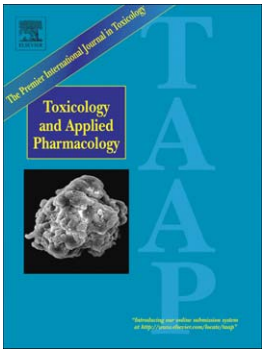

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The level of menadione redox-cycling in pancreatic β-cells is proportional to the glucose concentration: role of NADH and consequences for insulin secretion

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Abstract

Pancreatic β-cells release insulin in response to elevation of glucose from basal (4-7 mM) to stimulatory (8-16 mM) levels. Metabolism of glucose by the β-cell results in the production of low levels of reactive oxygen intermediates (ROI), such as hydrogen peroxide (H_2O_2), a newly recognized coupling factor linking glucose metabolism to insulin secretion. However, high and toxic levels of H_2O_2 inhibit insulin secretion. Menadione, which produces H_2O_2 via redox cycling mechanism in a dose-dependent manner, was investigated for its effect on β-cell metabolism and insulin secretion in INS-1 832/13, a rat β-cell insulinoma cell line, and primary rodent islets. Menadione-dependent redox cycling and resulting H_2O_2 production under stimulatory glucose exceeded several-fold those reached at basal glucose. This was paralleled by a differential effect of menadione (0.1-10 µM) on insulin secretion, which was enhanced at basal, but inhibited at stimulatory glucose. Redox cycling of menadione and H_2O_2 formation was dependent on glycolytically-derived NADH, as inhibition of glycolysis and application of non-glycogenic insulin secretagogues did not support redox cycling. In addition, activity of plasma membrane electron transport, a system dependent in part on glycolytically-derived NADH, was also inhibited by menadione. Menadione-dependent redox cycling was sensitive to the NQO1 inhibitor dicoumarol and the flavoprotein inhibitor diphenylene iodonium, suggesting a role for NQO1 and other oxidoreductases in this process. These data may explain the apparent dichotomy between the stimulatory and inhibitory effects of H_2O_2 and menadione on insulin secretion.

Keywords

Insulin secretion, redox cycling, NADH, cytosolic oxidoreductase, plasma membrane electron transport (PMET), NQO1

Introduction

Pancreatic β-cells, which comprise more than 75% of the mass of the islets of Langerhans, release insulin in response to elevation of blood glucose concentrations from fasting, basal levels (4-6 mM) to post-meal, stimulatory levels (7-16 mM) (reviewed in (Jitrapakdee *et al.*, 2010)). Insulin secretion from pancreatic β-cells is contingent upon glucose metabolism. Extracellular glucose is immediately sensed by the β-cell as it enters the cell via the low affinity high capacity GLUT-2 transporter (reviewed in (Thorens, 2004) and is rapidly metabolized via glycolysis. Pyruvate, the end product of glycolysis, enters the TCA cycle via both pyruvate carboxylation and decarboxylation routes, resulting in the increase in activity of cytosolic and mitochondrial metabolic pathways. This results in an increase of the ATP/ADP ratio, closure of the K_{ATP} channels, depolarization of the plasma membrane, calcium influx, and enhanced pyruvate cycling coupled to the production of NADPH (reviewed in (Jensen *et al.*, 2008)). All these processes work in synchrony to trigger insulin release from the pancreatic β-cell.

Glucose metabolism itself is responsible for the generation of low, physiological levels of reactive oxygen intermediates (ROI) (Bindokas *et al.*, 2003) such as H_2O_2 (Pi *et al.*, 2007), a pro-oxidant that is generated from superoxide by superoxide dismutase or spontaneous dismutation. β-cells express relatively low levels of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase when compared with other tissues (Tiedge *et al.*, 1997; Robertson and Harmon, 2007), suggesting that β-cells might be more sensitive than other cell types to both oxidant signaling as well as oxidative insult. Indeed, at high levels (.05 and 1 mM), H_2O_2 is known to interfere with glucose metabolism in β-cells, hyperpolarize the plasma membrane, lower the ATP/ADP ratio, and block glucose-stimulated insulin release (Krippeit-Drews *et al.*, 1999; Maechler *et al.*, 1999; Sakai *et al.*, 2003; Rebelato *et al.*, 2010) and it is thought that chronic exposure to nonphysiologically high levels of glucose causes β-cell toxicity through the generation of oxidative damage (Evans *et al.*, 2002; Piro *et al.*, 2002; Evans *et al.*, 2003; Robertson *et al.*, 2003). However, attempts to bolster antioxidant defense in β-cells through overexpression of antioxidant enzymes

or direct treatment with antioxidants reduced the ability of β-cells to release insulin in response to stimulatory glucose (Pi *et al.*, 2007; Leloup *et al.*, 2009) and treatment of β-cells with low doses of H_2O_2 (1-4 μM) stimulated insulin secretion in the presence of basal glucose levels (Pi *et al.*, 2007).

Menadione is an exogenous quinone, similar in structure to ubiquinone, the membrane-bound electron carrier. Like other quinones, menadione can undergo redox cycle, which involves enzymatic reduction by cellular oxidorecuctases, followed by autooxidation in the presence of molecular oxygen, generating superoxide ($O_2^{\bullet-}$) and subsequently H_2O_2 (Bolton *et al.*, 2000).To date, the effects of quinones such as menadione on β-cell metabolism and insulin secretion have not been investigated in sufficient depth. Although menadione (1 mM) was shown to stimulate insulin secretion at basal glucose (MacDonald, 1991b), this dose is quite toxic and the reported insulin secretion may represent lysis of the cells and subsequent release of insulin from damaged cells, as the viability of cells exposed to this level of menadione was not addressed in that study. In contrast, other studies reported that menadione at 10 and 50 μM caused a decrease in insulin secretion at stimulatory glucose (Malaisse *et al.*, 1978b; MacDonald, 1991b). Thus, these studies suggest that menadione exerts opposite effects on insulin release depending on the level of glucose present and this study was undertaken to elucidate basis of this dichotomy.

The effects of menadione on insulin secretion may be due in part to redox cycling and the consequent generation of H_2O_2. In contrast to the transient exposure to H_2O_2 that occurs following the direct addition of H_2O_2 to the cells, the exposure of cells to menadione allows the sustained generation of H_2O_2 in a dose dependent manner that might better reflect the physiological release of H_2O_2 during intermediary metabolism of glucose (Bindokas *et al.*, 2003). Therefore, a wide range of H_2O_2 levels can be produced dependent on the dose of menadione (Adam *et al.*, 1990), at levels similar to or greater than those involved in cellular signaling. In the current work, we employed menadione at the sub-toxic dose range (1-10 μM) (Tiedge *et al.*, 1998) to generate a controlled and sustained release of H_2O_2 in the INS-1 832/13 insulinoma cell line, which exhibits key metabolic features of native islet β-cells and presents robust insulin secretion in response to elevation in glucose levels from 2 (basal, non-stimulatory) to 16

(stimulatory) mM (Hohmeier *et al.*, 2000). We demonstrate that in both INS-1 832/13 cells and in isolated pancreatic islets, menadione-stimulated H_2O_2 formation is supported by the application of glucose, but not any other metabolic fuels shown to stimulate insulin secretion, such as methyl-succinate, methyl-pyruvate, or glutamine/leucine. Furthermore, we demonstrate that the level of applied glucose dictates the level of H_2O_2 produced via redox-cycling under a given dose of menadione. This information provides mechanistic insight into menadione's dichotomous effect on insulin secretion: under stimulatory glucose conditions, the greater amount of H_2O_2 produced negatively affects the insulin secretory pathway, whereas the lower amount produced at basal glucose stimulates insulin secretion.

Methods

Reagents. 10-acetyl-3,7-dihydroxyphenoxazine (AMPLEX-RED) was obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase, quinones, and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Cells. Clonal INS-1 832/13 cells were provided by Dr. Christopher Newgard (Duke University) and were maintained and cultured as described previously (Hohmeier and Newgard, 2004). KRB buffer (140 mM NaCl, 30 mM HEPES pH 7.4, 4.6 mM KCl, 1 mM $MgSO_4$, 0.15 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, and 2 mM $CaCl_2$), supplemented with glucose as indicated in each experiment, used in place of cell culture medium for most assays. For treatments greater than two hours, KRB was supplemented with 0.5% w/w bovine serum albumin. All experiments with INS-832/13 cells were performed at ~90% confluence in 96 well plates (H_2O_2 studies) or 48-well plates (insulin secretion studies).

Animals and Islet isolation. All procedures were performed in accordance with the Institutional Guidelines for Animal Care, in compliance with the United States Public Health Service Regulations. Male CD-1 mice (Charles River) were euthanized by Halothane. Pancreatic islets were isolated by collagenase (Roche) digestion, as previously described (Gray *et al.*, 2011) and used for experiments following overnight culture.

Hydrogen peroxide assay. The Amplex Red/horseradish peroxidase assay was used to quantify the production of H_2O_2 as previously described with minor modifications (Gray *et al.*, 2007). Reaction mixes contained 25 μM Amplex Red and 1 U/ml horseradish peroxidase in KRB buffer in the presence of glucose or other fuels as indicated in each experiment. Fluorescence was monitored using a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). The production of H_2O_2 was quantified by measuring the rate of increase in fluorescence (540 nm excitation, 595 nm

emission) due to conversion of Amplex Red to Resorufin by horseradish peroxidase in the presence of adherent cells. The rate of H_2O_2 formation was calculated based on a standard curve constructed with H_2O_2 in a concentration range of 0.5-16 μM.

PMET activity assay. PMET activity assays were performed as previously described with minor modifications (Gray *et al.*, 2011). Following a 40 min preincubation in KRB buffer supplemented with 2 mM glucose, INS-1 832/13 cells (96-well plates) were exposed to the indicated concentrations of glucose, menadione, or H_2O_2, and incubated in the presence of 250 mM WST-1 or 250 mM ferricyanide for 3 hr. The presence of the intermediate electron carriers 1-mPMS and CoQ1 was obligatory for WTS-1 and ferricyanide reduction, respectively (Gray *et al.*, 2011). Reduction of WST-1 to WST-1-formazan and the reduction of ferricyanide (Fe^{3+}) to ferrocyanide (Fe^{2+}) was monitored by the change in absorbance at 450 and 420 nm, with reference absorbances at 650 or 500 nm, respectively using a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).

Insulin secretion. INS-1 832/13 cells were pre-incubated for 1 hr in the presence of 2 mM glucose in KRB buffer. The amount of released insulin was determined after 60 min of static incubation in the presence of indicated amount of glucose and/or H_2O_2 or menadione using an ELISA kit (Alpco Diagnostics, Salem, NH). Data were normalized for protein content determined by the Micro-BCA Protein Assay kit (Pierce, Rockford, IL).

Cell viability and toxicity assays. All assays purchased from Promega (Madison, WI) were performed according to manufacturer's instructions using a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). CellTiter-Blue assays (cat # G8081) were performed by the addition of 20 μL of CellTiter-Blue dye to wells containing cell culture media, and measured by the increase in fluorescence (560 nm excitation, 590 nm emission). Increasing concentrations of glucose increased CellTiter-Blue activity (data not shown); therefore, all experiments were performed with equal concentrations of glucose. The trypan blue exclusion was performed as previously

described (Grankvist *et al.*, 1979). The GSH/GSSG-Glo assay (cat #V6611, Promega) was used to quantify oxidized glutathione and total glutatione (oxidized and reduced).

Statistical analysis. Data are expressed as means ± standard deviation. Significance was determined for multiple comparisons using 1 or 2-way Analysis of Variance (ANOVA) followed by the Bonferroni post test (Neter *et al.*, 1990).

Results

Insulin secretion is the primary function of pancreatic β-cells. A significant increase in insulin output occurs when glucose is raised from basal, non-stimulatory levels (2-4 mM) to stimulatory levels (10-16 mM). Previous studies showed that treatment of intact islets with either H_2O_2, or 10 μM menadione, whose intracellular metabolization generates H_2O_2, blunted insulin secretion under stimulatory glucose (Malaisse *et al.*, 1978a; Rebelato *et al.*, 2010). However, other studies showed that low levels of H_2O_2 stimulated insulin secretion at basal glucose levels (Pi *et al.*, 2007). No study has been performed so far in which menadione's effect was tested under the complete range of glucose concentrations and therefore consensus regarding the effect of menadione and its redox product H_2O_2 on β-cell metabolism are lacking. Because menadione induces the production of H_2O_2 in cells, we tested the effect of a dose response of menadione in INS-1 832/13 cells under both basal (2 mM) and stimulatory (16 mM) glucose levels. A basal glucose level of 2 mM was used since these cells are left-shifted in their glucose dose-response (Poitout *et al.*, 1996). As previously shown (Malaisse *et al.*, 1978b), elevated levels of menadione (10 uM) blunted secretion under stimulatory (16 mM) glucose levels (Figure 1A). In contrast, menadione (1-10 μM) stimulated insulin secretion at 2 mM glucose in a dose dependent manner (Figure 1A), consistent with the reported H_2O_2 –dependent insulin release at non stimulatory glucose (Pi *et al.*, 2007) (Figure 1B). To investigate if menadione-dependent formation of H_2O_2 is responsible for the observed increased secretion, cells were treated with pegylated catalase (PEG-CAT), a membrane permeable H_2O_2 scavenger. PEG-CAT blunted both glucose-stimulated insulin secretion (Figure 1C) and menadione-stimulated insulin secretion (Figure 1D), suggesting that H_2O_2 is a common factor required for insulin secretion.

Menadione redox cycling is dependent on both NADPH and NADH as substrates. Pancreatic islets β-cells respond to an increase in glucose concentration with an increase in the reduced-to-oxidized adenine nucleotide ratio ($NAD(P)H/NAD(P)^+$) (Malaisse *et al.*, 1979; Capito *et al.*, 1984; Sener and Malaisse, 1987; Heart *et al.*, 2007). Therefore we investigated the redox cycling of menadione and

similar quinones in β-cells under basal (2 mM) or stimulatory (16 mM) concentrations of glucose by measuring the rate of H_2O_2 formation. Menadione stimulated a dose-dependent increase in H_2O_2 formation at both basal and stimulatory glucose (Figure 2A). In a dose range of 1-30 μM, stimulatory (16 mM) glucose increased the rate of H_2O_2 formation as much as 4-fold over that of basal (2 mM) glucose. Similar results were found in isolated mouse islets, where menadione-dependent H_2O_2 production at 16 mM glucose reached 151 ± 15.5% of that at 4 mM glucose. Other quinones of similar chemical structure also showed elevated redox cycling at stimulatory glucose (Figure 2B-2F), while paraquat, a non-quinone redox cycling chemical, failed to stimulate H_2O_2 formation (data not shown). Higher passage number of INS-1 832/13 cells also resulted in decreased H_2O_2 formation in response to menadione treatment (data not shown). This is consistent with the passage-dependent decrease in metabolic function and glucose-stimulated insulin secretion reported for various β-cell lines (Zhang *et al.*, 1989; Clark *et al.*, 1990), including INS-1 cells (Ramanadham *et al.*, 2000).

The first step of glucose metabolism in β-cells is its phosphorylation by glucokinase, whose K_M for glucose is approximately 8.7 mM (Meglasson and Matschinsky, 1986). To test whether stimulation of the redox cycling of menadione by glucose demonstrates a similar K_m, INS-1 832/13 cells were treated with 10 μM menadione in the presence of glucose (2-84 mM) (Figure 3A). A dose-dependent increase in H_2O_2 was found with a K_m of 11.1 mM, similar to that of glucokinase. Treatment with the non-metabolizable glucose analog 2-deoxy-D-glucose failed to stimulate H_2O_2 formation (Figure 3A). Alloxan is a diabetogenic agent that functions in part through inhibition of glucokinase, blocking glycolysis in β-cells (Lenzen *et al.*, 1988). Pretreatment of β-cells with alloxan blunted menadione redox cycling at both basal and stimulatory levels of glucose, suggesting that glycolysis is required for menadione stimulated H_2O_2 formation (Figure 3B).

In addition to glucose, a number of other metabolic fuels have been shown to stimulate insulin secretion. These fuels primarily function through the direct stimulation of mitochondrial metabolism. Methyl succinate is metabolized via direct entry to mitochondrial complex 2 (MacDonald and Fahien, 1990); methyl pyruvate is thought to

be metabolized, after removal of the methyl group, directly by the mitochondria (Jijakli *et al.*, 1996); and the combination of glutamine and leucine activates mitochondrial glutamate dehydrogenase (Fahien *et al.*, 1988). While menadione redox cycling in the presence of 10 or 16 mM glucose was increased more than two-fold in INS-1 832/13 cells (Figure 4), all of the non-glycogenic secretagogues, applied at concentrations shown to stimulate metabolism and insulin secretion, failed to support menadione-mediated redox cycling. Since these other fuels are not metabolized via glycolysis, these data suggest that glycolytically-derived NADH, rather than mitochondrially-derived NAD(P)H, is important to mediate menadione redox cycling by glucose. Furthermore, methyl pyruvate, a membrane-permeable form of pyruvate, actually inhibited menadione-mediated redox cycling, consistent with pyruvate-mediated scavenging of H_2O_2 (reviewed in (Kao and Fink, 2010)).

Menadione-mediated redox cycling is dependent upon the availability of NADH or NADPH. NADH and NADPH are generated during glucose metabolism by reduction of their oxidized counterparts, NAD^+ and $NADP^+$, which results in an increase in reduced-to-oxidized nucleotides ratios ($NAD(P)H/NAD(P)^+$) following the rise in glucose concentration (Malaisse *et al.*, 1979; Capito *et al.*, 1984; Sener and Malaisse, 1987; Ivarsson *et al.*, 2005; Heart *et al.*, 2007). Any processes which further increase the reduced nucleotide pool have the potential to enhance redox cycling. The mitochondrial inhibitors cyanide (0.3-5 mM) and antimycin A (0.1-10 nM) stimulated menadione-mediated H_2O_2 formation at both basal and stimulatory glucose in a dose-dependent manner (Figure 5). Since glycolytically-derived NADH reoxidation is prevented when mitochondrial function is compromised, mitochondrial inhibitors may increase menadione redox cycling by increasing the available NADH pool. In addition to mitochondrial metabolism, plasma membrane electron transport (PMET) is another mechanism by which cytoplasmic NADH and NADPH can be oxidized. In PMET, electrons are passed from cytosolic NAD(P)H through the plasma membrane to extracellular acceptors (reviewed in (Principe *et al.*, 2011)). Previous work showed that glucose stimulated PMET activity in a dose-dependent manner (Gray *et al.*, 2011). Menadione was found to inhibit PMET in a dose-dependent manner (Figure 6A-6B). Since both PMET and menadione redox cycling consume NAD(P)H, menadione-

mediated inhibition of PMET activity may be due in part the reduced level of NAD(P)H available. Alternatively, H_2O_2 produced by menadione redox cycling might exert a toxic effect on the cells. Although H_2O_2 (50 uM) was able to inhibit PMET activity (Figures 6C and 6D), the dose required to do this was much greater than the concentration of H_2O_2 produced by menadione redox cycling, suggesting that it is direct competition for NAD(P)H that drives menadione-mediated inhibition of PMET activity.

Menadione redox cycling is mediated by the action of quinone oxidoreductase enzymes (Bolton *et al.*, 2000). Multiple oxidoreductases are capable of supporting redox cycling of quinones, and the identity of these enzymes varies by cell type. Pancreatic islet homogenates were previously shown to contain quinone reductase activity (Malaisse *et al.*, 1978a; MacDonald, 1991a). NQO1 is a quinone oxidoreductase that is a component of the plasma membrane electron transport system in β-cells (Gray *et al.*, 2011). Diphenylene iodonium (DPI), a flavoprotein inhibitor (Cross and Jones, 1986), was previously shown to block insulin secretion under stimulatory glucose levels in isolated rat islets (Morgan *et al.*, 2009). These authors suggested that DPI affected insulin secretion by modifying H_2O_2 levels. Therefore, DPI and dicoumarol, an inhibitor of NQO1 (Hosoda *et al.*, 1974), were tested for their ability to reduce menadione-mediated H_2O_2 formation in β-cells. Both inhibitors caused a dose-dependent decrease in H_2O_2 formation (Figure 7A and 7B).

Prolonged treatment with high and toxic levels of H_2O_2 are known to be detrimental to β-cells. INS-1 832/13 cells treated with H_2O_2 for 6 h experienced less toxicity in the presence of 16 mM glucose than at 2 mM glucose (Figure 8A), suggesting that glucose metabolism induces the production of biochemical pathways that protect against oxidative stress (Martens *et al.*, 2005). Glucose exhibited a similar protective effect against menadione toxicity (Figures 8B and 8C), suggesting that, although menadione produces more H_2O_2 in the presence of glucose (Figure 3A), the glucose-dependent increase in protection from oxidative stress also increases and protects against some of the H_2O_2 produced via redox cycling. Interestingly, menadione-induced glutathione oxidation was not affected by the presence of glucose (Figure 8D), suggesting an alternative biochemical pathway might be exhibiting this protection.

Discussion

Glucose metabolism in β-cells is prerequisite for insulin secretion. Glucose equilibrates rapidly across the β-cell plasma membrane via the GLUT-2 transporter, and the rate of glycolysis is largely regulated via glucokinase, whose Km (8.7 mM) acts as a glucose sensor (reviewed in (Deeney *et al.*, 2000)). A physiological transient increase in extracellular glucose following a meal therefore leads to an increase in intermediary metabolism, and the concomitant production of low levels of ROI, namely H_2O_2, from a number of intracellular sources, such as the mitochondrial electron transport chain and cytosolic oxidoreductases via PMET (Pi *et al.*, 2007; Leloup *et al.*, 2009; Gray and Heart, 2010; Gray *et al.*, 2011). In contrast to chronic hyperglycemia (constantly elevated glucose levels seen in uncontrolled diabetes), which leads to the generation of high levels of ROI, responsible for oxidative stress (Robertson *et al.*, 2003), low and physiological levels of H_2O_2 generated during normal postprandial glucose metabolism recently received attention in the β-cell field as a novel coupling factor linking glucose metabolism to insulin secretion (Pi *et al.*, 2007). Indeed, H_2O_2 is an important intracellular second messenger in many cell types (Kamata and Hirata, 1999).

The effect of H_2O_2 on cellular metabolism and signaling is dependent on its concentration: lower levels are generated as a consequence of intermediary metabolism under normal physiological situations and are involved in signaling, whereas higher levels are produced by oxidative insult such as exposure to high levels of ultraviolet radiation (Stone and Yang, 2006). This is supported by several indirect and direct observations: β-cells express low levels of antioxidant enzymes, and treatment of β-cells with antioxidants blunts insulin secretion (Mikawa *et al.*, 1997; Robertson and Harmon, 2007), suggesting that a low level of ROI, derived from the intracellular glucose metabolism is necessary to support insulin secretion. Likewise, low doses of H_2O_2 (1-4 μM) were shown to modestly stimulate insulin secretion at basal levels of glucose (Pi *et al.*, 2007)(Figure 1).

Previous studies of the effects of H_2O_2 on insulin secretion may have yielded conflicting results due to the nature of H_2O_2 generation and metabolism in cells and the experimental approaches used (reviewed in (Chance *et al.*, 1979)). A single addition of

H_2O_2 at non-toxic levels involved in signaling will not mimic the physiological generation of H_2O_2. Low doses of H_2O_2 given as a single addition (bolus) may be initially equivalent to physiological levels, but because H_2O_2 is rapidly degraded, the signal is immediately lost. Attempting to overcome the degradation of H_2O_2 by using an elevated dose can trigger toxicity. Neither method mimics the physiological situation in which a particular steady-state level of H_2O_2 is maintained through equal rates of production and degradation, such as that which occurs during elevation of postprandial glucose for 30-60 minutes following a meal. To mimic this physiological situation, H_2O_2 would have to be added in short intervals or delivered by perfusion to maintain steady state levels for that particular time period, an approach which is technically difficult to undertake. An alternative approach would be to employ a well-defined system capable of generating the desired levels of peroxide in a continuous fashion inside the cell.

Menadione is a redox cycling quinone shown by us (Figure 1) and others (Malaisse *et al.*, 1978b; MacDonald, 1991b) to impact insulin secretion. Like other quinones, menadione is reduced by cellular oxidoreductase enzymes, at the expense of NAD(P)H, and chemically reacts with molecular oxygen to produce O_2^- which is subsequently converted to H_2O_2, spontaneously or through the action of superoxide dismutase (Bolton *et al.*, 2000). Addition of menadione to β-cells therefore results in the production of H_2O_2 in a sustained and dose-dependent manner that may better reflect its natural production as a second messenger (Figure 2). The level of menadione-mediated H_2O_2 formation was also directly proportional to the level of glucose, reaching an apparent maximum at about 20 mM glucose (Figures 2 and 3). These results are consistent with the fact that pool size of reduced nucleotides NADH and NADPH, both of which serve as substrates for enzymes that redox cycle menadione (Bolton *et al.*, 2000), is dependent upon level of glucose. Furthermore, treatment of β-cells with the non-metabolizable glucose analog 2-deoxy-D-glucose or treatment with the glucokinase inhibitor alloxan abolished the enhancing effect of glucose on menadione redox cycling, suggesting that glycolysis is critical for this process. Likewise, several non-glycogenic insulin secretagogues which do not undergo glycolysis, methyl-succinate, methyl-pyruvate, and the combination of glutamine/leucine, did not stimulate menadione redox cycling (Figure 4). These data suggest that glycolysis is important for supporting fuel-

supported increases in menadione redox cycling and H_2O_2 generation. Interestingly, methyl pyruvate (10 mM and 16 mM), membrane permeable pyruvate analog, was found to suppress menadione redox cycling. Pyruvate has been shown to act as an antioxidant and to scavenge H_2O_2 via nonenzymatic oxidative decarboxylation (Giandomenico *et al.*, 1997). Indeed, we found that methyl pyruvate reduces the H_2O_2 levels in vitro, as determined by Amplex Red assay in cell-free solution (data not shown).

NADH is produced in glycolysis via by reduction of NAD^+ by glyceraldehyde phosphate dehydrogenase. In order for glycolysis to continue, NADH must be re-oxidized back to NAD^+. Transport of NADH to the mitochondria for its re-oxidation back to NAD^+ is accomplished via the malate/aspartate and glycerol phosphate shuttles (Eto *et al.*, 1999). We hypothesized that a glycolysis-dependent rise in NADH was responsible for enhanced redox cycling seen under stimulatory (16 mM) glucose. To test this, re-oxidation of glycolytically-derived NADH by the mitochondria was prevented by application of mitochondrial inhibitors. Application of antimycin A and cyanide, which block complex 3 and 4 of the mitochondrial electron transport chain, resulted in a potentiation of menadione redox cycling at both 2 and 16 mM glucose (Figure 5).

NADH may also be re-oxidized through trans-plasma membrane electron transport (PMET), a system that we've recently shown to be active within β-cells (Gray *et al.*, 2011). The PMET system oxidizes intracellular NADH and NADPH, while transferring electrons across the plasma membrane to extracellular targets (reviewed in (Gray and Heart, 2010)). PMET is stimulated by an increase in the concentration of glucose, due to a glucose-dependent increase in reducing equivalents NADH and NADPH. Menadione redox cycling, which consumes NAD(P)H, might be expected to inhibit PMET through competition for reduced substrates. Indeed, we found that even low micromolar concentrations of menadione were able to inhibit PMET activity (Figure 6). Altogether, these data suggest that glycolytically-derived NADH is a major source for menadione mediated H_2O_2 formation in β-cells (Figure 9).

NADH and NADPH are capable of supporting menadione-dependent redox cycling by serving as substrates for oxidoreductase enzymes; many oxidoreductase enzymes are capable of using both nucleotides (Lind *et al.*, 1990; Dicker and

Cederbaum, 1991; Jaiswal, 2000; Xia *et al.*, 2003). Earlier studies showed that islets have quinone reductase activity and that islet lysates consume NADPH and NADH in response to treatment with substrates such as CoQ_0 and menadione (Malaisse *et al.*, 1978a; MacDonald, 1991a). NQO1 is a prototypical menadione reductase found in many cell types, including the β-cell, where it is a component of the PMET pathway (Lind *et al.*, 1990; Gray *et al.*, 2011). We demonstrated that dicoumarol, an inhibitor of NQO1, blocked menadione redox cycling in a dose-dependent manner (Figure 7A). DPI, a non-specific flavoprotein inhibitor, exerted a similar effect.

While short exposure (30 min) to menadione did not adversely affect cell viability (data not shown), prolonged treatment (6 h) with either H_2O_2 or menadione caused toxicity, as assessed by several cell viability assays. Interestingly, in parallel to its capacity to facilitate redox cycling and menadione-dependent H_2O_2 production, 16 mM glucose increased cell viability in the presence of H_2O_2 or menadione (Figures 8A-8C), in agreement with the notion that glucose metabolism-dependent cytoprotective mechanisms are induced under physiologically elevated glucose (Martens and Pipeleers, 2009). Previous work showed that reduced glutathione is depleted in hepatocytes treated with menadione for 10 min (Gant *et al.*, 1988), and we also observed a depletion of reduced glutathione in INS-1 832/13 cells treated with menadione (Figure 8D). However, stimulatory glucose did not blunt this depletion, suggesting that glutathione is not responsible for the protection of INS-1 832/13 cells from menadione toxicity. Future studies are underway to further investigate the mechanism of glucose-dependent protection from oxidative stress.

Altogether, our data suggest a mechanism for both menadione-mediated stimulation and inhibition of insulin secretion, summarized in Figure 9. β-cells may require a physiological level of H_2O_2, generated via intermediary metabolism, to enable the secretion of insulin. However, the insulin secretory pathway is also sensitive to the toxic effects of H_2O_2. Our study outlines a mechanism by which menadione affects β -cell metabolism and insulin secretion, and demonstrates that the level of glucose is a critical determinant in the degree of redox cycling in the β-cell.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure Legends:

Figure 1. Effect of menadione, H_2O_2, and catalase on insulin secretion in INS-1 832/13 cells. Insulin secretion was measured in cells exposed to 1-10 μM menadione (A) or H_2O_2 (B) under both basal and stimulatory glucose. Effect of PEG-CAT on insulin secretion in response to 16 mM glucose and 10 μM menadione is shown in (C) and (D), respectively. Data, expressed as % insulin secretion obtained under 2 mM glucose (control), are means ± SE from 2-3 independent experiments performed in quadruplicate measurements.

Figure 2. Glucose enhances redox cycling of quinones in INS-1 832/13 cells. Cells were treated with basal (2 mM) or stimulatory (16 mM) concentrations of glucose in the absence or presence of 0.1-100 μM (A) menadione, (B) 1,4-naphthoquinone, (C) 1,2-naphthoquinone, (D) 2-methoxy-1,4-naphthoquinone, (E) 9,10-phenanthroquinone, and (F) acenaphthene. The rate of H_2O_2 formation was performed as described in Methods. Data are means ± SD (n=3) and are representative of 2 independent experiments. Asterisks (*) indicate a significant different ($p < 0.05$) between 2 mM and 16 mM glucose-treated samples as determined by ANOVA followed by a Bonferroni comparison.

Figure 3. Glucose-dependent enhancement of menadione redox cycling requires glycolysis. (A) INS-1 832/13 cells were treated with menadione (10 μM) and the indicated concentration of glucose or the non-metabolizable glucose analog 2-deoxy-D-glucose. H_2O_2 production was determined as described in Methods. 4 mM glucose and above are significantly different from 2 mM glucose-treated sample ($p < 0.05$). (B) Following a three hour pretreatment with alloxan, INS-1 832.13 cells were treated with glucose and/or menadione and analyzed for the production of H_2O_2 as in A. Data are means ± SD (n=3) and are representative of 2 independent experiments. Asterisk indicates significant difference for D-glucose treated samples from 2 mM treated controls ($p < 0.05$, one way ANOVA followed by a Bonferroni comparison).

Figure 4. Effect of insulin secretory fuels on menadione-dependent H_2O_2 formation. INS-1 832/13 cells were treated with 10 mM or 16 mM of the indicated fuel in the presence of menadione (10 μM) and production of H_2O_2 was measured as described in Methods. Values are expressed as fold change in the rate of H_2O_2 formation relative to the cells treated with 2 mM glucose in the absence of menadione (control). Data are means ± SD (n=3) and are representative of 2 independent experiments. Asterisks (*) indicate a significant increase from corresponding 2 mM control for each fuel. Pound symbols (#) indicate a significant decrease from control.

Figure 5. Mitochondrial inhibitors potentiate menadione-stimulated redox cycling in INS-1 832/13 cells. Cells were treated with glucose (2 mM or 16 mM) and menadione (10 μM) in the absence or presence of mitochondrial inhibitors cyanide (A) or Antimycin A (B). The rate of production of H_2O_2 was determined as described in Methods. Data are means ± SD (n=3) and are representative of 3 independent experiments.

Figure 6. Effect of menadione and H_2O_2 on PMET activity in INS-1 832/13 cells. Cells were treated with menadione (A,B) or H_2O_2 (C,D) in the presence of 2 mM or 16 mM glucose and analyzed for reduction of WST-1 (A,C) or ferricyanide (B,D). Data are means ± SD (n=3) and are representative of 2 independent experiments. Pound symbols (#) and asterisks (*) indicate significant difference from 2 mM and 16 mM glucose-treated samples without menadione or H_2O_2.

Figure 7. Dicoumarol and DPI inhibit menadione-mediated H_2O_2 formation. INS-1 832/13 cells exposed to 2 or 16 mM glucose were treated with dicoumarol (A) or DPI (B) in the presence of 10 μM menadione. Rate of H_2O_2 formation was determined as described in Methods. Data are means ± SD (n=3) and are representative of 3 independent experiments.

Figure 8. Glucose protects against H_2O_2 and menadione-induced toxicity in INS-1 832/13 cells. Cells were treated with H_2O_2 (A) or menadione (10 μM) or vehicle control (B) in the presence of basal (2 mM) or stimulatory (16 mM) glucose for 6 h. Cell survival was quantified by CellTiter-Blue. (C) Cells were treated as in (B) and analyzed

for viability by trypan blue exclusion after 6 h. (D) Oxidized and total glutathione was measured after 30 min exposure of cells to menadione or vehicle control in the presence of basal (2 mM) or stimulatory (16 mM) glucose.

Figure 9. Mechanism for menadione-stimulated insulin secretion. In pancreatic β-cells, NADH is produced via glycolysis and is re-oxidized by mitochondria (1) and plasma membrane electron transport (2). Treatment with menadione leads to the consumption of NADH and NADPH as menadione is reduced by oxidoreductases (3) to its semiquinone or hydroquinone form. These reduced forms chemically react with molecular oxygen to produce O_2^- and the parent compound, allowing sustained production of H_2O_2 which induces insulin secretion. Under stimulatory glucose, the rate of glycolysis is increased, resulting in greater production of NADH (via glycolysis) and NADPH (via pyruvate cycling (4), both of which increase the redox cycling of menadione and production of H_2O_2 to levels that inhibit insulin secretion.

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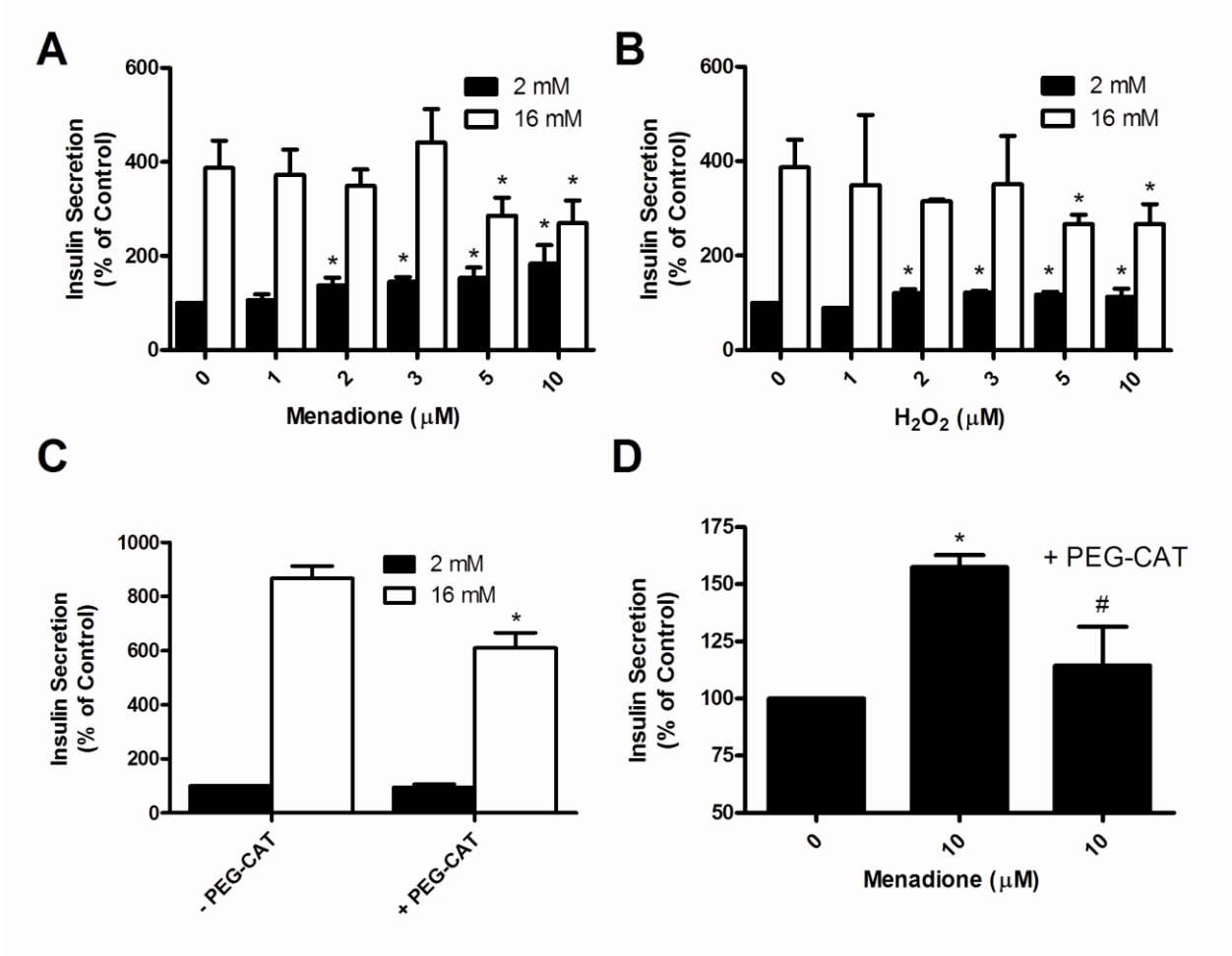
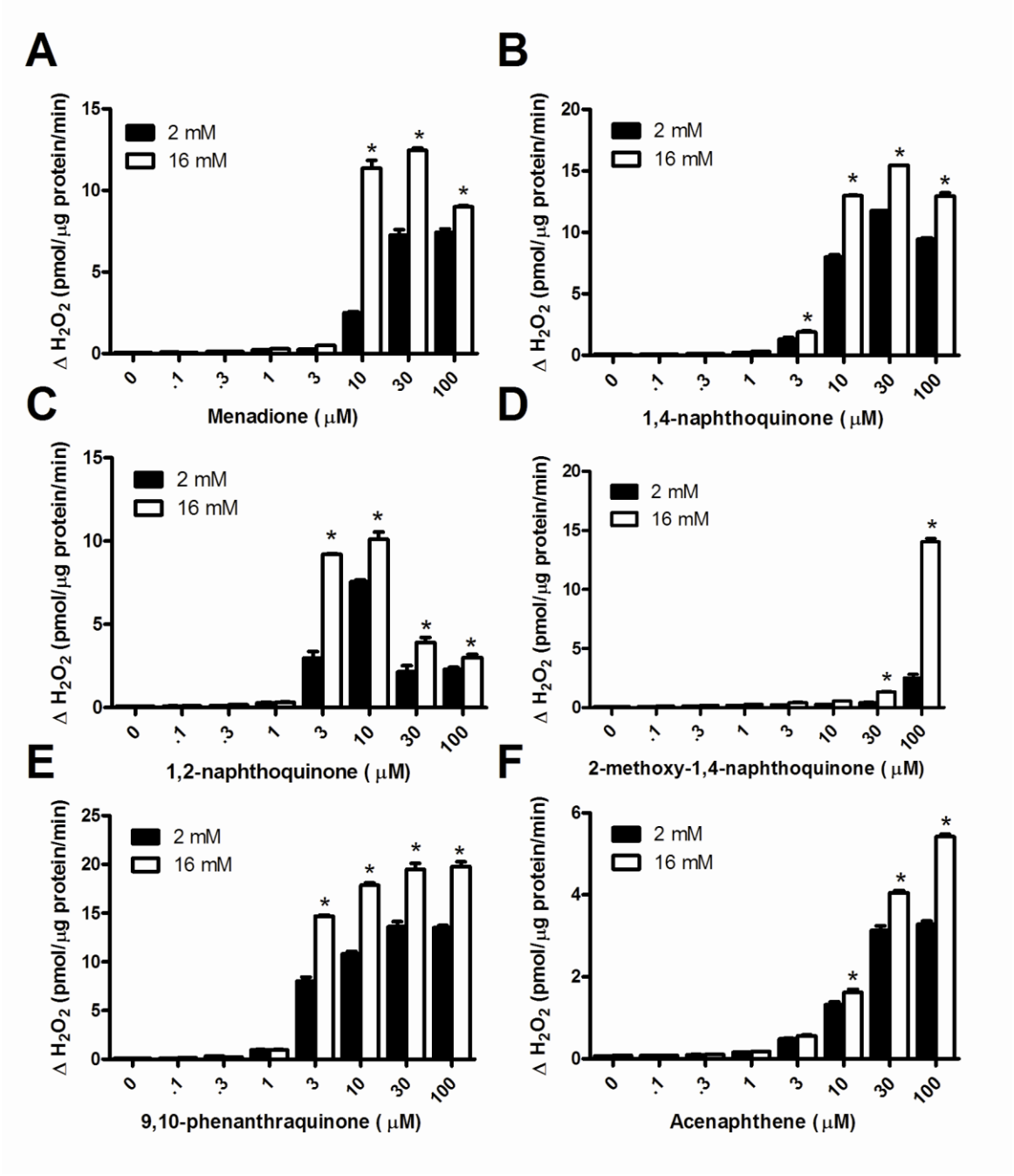


Fig. 1



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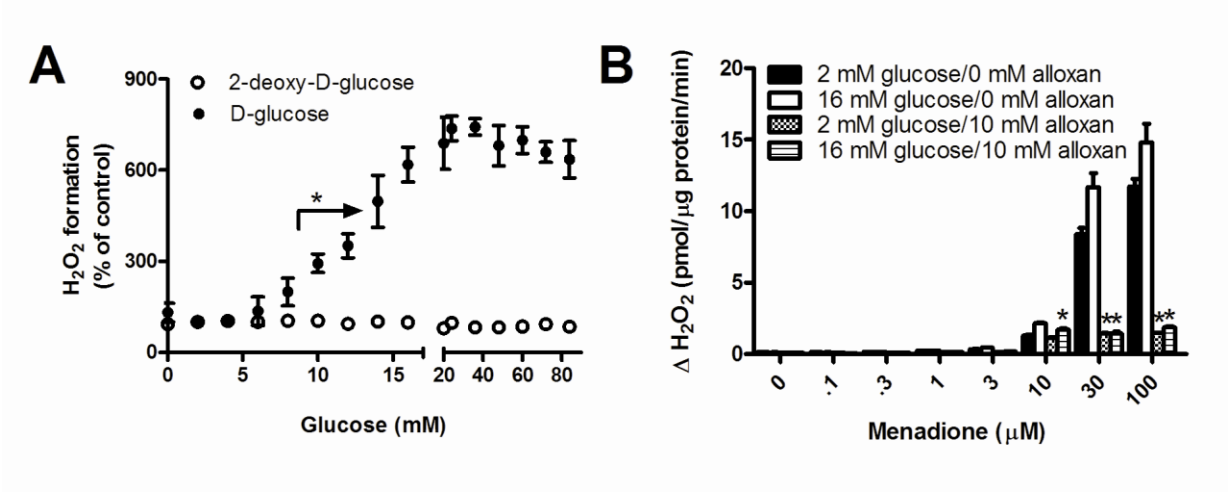

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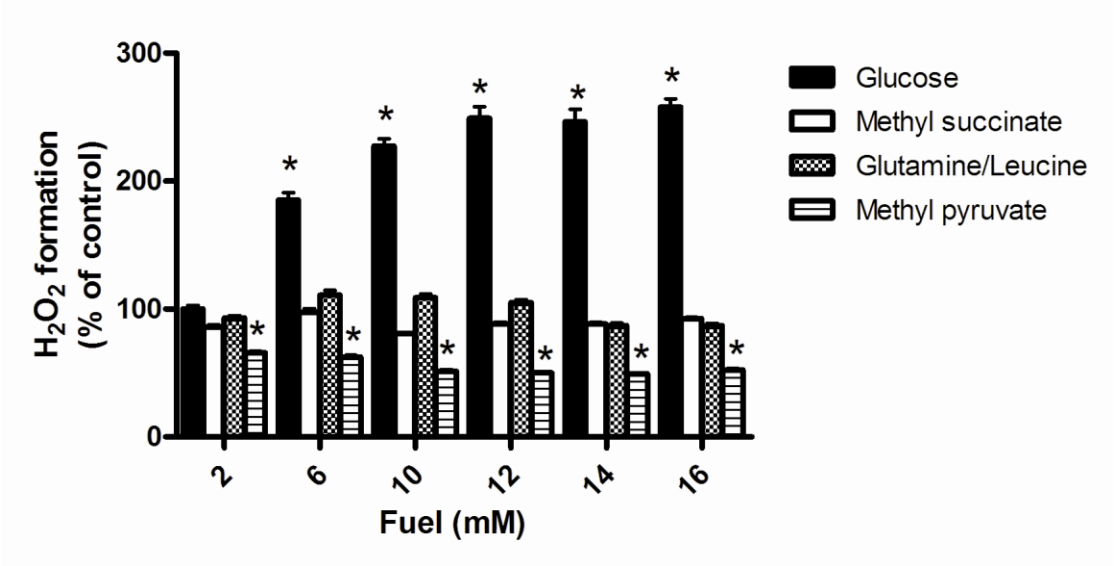

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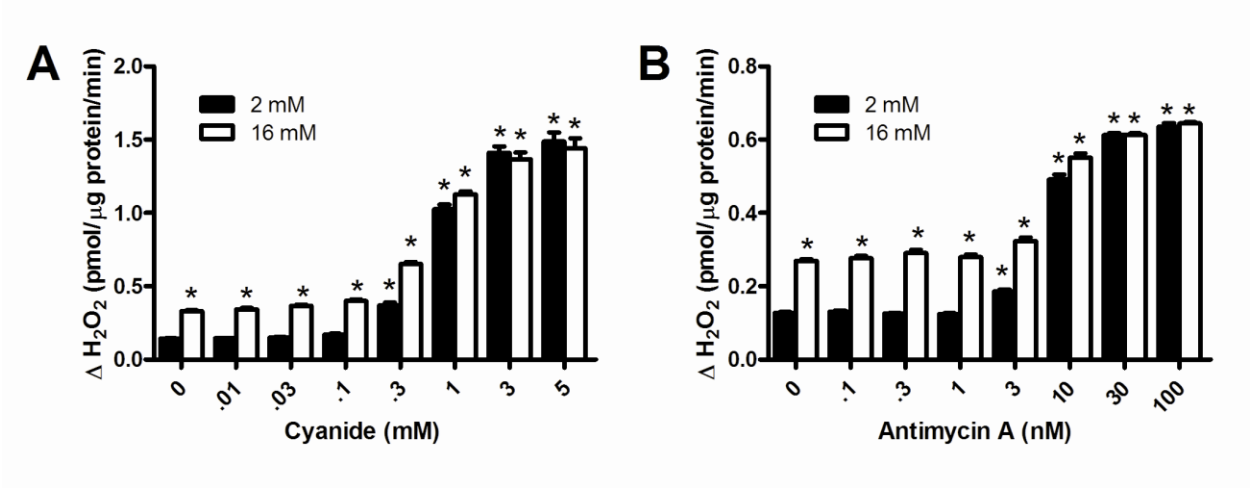

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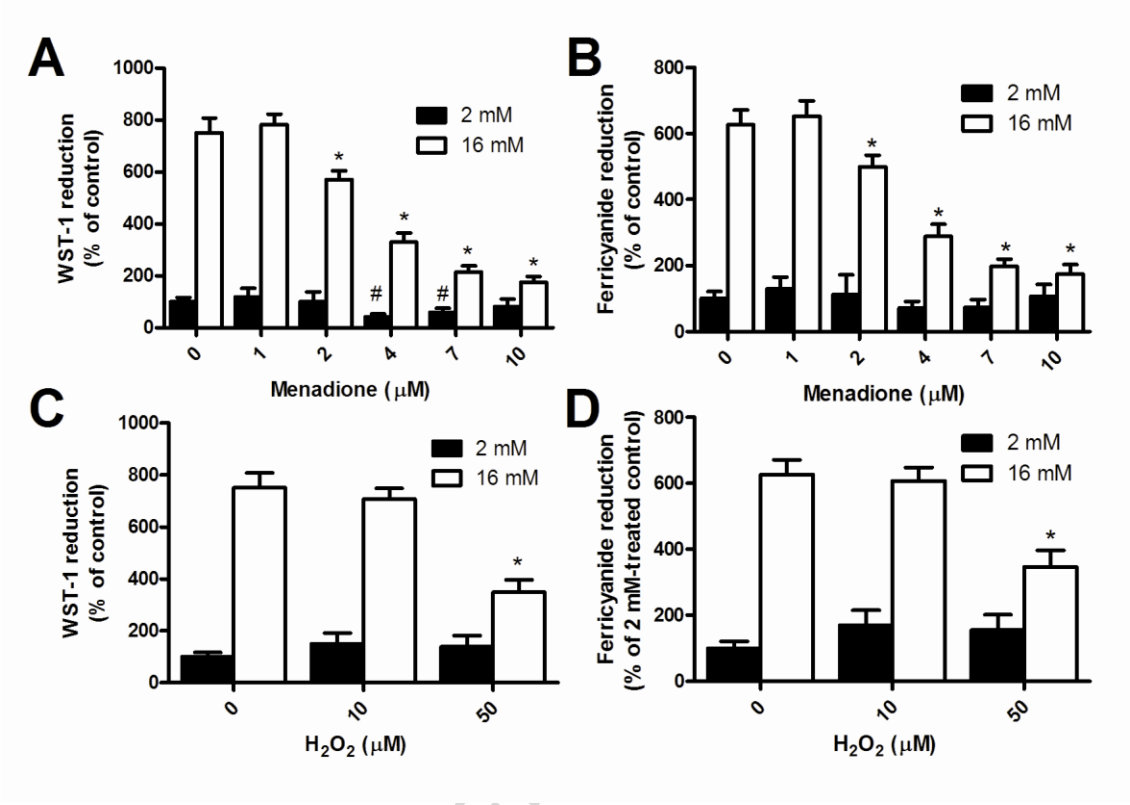

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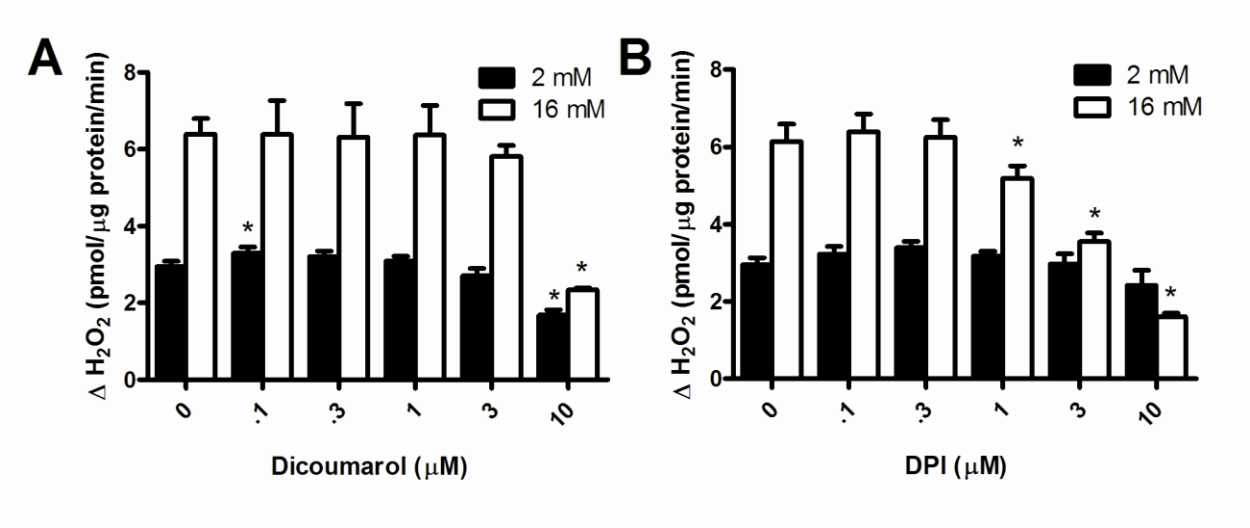

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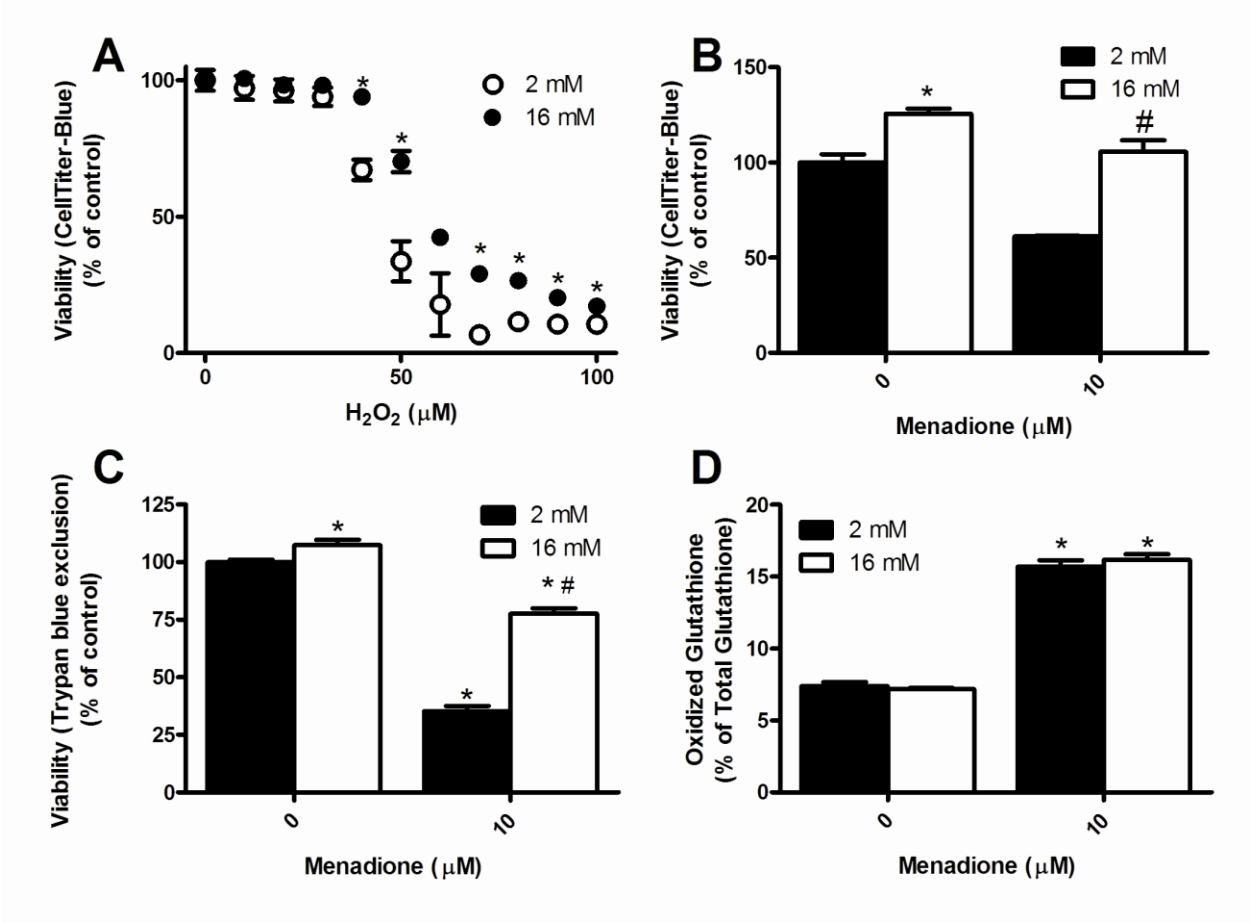

Fig. 8

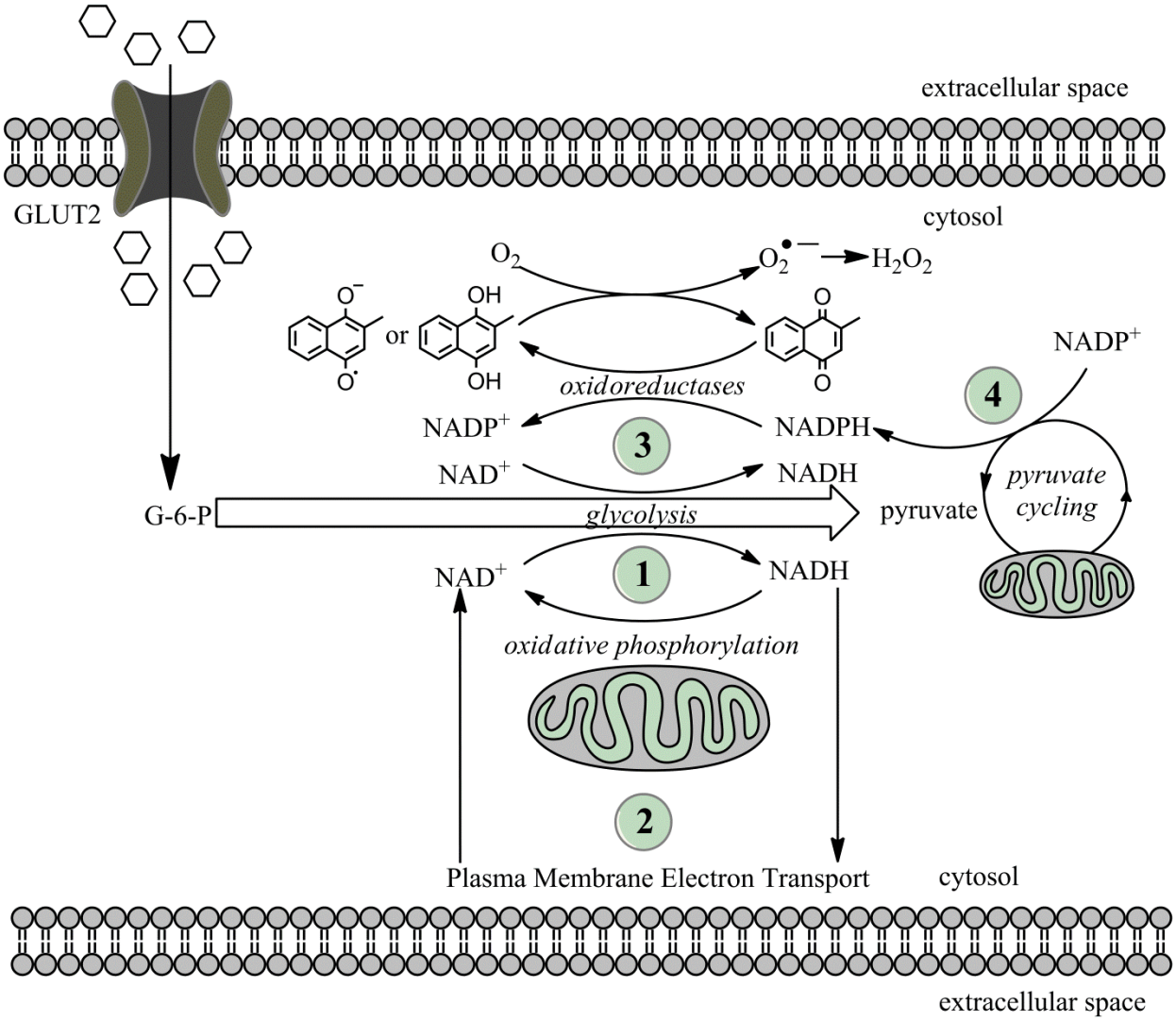

Fig. 9

Highlights

Menadione stimulation or inhibition of insulin secretion is dependent upon applied glucose levels.

Menadione-dependent H_2O_2 production is proportional to applied glucose levels

Quinone-mediated redox cycling is dependent on glycolysis