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ORIGINAL ARTICLE

Diabetogenic milieus induce specific changes in mitochondrial transcriptome and differentiation of human pancreatic islets

Thierry Brun[1,*], Ning Li[1], Alexis A. Jourdain[2], Pascale Gaudet[3,4], Dominique Duhamel[1], Jérémy Meyer[5], Domenico Bosco[5] and Pierre Maechler[1,*]

[1]Department of Cell Physiology and Metabolism, University of Geneva, Medical Center, Geneva, Switzerland, [2]Department of Cell Biology, University of Geneva, Sciences III, Geneva, Switzerland, [3]Swiss Institute of Bioinformatics (SIB), Geneva, Switzerland, [4]University of Geneva, Medical Center, Geneva, Switzerland and [5]Cell Isolation and Transplantation Center, Department of Surgery, Geneva University Hospital, Geneva, Switzerland

*To whom correspondence should be addressed at: Department of Cell Physiology and Metabolism, University of Geneva Medical Center, rue Michel-Servet 1, 1211 Geneva 4, Switzerland. Tel: +41 223795485; Email: thierry.brun@unige.ch (T.B.); Tel: +41 223795554; Email: pierre.maechler@unige.ch (P.M.)

Abstract

In pancreatic β-cells, mitochondria play a central role in coupling glucose metabolism to insulin secretion. Chronic exposure of β-cells to metabolic stresses impairs their function and potentially induces apoptosis. Little is known on mitochondrial adaptation to metabolic stresses, i.e. high glucose, fatty acids or oxidative stress; being all highlighted in the pathogenesis of type 2 diabetes. Here, human islets were exposed for 3 days to 25 mM glucose, 0.4 mM palmitate, 0.4 mM oleate and transiently to H_2O_2. Culture at physiological 5.6 mM glucose served as no-stress control. Expression of mitochondrion-associated genes was quantified, including the transcriptome of mitochondrial inner membrane carriers. Targets of interest were further evaluated at the protein level. Three days after acute oxidative stress, no significant alteration in β-cell function or apoptosis was detected in human islets. Palmitate specifically increased expression of the pyruvate carriers MPC1 and MPC2, whereas the glutamate carrier GC1 and the aspartate/glutamate carrier AGC1 were down-regulated by palmitate and oleate, respectively. High glucose decreased mRNA levels of key transcription factors (HNF4A, IPF1, PPARA and TFAM) and energy-sensor SIRT1. High glucose also reduced expression of 11 mtDNA-encoded respiratory chain subunits. Interestingly, transcript levels of the carriers for aspartate/glutamate AGC2, malate DIC and malate/oxaloacetate/aspartate UCP2 were increased by high glucose, a profile suggesting important mitochondrial anaplerotic/cataplerotic activities and NADPH-generating shuttles. Chronic exposure to high glucose impaired glucose-stimulated insulin secretion, decreased insulin content, promoted caspase-3 cleavage and cell death, revealing glucotoxicity. Overall, expression profile of mitochondrion-associated genes was selectively modified by glucose, delineating a glucotoxic-specific signature.

Introduction

In pancreatic β-cells, mitochondria participate to glucose-stimulated insulin secretion (GSIS) by generating metabolic signals, thereby connecting glucose usage to corresponding regulated insulin exocytosis (1–4). Mitochondrial dysfunction impairs GSIS and may promote dedifferentiation and ultimately death of the β-cells, two events central in the pathogenesis of type 2 diabetes (T2D) (5,6). Such defects are favored by chronic exposure to elevated concentrations of glucose and fatty acids (7–11). The respective contributions of specific diabetes-associated stresses

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to human β-cell dysfunction remain to be delineated through the associated changes in specific gene expression. In contrast to the acute potentiation of GSIS by fatty acids, chronic exposure induces β-cell lipo-dysfunction characterized by elevated basal insulin release and impaired glucose response, both *in vitro* (12) and *in vivo* (13). Indeed, deleterious effects of fatty acid infusion have been reported in individuals genetically predisposed for T2D (13). In most *in vitro* studies, unsaturated fatty acids (e.g. oleate) do not affect cell viability, whereas saturated fatty acids (e.g. palmitate) may promote ER stress and apoptosis (11,12,14–16). The cytotoxicity of palmitate increases with the exposure time and concomitant high glucose concentrations, whereas it decreases with the elevation of serum in the culture medium. The global glucolipotoxicity concept proposes that high glucose plus fatty acids induce pleiotropic alterations accompanying obesity, the metabolic syndrome and ultimately T2D (17,18). While the role of glucolipotoxicity in human physiopathology is still debated, its molecular basis requires active nutrient metabolism, in turn altering lipid partitioning and mitochondrial function with potential production of reactive oxygen species (ROS) (19). Mitochondria are both a source of ROS and a sensitive target of oxidative attacks (20–22). Accordingly, mitochondrial defects and oxidative stress have been proposed to contribute to the diabetic state (19,23).

Candidate genes have been associated with both monogenic diabetes (neonatal diabetes and Maturity-Onset Diabetes of the Young, MODY) and polygenic forms of T2D (24,25). Among them, mutations in the transcription factors HNF4A and IPF1/PDX1 are associated with MODY1 and MODY4, respectively. Their function is required for correct β-cell development, differentiation and function (26). Recently, a point mutation in the NADH-dependent histone deacetylase sirtuin *SIRT1* gene was identified in a family with T1D, revealing a monogenic form of this autoimmune disease (27). Mitochondrial DNA (mtDNA) carries only 37 genes that encode 13 polypeptides, 22 tRNAs and 2 ribosomal RNAs (3,5). The polypeptides encoded by the mtDNA are all subunits of the OXPHOS complexes composing the electron transport chain, with complementary contribution of the nuclear genome. Ablation in the β-cell of the nuclear gene encoding the mitochondrial transcription factor A (Tfam) results in mtDNA depletion and deficient oxidative phosphorylation (28). These mice are hyperglycemic, their islets exhibit impaired GSIS and increased β-cell death; reproducing some characteristics of human diabetes. Moreover, Pdx1 suppression in adult mice reduces Tfam expression, coinciding with mitochondrial dysfunction and defective GSIS (29). These mouse models with selected gene alterations call for better delineation of the expression profile of the mitochondrial machinery in human islets under both normal and diabetogenic conditions.

Beside components of the TCA cycle and the electron transport chain, the mitochondrial carriers are of particular interest, although still poorly characterized, in particular regarding stress-responses. These solute carriers transport a selection of metabolites across the inner mitochondrial membrane and are encoded by the nuclear *SLC25* gene family (30,31). To date, ∼14 inherited diseases (mostly neurological disorders) have been associated with alterations in *SLC25* genes, including the carriers for citrate/isocitrate CIC (*SLC25A1*), aspartate/glutamate AGC1 (*SLC25A12*) and AGC2 (*SLC25A13*), carnitine/acylcarnitine CAC (*SLC25A20*) and glutamate GC1 (*SLC25A22*) (31). UCP2 (SLC25A8), originally referred to as an uncoupling protein, has recently been characterized as a mitochondrial C4 metabolite transporter (32). Regarding β-cells, the role of only some mitochondrial carriers has been substantiated over the last years (33). For instance, we reported that down-regulation of AGC1 or GC1 reduces GSIS (34,35). Using pharmacological and siRNA approaches, similar conclusions were raised for CIC, phosphate PiC (*Slc25a3*), malate (dicarboxylate) DIC (*Slc25a10*), 2-oxoglutarate OGC (*Slc25a11*), as well as for the newly identified mitochondrial pyruvate carriers MPC1 and MPC2 (33,36–41). Conversely, overexpression of the NADH shuttle component AGC1 enhances mitochondrial metabolism and GSIS (42). Recently, we characterized mitochondrion-associated genes in INS-1E β-cells exposed to glucose or fatty acids, revealing stress-specific signatures (43). Based on this previous work using a rat cell line, we are now reporting identification of mitochondrial molecular targets and their respective gene expression in human islets in response to specific metabolic stresses, in order to deconstruct global glucolipotoxicity effects and uncover the specific contributions of different stressors. These diabetogenic conditions applied side by side in the same study include exposure to high glucose concentrations, to saturated versus unsaturated fatty acids, and to transient oxidative stress.

Results

Effects of diabetogenic milieus on expression of differentiation factors and energy sensors

Chronic effects of metabolic stresses on β-cell differentiation and function were determined in human islets at the end of a 3-day exposure to diabetogenic milieus, the physiological 5.6 mM glucose (G5.6) condition serving control. In an initial step to validate the control islet transcriptome, this one was compared with non-endocrine human HeLa and 143B cells. Messengers of the β-cell-specific transcription factors *HNF4A*, *IPF1* and *MAFA* were undetectable in non β-cells (Fig. 1A). In contrast, transcript levels of *TFAM* and pyruvate carboxylase *PC* were higher in HeLa and 143B cells compared with islets. Interestingly, in control conditions, we observed a negative correlation between the BMI of donors (ranging from 23.2 to 28.6 kg/m^2) and both *IPF1* and *MAFA* transcript levels ($P < 0.05$, Supplementary Material, Fig. S1A–D). Expression of essential β-cell genes were repressed by high glucose (G25), such as *HNF4A* and *IPF1* involved in β-cell differentiation (44), *TFAM* for mitochondrial biogenesis, the energy-sensor *SIRT1* and the nuclear receptor *PPARA* regulating fat metabolism (Fig. 1A). Analysis at the protein level in the same islet batches revealed that for some genes protein levels did not correlate with corresponding mRNA, suggesting gene-specific post-transcriptional regulation. IPF1, TFAM and PGC1α proteins were not changed in G25 condition, while SIRT1 and SIRT4 were increased (Fig. 1B–D; Supplementary Material, Fig. S1E–G). In agreement with previous report (45,46), G25 induced a strong increase in the lipogenic enzyme acetyl-CoA carboxylase ACC, whereas the levels of PC and glutamate dehydrogenase *GLUD1* were unchanged. Regarding fatty acid exposure, palmitate (and not oleate) up-regulated SIRT1, both at transcript and protein levels (Fig. 1C). Oleate treatment decreased expression of *MAFA* and *IPF1* (Fig. 1A), the latter being also down at protein level (Fig. 1B, D; Supplementary Material, Fig. S1E–G). Hydrogen peroxide exposure caused a specific repression of *MAFA*, confirming the high sensitivity oh this transcription factor to oxidative stress (47). Expressions of other transcription factors and sirtuins were not altered by H_2O_2 treatment, indicating selective effects induced by oxidative stress. Taken together, data show that high glucose modified expression of major differentiation factors and energy sensors, while fatty acids modestly affected the same genes, thereby showing stress-specific responses.

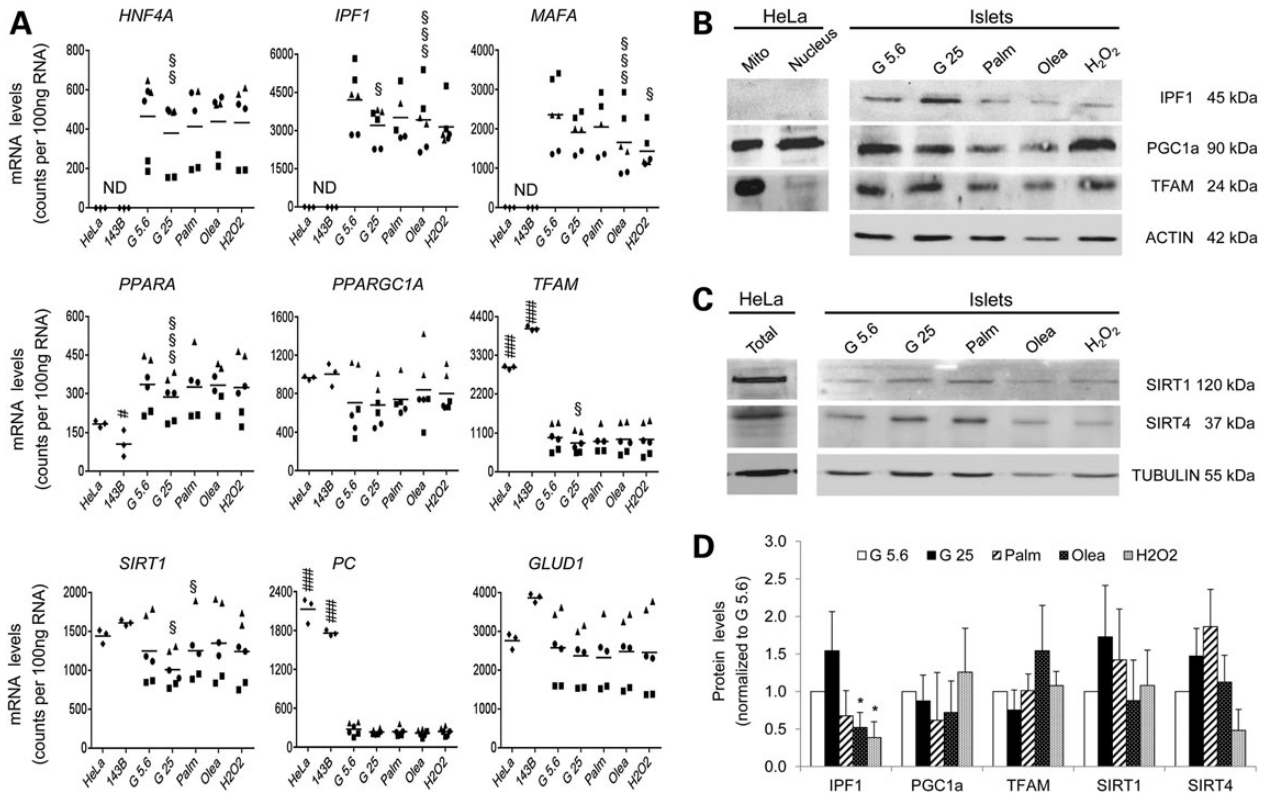


Figure 1. Transcriptome of β-cell differentiation factors and energy sensors in human islets after stress exposure. Islets obtained from three donors were exposed for 3 days to defined culture conditions: 25 mM glucose (G25), 0.4 mM palmitate (Palm), 0.4 mM oleate (Olea) and transient oxidative stress at Day 0 (200 μM H_2O_2 for 10 min). Culture at physiological 5.6 mM glucose (G5.6) served as no-stress control. (**A**) Transcript levels of major transcription factors, of the energy-sensor sirtuin *SIRT1*, of the mitochondrial enzymes pyruvate carboxylase *PC* and glutamate dehydrogenase 1 *GLUD1* were quantified as described in Materials and Methods. The values represent absolute numbers of transcript molecules measured in two human non-endocrine cell lines of reference (HeLa and 143B cells) and in three human islet preparations (filled circle represents donor 1, triangle donor 2 and square donor 3), both in physiological (G5.6) and metabolically stressful conditions. Transcript levels were normalized to those of *NUP155*, *HDAC1* and *TUBULIN* and expressed as counts per 100 ng total RNA. Means of three independent experiments each done in duplicate are represented (horizontal bars). ND not detectable (below detection limit) $^{\#}$ $P < 0.05$, $^{\#\#\#}$ $P < 0.005$, comparing non-endocrine cells versus islets (G5.6); § $P < 0.05$, §§§ $P < 0.005$ versus corresponding G5.6 control islets. (**B** and **C**) Representative immunoblottings showing levels of (B) the transcription factors IPF1, TFAM, the co-activator PGC1α, (C) the energy-sensors SIRT1 and SIRT4 from donor 2 (total extracts) under different experimental conditions. Total cell extracts as well as mitochondria (Mito) and nuclei isolated from HeLa cells were used as additional controls. (**D**) Quantitative analysis of relative band densities normalized to ACTIN and TUBULIN as shown in (B, C) is presented as means ± SEM of at least four independent experiments. Results are expressed as protein levels normalized to G5.6 values. *$P < 0.05$ versus G5.6 control islets.

Adaptation of mitochondrial respiratory chain subunits and associated proteins

Regarding islet specificity, we first observed that transcript levels of numerous respiratory chain subunits were lower in human islets compared with non-endocrine cells (Figs 2A and 3A). High glucose (G25), but not fatty acids, altered islet expression of respiratory chain subunits II (*SDHB*) and III (*UQCRC2*); while all metabolic stress conditions preserved expression of subunits I (*NDUFA1*), IV (*COX4*) and V (*ATP5C1*); of carriers associated with ATP production (*PiC* for phosphate import or *NCLX* controlling mitochondrial calcium homeostasis) and of mitochondrial structure (*STOML2*, *PHB1*) (Fig. 2A and B). G25-reduced expression of 11 mtDNA-encoded (MT) subunits of OXPHOS complexes: 7 subunits of complex I NADH dehydrogenase (*MT-ND1*/*ND6*), 2 subunits of complex IV cytochrome c oxidase (*MT-CO1*, *MT-CO2*) and 2 subunits of complex V ATP synthase (*MT-ATP6*, *MT-ATP8*). Consistent with moderate effects induced by fatty acids on transcription factors, oleate (not palmitate) decreased transcript levels of *MT-ND5*, *MT-ND6* and MT-CO3 (Fig. 3A). Interestingly, all the metabolic stresses down-regulated the complex V subunit *MT-ATP8*. Immunoblotting revealed preservation at the protein level of the respiratory chain complex subunits (Fig. 3B; Supplementary Material, Fig. S2A–C). Of note, expressions of the OXPHOS subunits and associated proteins were not altered by H_2O_2 treatment. Our results show that chronic exposure to high glucose caused a marked and selective down-regulation of transcript levels encoded by mitochondrial genome.

Adaptation of mitochondrial solute carriers responsible for metabolite transport

The second group of the mitochondrial components we analyzed mediates metabolite transport across the inner mitochondrial membrane. For those known to be required for GSIS in rodent β-cell lines (33–37,39–43), expression was assessed both at mRNA (Fig. 4A) and protein levels (Fig. 4B; Supplementary Material, Fig. S2A–C). As for respiratory chain subunits, transcript levels of some solute carriers were lower in human islets compared with non-endocrine cells. G25 increased transcript levels (Fig. 4A) of the carriers for aspartate/glutamate (*AGC2*) and malate (*DIC* and *UCP2*), the latter being also

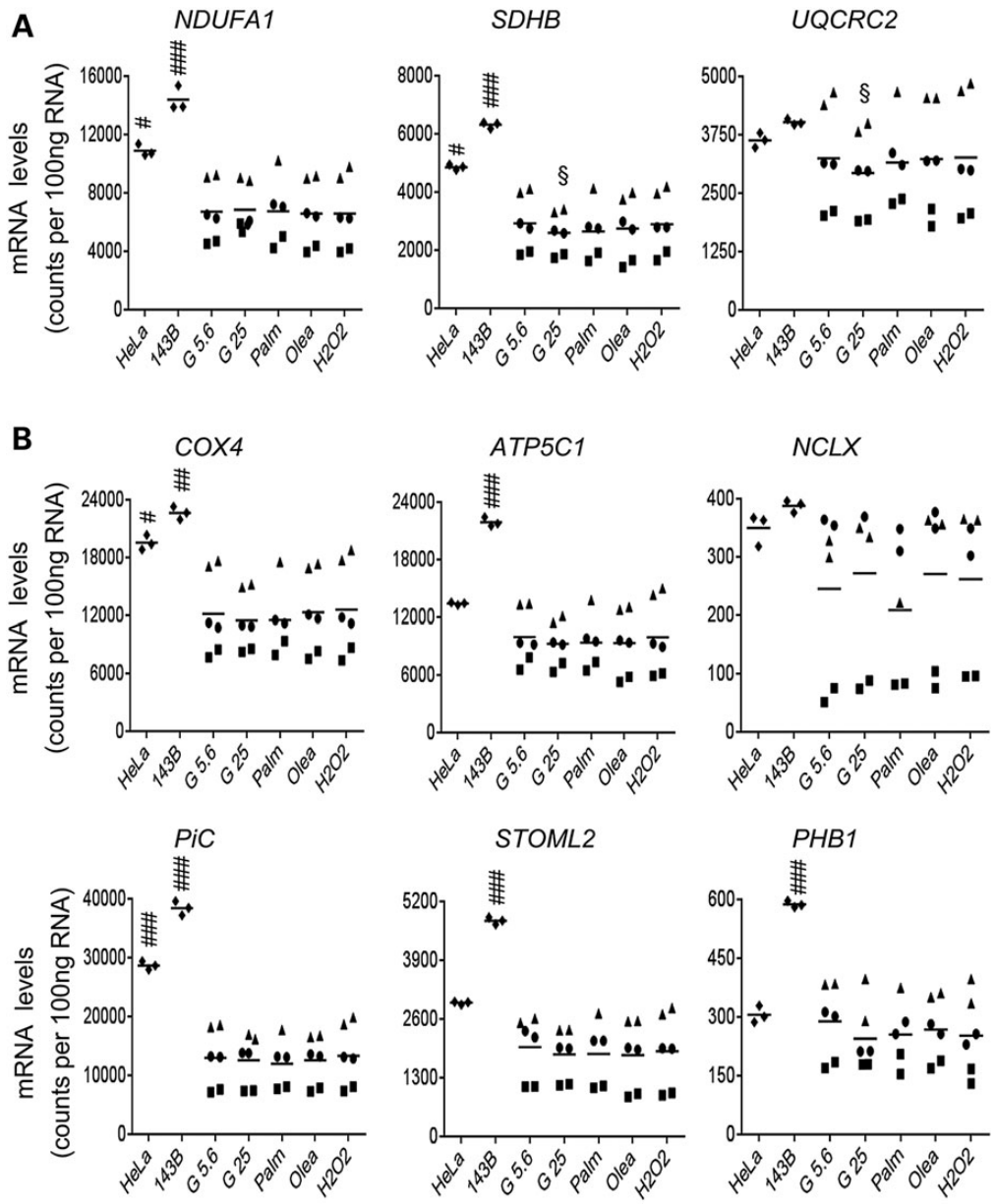
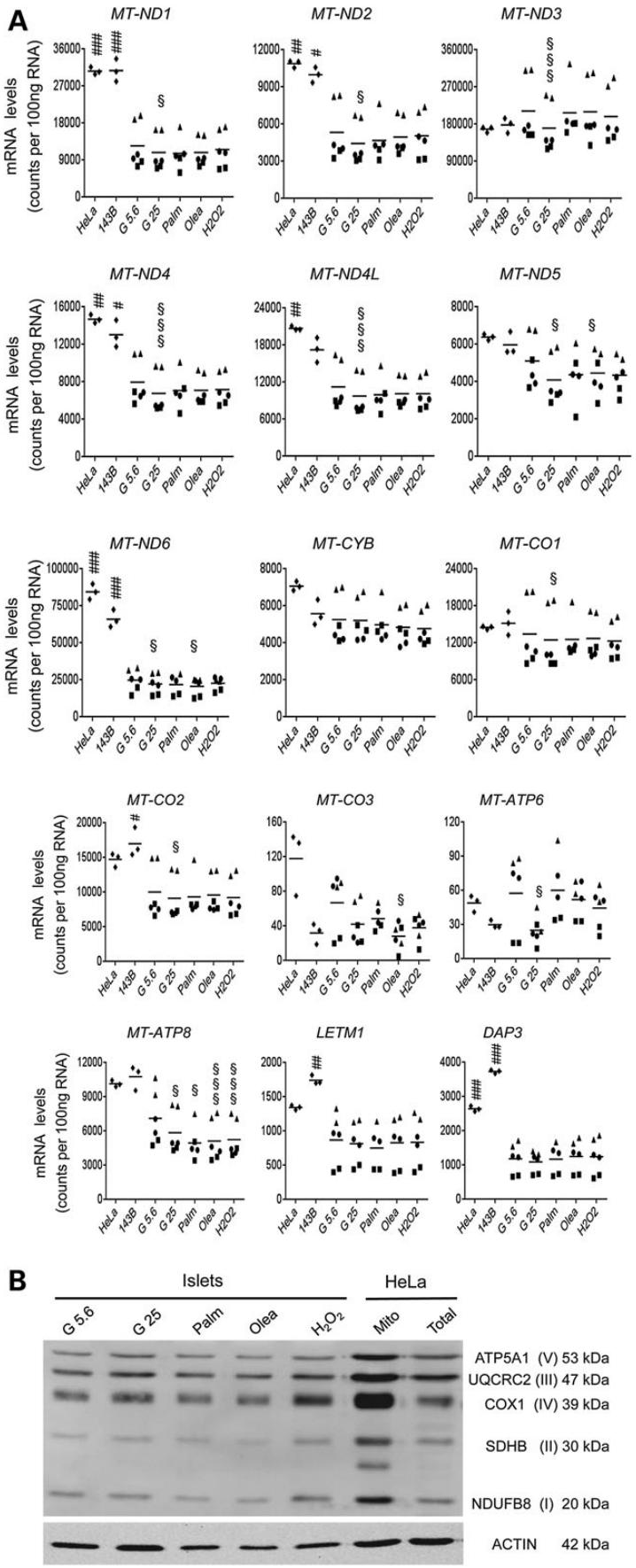


Figure 2. Transcriptome of mitochondrial respiratory chain subunits and associated proteins in human islets after stress exposure. Transcript levels of (**A**) nuclear-encoded subunits of the five OXPHOS complexes: *NDUFA1* (complex I), *SDHB* (complex II), *UQCRC2* (complex III), *COX4* (complex IV) and *ATP5C1* (complex V); (**B**) the mitochondrial sodium/calcium antiporter *NCLX*, the phosphate carrier *PiC*, the stomatin-like protein 2 *STOML2*, the prohibitin *PHB1* were quantified as described in Figure 1A. Means of three independent experiments done in duplicate are represented (horizontal bars). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.005$ comparing non-endocrine cells versus islets (G5.6); $^{\S}P<0.05$ versus corresponding G5.6 control islets.

up-regulated at protein level (10) (Figs 4B and 5D; Supplementary Material, Figs S2C and S3B). Palmitate increased expression of pyruvate carriers (*MPC1* and *MPC2*) and decreased the glutamate carrier (*GC1*), whereas oleate only down-regulated *AGC1*, showing specific responses according to the nature of fatty acids. Oxidative stress decreased *UCP2* and *GC1* transcripts. We also measured protein levels of carnitine/acylcarnitine carrier CAC, the rate-limiting mitochondrial transporter for β-oxidation, which was not modified by metabolic stresses (Fig. 4B; Supplementary Material, Fig. S2B and C). Results show that glucose up-regulated some key mitochondrial carriers, in particular AGC2 and DIC.

Effects of diabetogenic milieus on islet viability

To investigate whether the observed changes in gene expression were associated with cytotoxic effects, cell death was quantified at the end of the 3-day stress challenge. Control G5.6 cells exhibited low basal apoptotic rate (Supplementary Material, Fig. S4C), in the expected range of 1–3% (48). High glucose-induced apoptosis (2.5-fold cleaved CASPASE 3 in G25 versus control G5.6, Fig. 5A). Lipotoxicity with apoptosis is regularly associated with saturated fatty acids, whereas unsaturated fatty acids promote mainly dysfunctional GSIS (11,12). Fatty acid treatments reported to trigger apoptosis have usually been performed at low fetal calf serum (FCS) (1%) or even in absence of serum (16). When cultured



with standard FCS concentrations (5–10%), longer incubation periods are required to observe cytotoxic effects. We opted for 5% FCS in order to uncover the intrinsic effects of saturated versus unsaturated fatty acids, without changing the standard culture conditions. In accordance with previous reports (11,12), oleate did not increase cell death. In our hands, palmitate did not induce noticeable cytotoxicity in the different islet batches, exhibiting resistance to oxidative stress (Fig. 5A; Supplementary Material, Fig. S4C).

Among the different metabolic stresses tested side by side, high glucose induced the main changes. Therefore, we investigated further sugar effects on islet function and survival by varying glucose concentrations and exposure time periods. In agreement with previous results obtained after 72 h at G25, SIRT1 and DIC were already up-regulated at protein level after 24 h at G16.7 and G30 (Supplementary Material, Fig. S3A and B). In parallel, G16.7 and G30 markedly increased cleaved CASPASE 3 after 24 and 48 h (Fig. 5B). Of note, when islets were exposed to high glucose for 24 h, apoptosis, CASPASE 3 cleavage and the autophagy markers LC3-I and LC3-II were not systematically increased in the different islet batches (Fig. 5C; Supplementary Material, Fig. S3C). Interestingly, over short time periods ranging from 6 to 12 h, apoptosis was induced in islets cultured at G5.6 (Fig. 5B), indicating that varying glucose concentrations in the culture medium right after isolation is critical for the maintenance of islets prior to transplantation. In agreement with the observed activation of CASPASE 3 cleavage, G30 for 48 h induced a 2.0-fold increase in cell death (Fig. 5C) and increased expression of LC3-I and LC3-II (Fig. 5D). Overall, exposure of human islets for >24 h to high glucose increased autophagy markers and promoted cell death, exhibiting prototypic glucotoxicity. In contrast, no significant cytotoxic effects were observed with fatty acids and oxidative stress.

Effects of glucotoxicity on islet function

To examine whether the observed cytotoxic effects of high glucose were associated with ROS production and β-cell dysfunction, superoxide generation and insulin secretion were determined in parallel in the same islet batches. Superoxide levels measured directly at the end of the 48 h culture were lower in G30 islets compared with control G5.6 islets (Fig. 6A). These islets further acutely stimulated with 16.7 mM glucose for 1 h did not exhibit significant differences in superoxide generation (Fig. 6B). In parallel, insulin contents and the secretory responses were assessed at the end of different culture periods. Islets exposed to G30 over days exhibited gradual loss of their insulin contents, with only 20% left after 1 week compared with contemporary G5.6 controls (Fig. 6C). Although islets cultured at control G5.6 also lost part of their insulin contents, their secretory responses were progressively enhanced with time in culture (Fig. 6D and E; Supplementary Material, Fig. S4A). In contrast, islets at G30 were not responding to glucose stimulation already after 48 h and without any recovery over the 1-week period. Consistent with the absence of persistent cytotoxic effects of H_2O_2, no significant alteration in insulin contents, GSIS and mitochondrial network was observed in islets 3 days after acute oxidative stress (Supplementary Material, Fig. S4A and B). Overall, chronic exposure to high glucose markedly decreased insulin content and impaired GSIS in human islets, leading to severe β-cell dysfunction.

Chronic effects of glucotoxicity on islet protein levels

Correspondence of gene expression at mRNA versus protein level was rare after 3 days, possibly explained by rather long half-life of human proteins. We then extended the analysis in islets exposed to high glucose for 7 days. CASPASE 3 cleavage was enhanced by high glucose, starting already at 24 h and over the 7 days of culture (Fig. 6F). IPF1 and TFAM protein levels were up-regulated by chronic high glucose. This was also the case for adenosine mono-phosphate kinase (AMPK), whereas its active phosphorylated form (pAMPK) was decreased. These data indicate that islet cells escaping glucose-induced programmed cell death maintained β-cell differentiation, insulin expression, mitochondrial biogenesis and AMP sensing.

Discussion

In most T2D, hyperglycemia appears as a result of significant loss of functional β-cells secondary to the exposure to a pathophysiological environment, commonly referred to as glucolipotoxicity (6,9,10,18,24,49). However, the respective contribution of the different components of the diabetogenic milieus to β-cell dysfunction, dedifferentiation and ultimately death remains unclear; driven by specific changes in gene expression. Here, we explored the specificity of molecular targets in response to the different metabolic stresses investigated side by side in standardized conditions. Figure 7A summarizes the changes in gene expression in human islets following chronic exposure to high glucose, palmitate or oleate, and oxidative stress; demonstrating stress-specific signatures. Of note, the reported stress-responses were observed on intact islets, being by essence composed of different cell types. Some markers are specific for β-cells, such as IPF1 or insulin. Correspondence with pure fraction of insulin-secreting INS-1E cells is displayed on Figure 8, confirming good correlation between this rat cell line and human islets when exposed to diabetogenic milieu (48). However, the respective contribution of each islet cell type to the stress–response remains to be delineated.

Markers of oxidative stress

Transient exposure of INS-1E cells and rat islets to oxidative stress alters mitochondrial gene expression and metabolism and impairs β-cell function and survival (20,21,43). In human islets, at day 3 post-stress, we observed preservation of GSIS and no changes in cell death. Interestingly, transcript levels of the transcription factor *MAFA* (47) and the C5 and C4 mitochondrial carriers *GC1* and *UCP2*, respectively, were down-regulated as reported in INS-1E cells (43), conferring to these targets a stress-specific signature (Fig. 7A).

Figure 3. Expression of mtDNA-encoded respiratory chain subunits in human islets after stress exposure. (**A**) Transcript levels of mtDNA-encoded (MT) subunits of OXPHOS complexes: NADH dehydrogenase *ND1*-–*ND6* (complex I), ubiquinol:cytochrome c oxidoreductase *CYB* (complex III), cytochrome c oxidase *CO1*–*CO3* (complex IV), ATP synthase *ATP6* and *ATP8* (complex V), as well as the nuclear-encoded protein *LETM1* and the mitochondrial ribosome small subunit protein S29 *DAP3* were quantified as described in Figure 1A. Means of three independent experiments done in duplicate are represented (horizontal bars). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.005$ comparing non-endocrine cells versus islets (G5.6); $^{\S}P<0.05$, $^{\S\S\S}P<0.005$ versus corresponding G5.6 control islets. (**B**) Representative immunoblotting showing protein subunits of the 5 OXPHOS complexes from donor 2 (total extracts) under different experimental conditions. Total cell extracts and mitochondria (Mito) isolated from HeLa cells were used as additional controls.

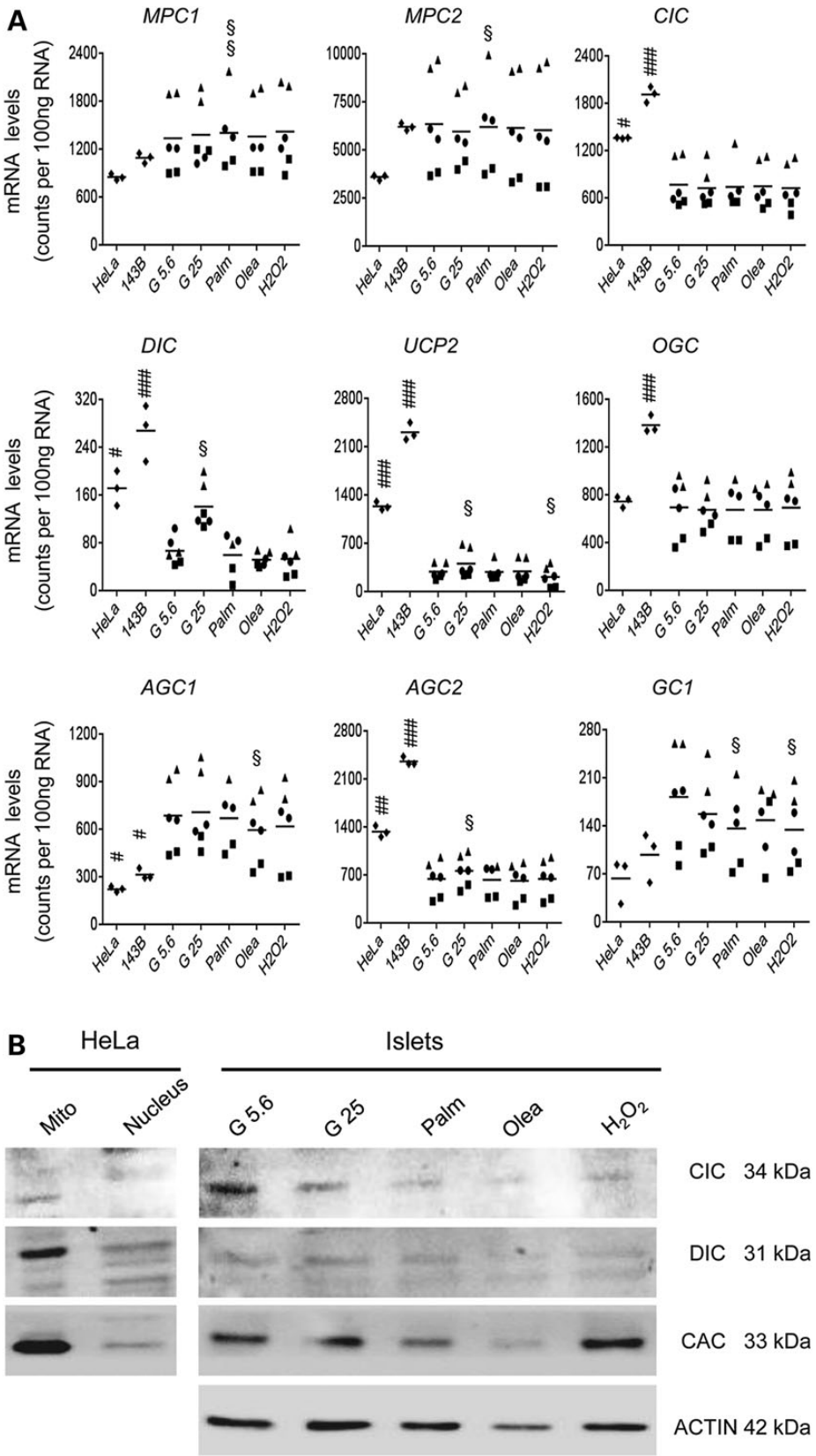


Figure 4. Expression of mitochondrial metabolite carriers in human islets after stress exposure. (**A**) Transcript levels of the carriers for pyruvate *MPC1* and *MPC2*, citrate/isocitrate *CIC*, malate *DIC*, malate/oxaloacetate/aspartate *UCP2*, 2-oxoglutarate/malate *OGC*, aspartate/glutamate *AGC1* and *AGC2*, glutamate *GC1* were quantified as detailed in Figure 1A. Means of three independent experiments done in duplicate are represented (horizontal bars). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$ comparing non-endocrine cells versus islets (G5.6); $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ versus corresponding G5.6 control islets. (**B**) Representative immunoblotting showing protein levels of the metabolite carriers CIC, DIC, carnitine/acylcarnitine CAC (total extracts) in islets and HeLa subcellular fractions used as control.

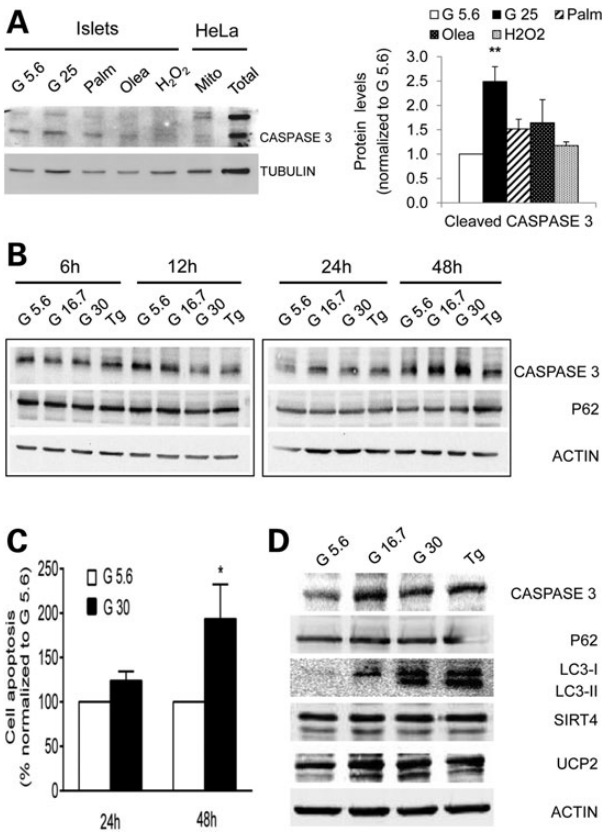


Figure 5. Apoptosis in human islets after stress challenge. (**A**) Islets were exposed for 3 days to different culture conditions as detailed in Figure 1. Representative immunoblotting showing the cleaved fragment of CASPASE 3 and TUBULIN from treated islets and HeLa cells used as non-endocrine controls. Quantitative analysis of band densities normalized to TUBULIN from immunoblots is presented as means ± SEM of three independent experiments. Results are expressed as protein levels normalized to the control value of G5.6. $^{**}P < 0.01$ versus G5.6 control islets. (**B–D**) Human islets were exposed for different time periods to the indicated glucose (G) concentrations. (B) Immunoblotting over time course of stress exposure showing cleaved CASPASE 3, the nuclear pore glycoprotein P62 and ACTIN. Thapsigargin (Tg, 1 μM) served as positive control. (C) Cell apoptosis was measured by the Cell Death Detection ELISA^{PLUS} assay. Values are means ± SEM of four independent experiments, each done in triplicate. $^{*}P < 0.05$ versus corresponding G5.6 controls. (D) Islets were exposed for 48 h to the indicated glucose concentrations or 1 μM thapsigargin. Immunoblotting showing levels of the cleaved CASPASE 3, P62, the autophagosome markers LC3-I and LC3-II, SIRT4 and UCP2 from treated islets.

Mitochondrial markers of saturated and unsaturated fatty acids

Previous studies reported that human islets exposed to palmitate and oleate increase their basal insulin release and fail to respond to stimulatory glucose (11,14,16). The absence of serum in the culture medium worsens the effects of palmitate, inducing cell death (16). Here, in the presence 5% FCS, neither palmitate nor oleate did promote cytotoxic effects at physiological glucose concentration. Thus, the lipo-dysfunction developed by isolated human islets was similar to the ones reported for primary rat islets and INS-1E cells (43,50), as well as for islets from patients with T2D (13). Discriminating fatty acids in the treatments uncovered specific targets associated to the nature of the lipid (Fig. 7A). Oleate decreased expression of *MAFA*, *IPF1* and the aspartate/glutamate carrier *AGC1*, whereas palmitate up-regulated *SIRT1* and the pyruvate carriers *MPC1*/*MPC2* while down-regulating *GC1*. Palmitate was reported to repress expression of genes involved in ATP production, including citrate synthase and mitochondrial ATP synthase (16). Here, we observed that both fatty acids down-regulated *MT-ATP8*, possibly contributing to the loss of GSIS. Overall, present data show modest impairment of mitochondrial machinery and islet integrity by palmitate and oleate. According to Robertson *et al.* (9), the relentless decline in TD2-associated β-cell function and mass ascribed to lipotoxicity occurs only in a context of preexisting hyperglycemia, whereas glucotoxicity can occur in the absence of hyperlipidemia. Defining the extent to which hyperglycemia *per se* underlies these alterations is crucial for the prevention of TD2. Recent studies in mice have shown that many changes in islet differentiation and function associated with diabetes are due to hyperglycemia only and can be reversed by blood glucose normalization (51,52).

Delineation of nuclear and mitochondrial markers of glucotoxicity

Hyperglycemia is associated with β-cell dedifferentiation, altered GSIS and increased cell death (5,7,9,10). A progressive deterioration in β-cell function is a common finding in patients with T2D. Similarly, *in vitro* exposure to high glucose markedly impairs INS-1E cell function and survival (43). Here, chronic exposure of human islets to G30 decreased insulin content, impaired GSIS and induced apoptosis. Incidentally, our data validate the use of 5.6 mM as the optimal glucose concentration for human islets in culture regarding insulin content and GSIS (Fig. 6B–D) and viability (Fig. 6E).

Figure 7B shows the expression of key components of nuclear differentiation factors and mitochondrial machineries being expressed in human islets and the ones being changed by chronic

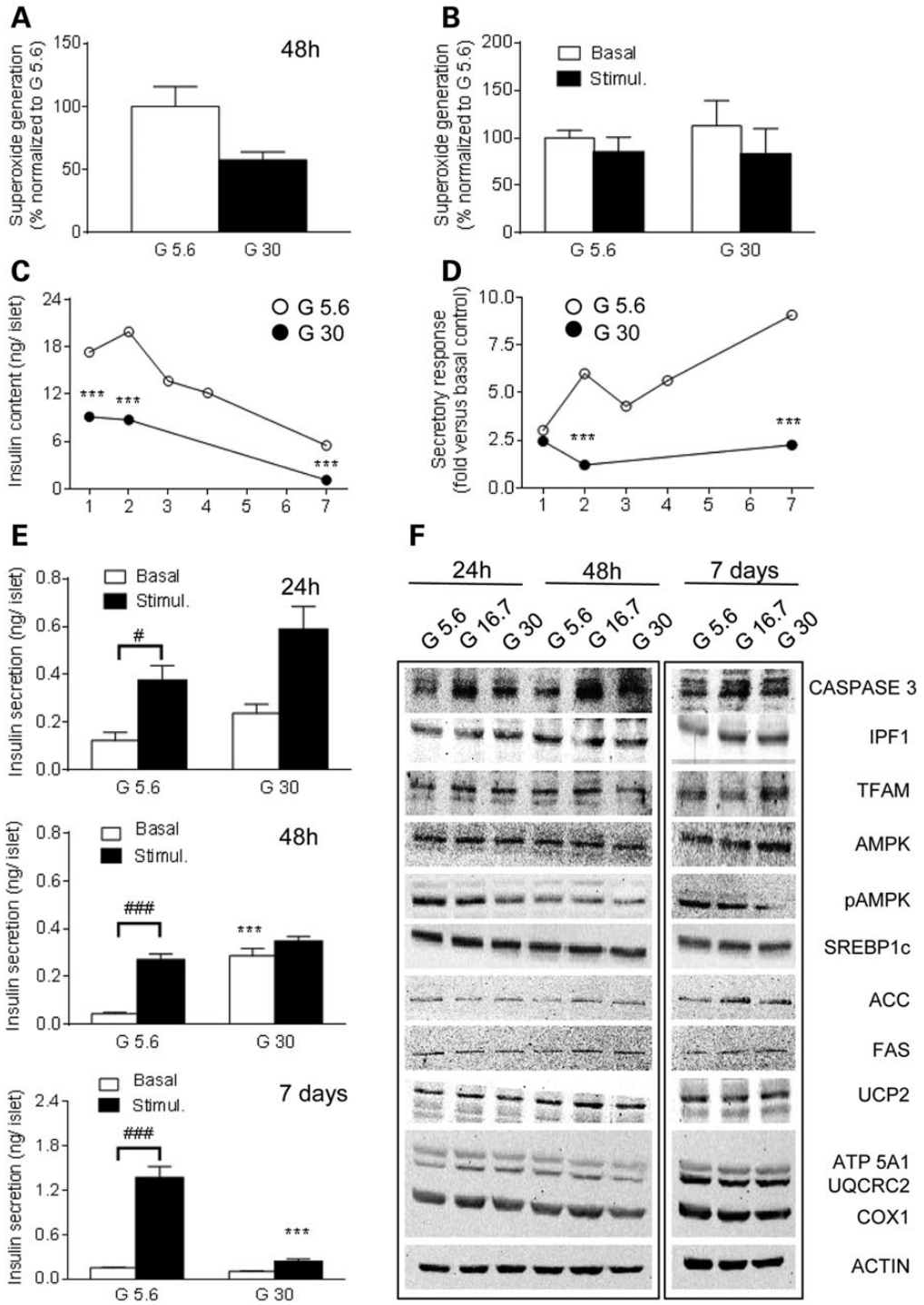


Figure 6. Effects of glucotoxicity on islet function and protein levels. Islets were exposed for the indicated time periods to the various glucose (G) concentrations. Culture at G5.6 served as no-stress control. Then, the responses of chronically treated islets were acutely challenged for 60 min with 2.8 mM (Basal) and 16.7 mM (Stimul.) glucose concentrations. (**A** and **B**) Superoxide generation measured in islets directly after the chronic 48 h G30 culture (A) or following further acute glucose stimulation (B; basal, white bars; Stimul., black bars). (**C**) Time course of insulin contents in islets cultured at G5.6 (open circle) or exposed to G30 (filled circle). (**D**) At the end of the indicated culture periods, insulin release was measured under Basal (white bars) and Stimul. (black bars) conditions. (**E**) Time course of secretory responses of islets cultured at G5.6 (open circle) or G30 (filled circle). Values are means ± SEM of at least four independent experiments, each done in triplicate. ***$P < 0.005$ versus corresponding G5.6 controls; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$ versus corresponding basal secretion. (**F**) Representative immunoblotting showing levels of the indicated proteins from treated islets.

high glucose. In line with rodent data (43,44), we observed down-regulation of the transcription factors *PPARA*, *TFAM*, *HNF4A* and *IPF1* in human islets. The two latter are master regulators of genes implicated in β-cell development and function (24–26), such as the glucose transporter *GLUT2*, the glucose sensor glucokinase *GCK* and insulin. TFAM, which is involved in the maintenance and transcription of mtDNA, is a direct target of IPF1/Pdx1 that binds to its promoter (29). In rat islets, defective Pdx1

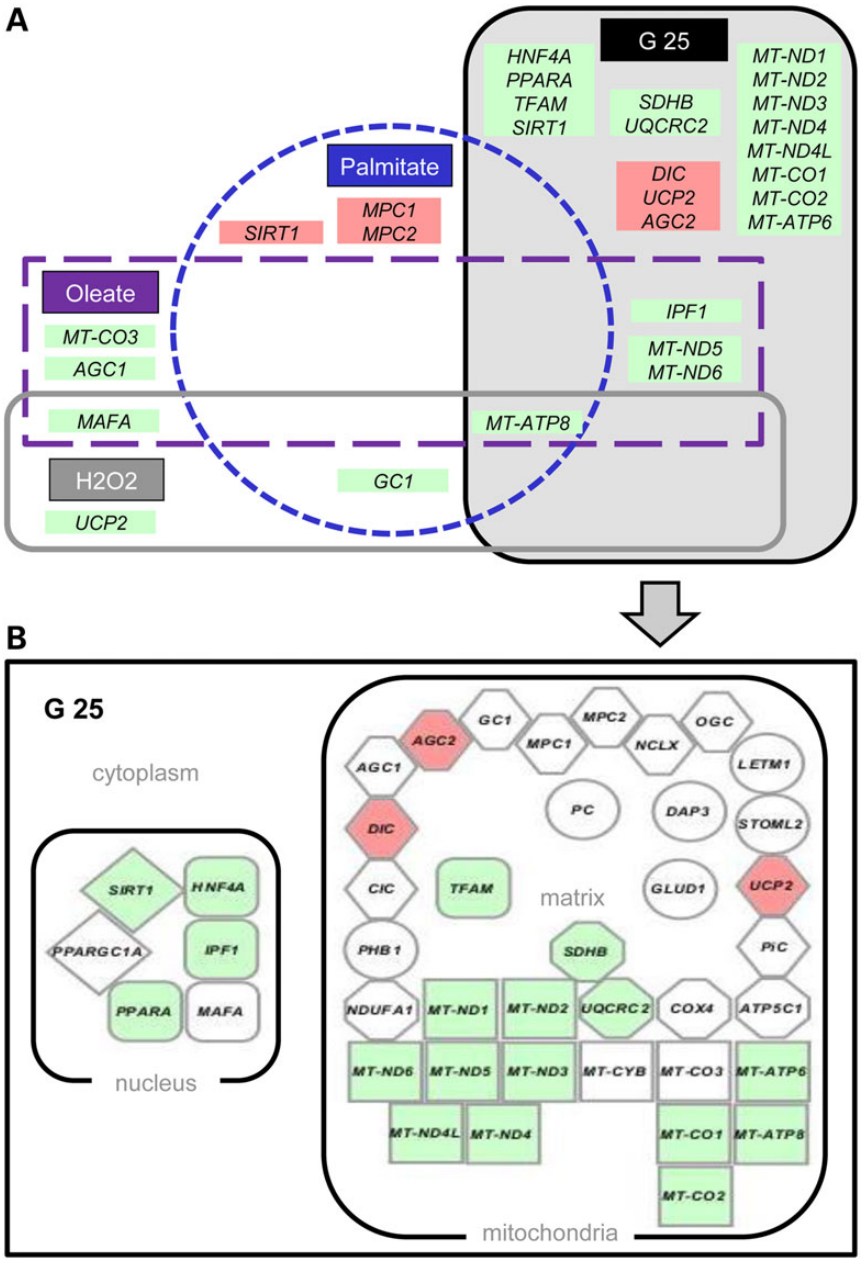


Figure 7. Stress-specific transcriptome of human islets after chronic exposure to diabetogenic milieus. (**A**) Changes in gene expression in human islets induced by the different metabolic stresses, delineating stress-specific signatures. The mRNA levels of the genes labeled in green and red colors were found to be significantly ($P < 0.05$ versus G5.6) down- and up-regulated, respectively. (**B**) Global view of the expression of the genes at transcript level (node core) in islets exposed to 25 mM glucose (G25). The expressed genes were grouped using the Cytoscape software according to their protein subcellular localization (from the human database neXtProt); cytoplasm, nucleus, mitochondrial inner membrane, matrix. Node shape: octagons show nuclear-encoded subunits of the five OXPHOS complexes, rectangles represent mtDNA-encoded (MT) respiratory chain subunits, hexagons are mitochondrial carriers, circles are enzymes or other proteins, rounded rectangles are transcription factors, and rhombi are energy-sensors. Colors reflect changes in expression levels in G25 versus G5.6 controls: green and red for significant ($P < 0.05$) down- and up-regulation, respectively; white no change.

down-regulates Tfam and mt-Nd1 and up-regulates Dic, along with impaired mitochondrial activity (53). A striking observation of the present study on human islets was the global down-regulation of mtDNA-encoded respiratory chain subunits by high glucose (Fig. 7B). Transcription of mitochondrial genes is also controlled by TFB1M and TFB2M, loss of the former being associated with T2D (54). In mice, Tfb1m deficiency in β-cells causes mitochondrial dysfunction and subsequently diabetes owing to combined loss of β-cell function and mass (55).

High glucose also altered the expression of *SIRT1* in human islets. As sirtuin activity depends on NAD^+/NADH ratio, it serves as redox energy-sensor acting as a transcriptional activator via deacetylase activity. In β-cells, the main SIRT1 targets are HNF-1α and IPF1/Pdx1 (56). Taken together, our data suggest interdependence between transcription regulators both in INS-1E β-cells and human islets, represented in Figure 8, for which chronic high glucose decreases *SIRT1* and *IPF1* expression, in turn down-regulating *TFAM* and mtDNA-encoded respiratory

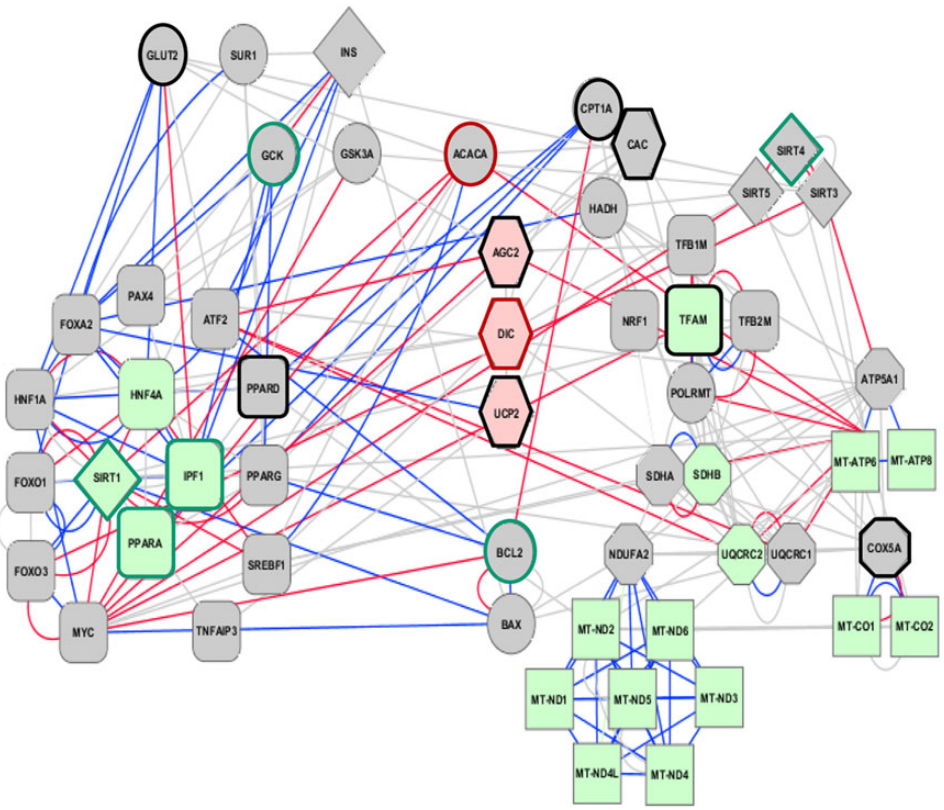


Figure 8. Putative molecular targets and related network in human islets and INS-1E β-cells secondary to glucotoxic insult. Network of genes with altered expression upon G25 treatment, either up- (red) or down-regulated (green). Gray nodes represent molecular targets that were obtained from network expansion using GeneMANIA (http://www.genemania.org/), providing network links between the initial 21 altered human genes. Border colors shows the identified transcriptional alterations in INS-1E cells (43): black no change; gray not tested. The connections are represented as follows: blue lines show proteins acting in the same pathway; red lines physical interactions; gray lines genes that are co-expressed or sharing same domains.

chain subunits. This alters mitochondrial ATP production and consequently insulin secretion. In the long term, altered respiratory activity secondary to repressed mitochondrial gene expression may lead to increased susceptibility of β-cells to apoptosis.

Insulin secretion is triggered and maintained via integration and production of signals by the mitochondria (3,4). In particular, pyruvate couples glycolysis to mitochondria, fueling the TCA cycle and resulting in the generation of metabolic signals such as ATP, ROS, citrate and glutamate (30,31). High glucose modified neither *MPC1* nor *MPC2* expression, suggesting that pyruvate transport was preserved in human islets. The associated decrease of respiratory chain subunits upon high glucose might favor saturation of the electron transport chain, promoting citrate export and consequently *de novo* lipid synthesis, an effect substantiated by the observed increase of ACC protein (Figs 6E and 8). Another unprecedented observation was the glucose-dependent up-regulation of mitochondrial carriers UCP2, AGC2 and DIC at the transcript level in human islets (Fig. 7). UCP2 has been implicated in numerous physiopathological conditions including hyperglycemia and diabetes (5,10). It was proposed that these effects of UCP2 were mediated by mitochondrial uncoupling activity, regulating ROS production. However, the recent demonstration of C4 metabolite transport properties of UCP2 shed a new light on the observed up-regulation of UCP2 secondary to hyperglycemia (32). Increasing mitochondrial export capacity of oxaloacetate and malate would reduce TCA cycle activity, thereby lowering the redox pressure and ATP production. The mitochondrial redox pressure is also contributed by NADH shuttles, having redundant routes in β-cells (33), AGC2 being involved in the malate-aspartate shuttle (34,42) as well as glutamate metabolism (57). By up-regulating AGC2 upon high glucose exposure (Fig. 7B), human islets could increase the capacity of aerobic glycolysis. Finally, the malate carrier DIC is a critical shuttle component for cytosolic NADPH production mediated by pyruvate cycling (37,58). In neurons, DIC mediates glutathione transport and the mitochondrial GSH pool, which participates to ROS homeostasis (59). In β-cells, the NADPH oxidase NOX2 negatively modulates GSIS by promoting ROS generation and reducing cAMP (22). According to these data, up-regulation of both DIC and UCP2 in human islets exposed to high glucose (Fig. 7B) could decrease ROS production within mitochondria, while generating cytosolic ROS, in turn inhibiting insulin release. Collectively, the activity of each of these mitochondrial carriers lowers the contribution of glucose to mitochondrial oxidative metabolism, suggesting a mitohormetic response implicating UCP2, AGC2 and DIC. Drugs acting on mitochondrial carrier activity such as metformin and thiazolidinediones, which regulate UCP2 and MPC, respectively, could be beneficial to restore metabolic function (60,61).

Conclusion

Hyperglycemic conditions induced down-regulation of 18 genes and up-regulation of 3 genes, uncovering a stress-specific signature in human islets. Bioinformatics analysis of the expression profiles delineated comprehensive network and established putative molecular targets as well as related network in human

islets secondary to glucotoxic insult. Thus, high glucose impedes primarily islet function through mitochondrial metabolism, leading to impaired GSIS and islet mass.

Materials and Methods

Cell culture and treatments

HeLa and 143B cells, used as control human non-endocrine cells, were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in 5% CO_2 at 37°C (62). Human islets were isolated from pancreases of deceased multiorgan donors ($n = 14$), who had provided written informed consent (ECIT consortium). None of the donors were diagnosed with metabolic syndrome or diabetes (see description in Supplementary Material, Table S1). Donors had an average BMI of 25.1 ± 3.6 kg/m^2 and age of 53.1 ± 7.5 years. Islets were maintained for standard recovery period of time (1–4 days) in CRML-1066 medium at 5.6 mM glucose supplemented with 10% heat-inactivated FCS and used for experiments straight away without shipping maneuver (isolation and experiment being performed in the same institution). Then, islets were hand-picked, washed and further cultured for the indicated culture period in 6-well plates (Falcon, OmniLab, Mettmenstetten, Switzerland) in the presence of 5% FCS at either physiological 5.6 mM glucose (G5.6, control) or exposed to high glucose (25 mM, G25 or 30 mM, G30). In parallel, islets were also exposed to 0.4 mM palmitate (saturated fatty acid C16:0; Palm) or with 0.4 mM oleate (unsaturated fatty acid C18:1; Olea) in the presence of 0.5% BSA in control medium as detailed previously (14,43). Stock solutions of fatty acids (palmitate and oleate; Sigma-Aldrich, St Louis, MO, USA) bound to BSA were adjusted to 10 mM fatty acids using 1.8 mM fatty acid-free BSA before storage at –20°C under nitrogen. Additionally, islets were acutely treated at Day 0 of experiment with single transient oxidative stress (200 μM H_2O_2 for 10 min) (21). Islets exposure to each type of stresses was performed in parallel with each batch of human islets.

Establishment of mitochondrial transcriptome

We designed a human molecular screen based on NanoString® nCounter system (Seattle, WA, USA) allowing captures and counts of individual cellular mRNA transcripts not requiring cDNA synthesis and more sensitive than micro-arrays (62–64). After a pilot experiment in order to validate our probes using HeLa cells (data not shown; probes ~100 bases long are described in Supplementary Material, Table S2), human islets (200 islets/well) were exposed to the different stresses as described above. Three independent experiments with the same culture conditions were performed (donors 1–3 described in Supplementary Material, Table S1) and for every experiment each culture condition was done in duplicate. Total RNA was extracted with TRIzol reagent and all samples were quantified (QuBit from Invitrogen) at high precision level using fluorescent specific dyes. Quality controls of RNA samples were performed before each analysis (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA). Then, 100 ng of total RNA were mixed with nCounter fluorescence-reporter and biotin-capture probes in hybridization buffer before overnight incubation at 65°C, allowing quantification of 45 gene transcripts in a single run. Following hybridization, samples were immediately processed with the PrepStation and subsequently analyzed on an nCounter Digital Analyzer (iGE3 genomics platform, University of Geneva) (62). Reporter probes, representing individual copies of mRNA, were scanned for quantification and tabulated as counts of individual mRNA transcripts (63,64). For this experimental design, statistics have been performed on the mean values obtained from the duplicate for each independent experiment ($n = 3$). Data were normalized to the following housekeeping genes: nuclear pore complex protein *NUP155*, histone deacetylase *HDAC1* and *TUBULIN* to normalize the data; showing no statistical differences between conditions. Transcript levels of gene of interest were expressed as mRNA molecule counts per 100 ng total RNA.

Immunoblotting

At the end of the culture period, protein extracts were harvested from 200 islets/well in lysis buffer (56). Total cell extracts as well as mitochondria and nuclei isolated from HeLa cells were prepared as described (21) and used as human non-endocrine controls. Proteins from total cell extracts (15 μg/lane) and mitochondria (10 μg/lane) were separated by 10–12% SDS–PAGE before transfer onto polyvinylidene fluoride membrane. The membrane was blocked with polyvinyl alcohol and then probed overnight at 4°C with: goat polyclonal antibodies against IPF1 (1:50 000, provided by C. Wright, Vanderbilt University, USA), PGC1α, TFAM, SREBP1c (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), UCP2 (1 : 1000, Alpha Diagnostic, San Antonio, TX, USA) and SIRT4 (1 : 1000, Abcam Inc., Cambridge, MA, USA); mouse monoclonal antibodies against SIRT1 (1 : 1000), ACTIN (1 : 20 000, Chemicon-Millipore, Zug, Switzerland), TUBULIN (1 : 100 000, Sigma-Aldrich) and five subunits of OXPHOS complexes (1 : 15 000, MitoSciences, Eugene, OR, USA); rabbit polyclonal antibodies against CIC, DIC, CAC (1 : 10 000, provided by F. Palmieri, University of Bari, Italy), P62 (1 : 1000, Proteintech, Chicago, IL, USA), LC3 (1 : 1000, Abcam Inc., Cambridge, MA, USA), AMPK, p-AMPK, ACC, FAS and cleaved CASPASE-3 (1 : 1000 dilution, Cell Signaling Technology, Danvers, MA, USA). After washing, membranes were incubated for 1 h at RT with secondary horseradish peroxidase-conjugated anti-goat (1 : 10 000, Amersham Biosciences, UK), anti-mouse or anti-rabbit antibodies IgG (1 : 10 000, Sigma-Aldrich) according to primary antibodies. Proteins were visualized by chemiluminescence (ECL #RPN2135, Amersham), analyzed with the ChemiDoc XRS System (Bio-Rad, Hercules, CA) and bands were quantified with Scion Image software (Scion Corporation, Frederick, MD, USA).

Apoptosis, insulin secretion and superoxide measurements

After recovery, human islets were cultured (100 islets/well) in 6-well plates and exposed for 24 and 48 h at G5.6 and G30. Apoptosis was quantified using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Alternatively, cell death was estimated by CASPASE 3 cleavage activation by immunoblotting (see above). For superoxide measurements, islets were cultured at G5.6 and G30 for 48 h. Then, islets were hand-picked, washed and challenged for insulin secretory response at basal 2.8 mM and stimulatory 16.7 mM glucose concentrations for 60 min as detailed previously (56). Secreted and total cellular insulin contents were quantified by radioimmunoassay (Linco Research Inc., St. Charles, MO, USA). Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL, USA). For superoxide generation, groups of 20 islets were loaded with dihydroethidine (DHE 25 μM, Sigma-Aldrich) at either 5.6 or 30 mM glucose. Fluorescence was monitored in a thermostated plate reader (Fluostar

Optima, BMG Labtechnologies, Offenburg, Germany) for 60 min with excitation at 544 nm and emission at 590 nm and slope served to calculate superoxide generation as described (22).

Cytoscape

Gene expression data were analyzed by integrating knowledgebase (neXtProt) data (65) with differential subcellular expression information in order to establish gene associations. Comprehensive map of putative links between identified genes and molecular targets obtained from GeneMANIA-based network expansion was drawn with Cytoscape software version 2.8.2 (66).

Statistical analysis

All data were analyzed with the IBM SPSS Statistics 20.0 software (SPSS Inc., Chicago, IL, USA). Statistical tests between each stress condition and the control G5.6 values were performed using one-way ANOVA analysis followed by Dunnet *post hoc* test. Differences between human cell lines and control islets (G5.6) values were assessed by a two-tailed paired *t*-test using Prism software. Results are presented as means ± SEM. A *P*-value of <0.05 was considered statistically significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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