQ for the present study due to its biocompatibility and safety at levels that have shown efficacy. Previous studies have shown that TPP^{+} compounds, such as MitoQ, when used at typical culture concentrations, can affect mitochondrial function depending on the linker group but not on antioxidant properties [32] and under our conditions, MitoQ did not disrupt mitochondrial function and the decyl-TPP control compound was not protective.

HG and hyperlipidaemia are related to mitochondrial ROS production in pancreatic β cells during T2D [7,31]. In fact, β cells are particularly susceptible to damage by ROS because of their reduced expression of antioxidant enzymes [33]. In

relation to this, the results of the present study show that treatment with MitoQ reduces mitochondrial O_2 consumption under HG conditions, as well as total and mitochondrial ROS, and enhances GSH levels, thus exhibiting an antioxidant effect. These results are in line with those of previous studies showing that MitoQ reduces ROS and modulates antioxidant activities such as glutathione peroxidase (GPX1) under oxidative stress conditions in propionic acidemia patient-derived fibroblasts [34] and in leukocytes from T2D patients [30]. Moreover, it is important to highlight that mitochondria-targeted antioxidants have also been demonstrated to maintain mitochondrial morphology, to restore intracellular ATP levels and to increase β cell survival under glucotoxic and glucolipotoxic conditions [31].

It is well known that ER stress and oxidative stress are related [27,35], which in turn links ER stress to mitochondrial ROS. In this sense, several studies have focused their research on the contribution of ER stress to the development of insulin resistance and T2D [36, 37]. In addition, ER stress has been implicated in apoptosis of pancreatic β cells in a diabetic mouse model [38], and of leukocytes in T2D patients [27, 39]. Furthermore, Sage *et al*. [40] showed GRP78, sXBP1 and CHOP levels to be positively correlated with glucose levels in leukocytes from patients with metabolic syndrome. In accordance with these data, our present findings demonstrate that HG enhances levels of ER stress markers such as GRP78, P-eif2α, and intracellular calcium levels. Interestingly, MitoQ treatment reverses these effects, suggesting that this molecule ameliorates the mitochondrial disruption that leads on to ER stress.

It is well known that oxidative stress leads to pro-inflammatory responses. Specifically, it has been reported that the increase of ROS production may activate the pro-inflammatory nuclear factor NFκB, thus contributing to insulin resistance [41,42]. In this sense, we have previously reported a significant increase in NFκB expression in

leukocytes of T2D patients [30]. For this reason, we decided to explore whether the antioxidant MitoQ has an effect on p65-NFκB protein expression in pancreatic β cells under HG. Our results show an increase in the protein NFκB-p65 under HG, which was reverted in the presence of MitoQ, thus identifying this molecule as a mitochondrial antioxidant capable of modulating not only oxidative stress and ER stress, but also inflammatory response. Furthermore, previous studies have demonstrated that glucose intolerance in obese mice is attenuated after inhibition of the IKKβ/NFκB pathway [43], which suggests this signaling pathway is a central player in the development of T2D. For this reason, we foresee MitoQ treatment as an option for the prevention and/or treatment of insulin resistance and, hence, T2D.

Overall, our findings provide a better understanding of the pathophysiological mechanisms occurring in pancreatic β cells under HG. Importantly, our data show that treatment with MitoQ modulates mitochondrial function, ER stress and the NFκB signalling pathway, suggesting that this compound exerts beneficial effects that can be used to treat insulin resistance-related diseases such as T2D and/or obesity.

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Disclosure Statement

We declare that there is no conflict of interests.

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Figure 1

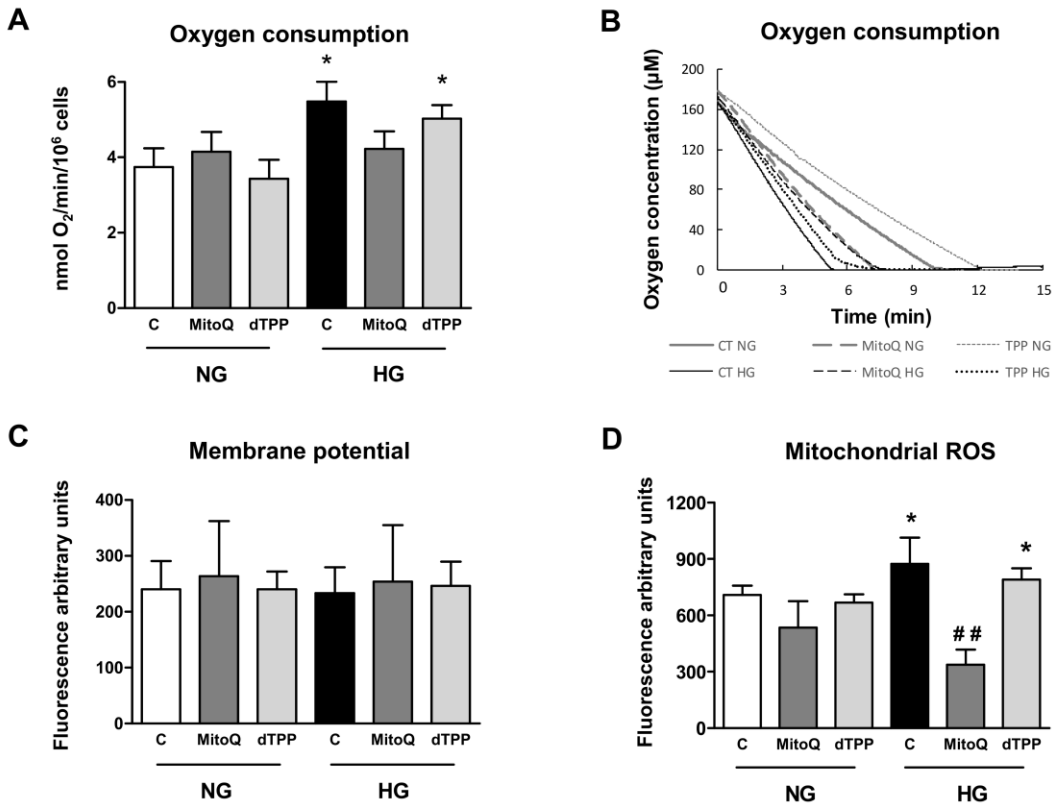

Figure 1. Effects of MitoQ (30 min, 0.5 μM) on mitochondrial function in pancreatic β cells under normoglycaemia and hyperglycaemia. (A) Oxygen consumption in a closed respiration chamber measured as nmol O_2/min/10^6 cells. **(B)** Representative traces showing the rate of O_2 consumption in a closed respiration chamber. **(C)** Mitochondrial membrane potential measured as TMRM fluorescence by fluorometry. **(C)** Mitochondrial ROS production measured as MitoSOX fluorescence by fluorometry. *$p<0.05$ with regard to control in normoglycaemia. $^{\#\#}p<0.01$ when comparing groups treated and not treated with MitoQ in the hyperglycaemic condition (data represented as mean ± SEM, n=6).

Figure 2

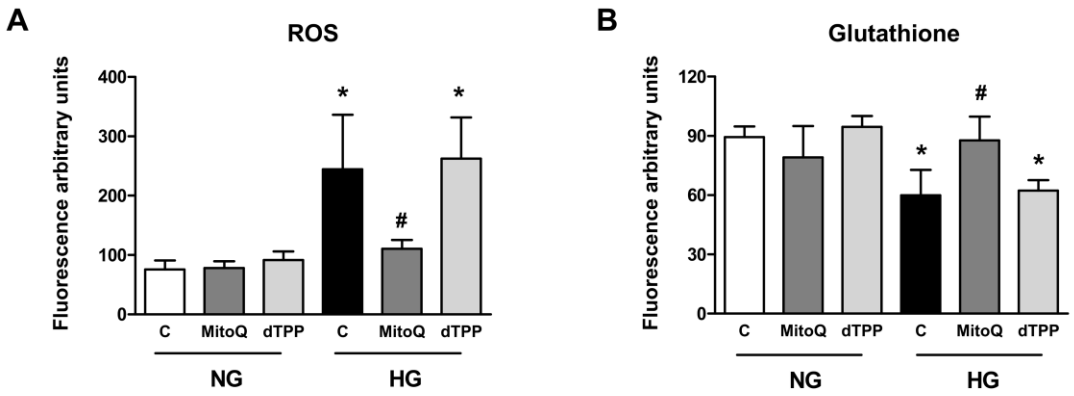

Figure 2. Effects of MitoQ (30 min, 0.5 μM) on oxidative stress parameters in pancreatic β cells under normoglycaemia and hyperglycaemia. (A) ROS production measured as DCFH-DA fluorescence by fluorometry. **(B)** GSH levels measured as CMFDA fluorescence by fluorometry. *$p<0.05$ with regard to control in normoglycaemia. $^{\#}p<0.05$ comparing groups treated and not treated with MitoQ in the hyperglycaemic condition (data represented as mean ± S.E.M, n=6).

Figure 3

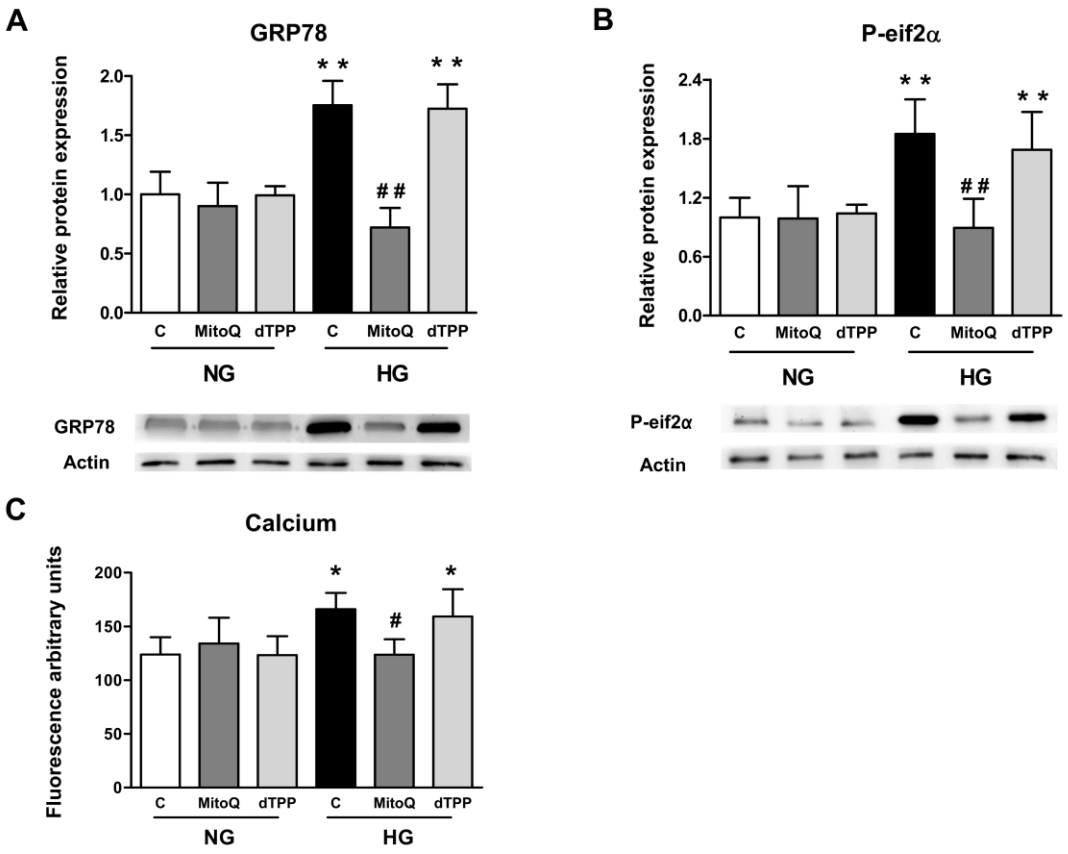

Figure 3. Evaluation of endoplasmic reticulum stress markers in pancreatic β cells under normoglycaemia and hyperglycaemia in the presence and absence of MitoQ (30 min, 0.5µM). (A) Protein levels of GRP78 and representative WB images. **(B)** Protein levels of P-eif2α and representative WB images. **(C)** Levels of intracellular calcium measured as Fluo-4 AM fluorescence by fluorometry. *p<0.05 and **p<0.01 with regard to control in normoglycaemia. $^{\#}$p<0.05, $^{\#\#}$p<0.01 when comparing groups treated and not treated with MitoQ in the hyperglycaemia condition (data represented as mean ± S.E.M, n=6).

Figure 4

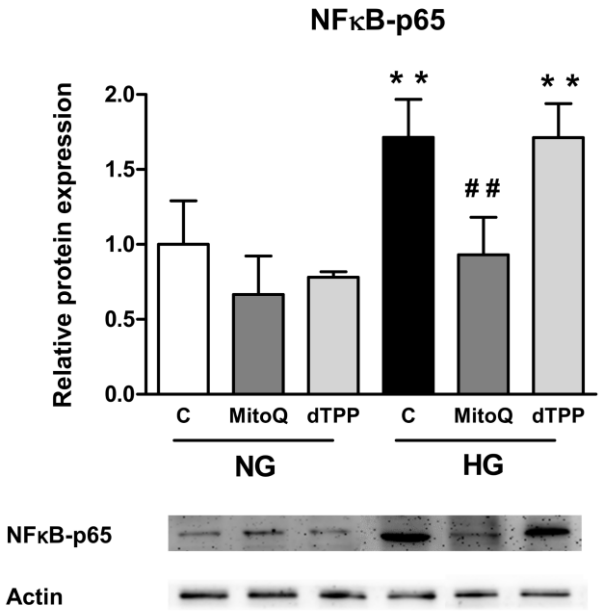

Figure 4. Evaluation of NFκB-p65 protein expression in pancreatic β cells under normoglycaemia and hyperglycaemia in the presence and absence of MitoQ (30 min, 0.5 μM). Protein levels of NFκB-p65 and representative WB images. **p<0.01 with regard to control in normoglycaemia. ##p<0.01 comparing groups treated and not treated with MitoQ in the hyperglycaemia condition (data represented as mean ± S.E.M, n=6).