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ARTICLE

Identification of an intracellular metabolic signature impairing beta cell function in the rat beta cell line INS-1E and human islets

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Abstract
Aims/hypothesis Chronic hyperglycaemia promotes the progressive failure of pancreatic beta cells in patients with type 2 diabetes mellitus, a clinically highly relevant phenomenon known as glucotoxicity. The intracellular metabolic consequences of a chronically high availability of glucose in beta cells are, as yet, poorly understood in its full complexity.
Methods An unbiased metabolite profiling analysis (GC-time-of-flight-MS) was used to identify the time course of core metabolite patterns in rat beta cell line INS-1E during exposure to high glucose concentrations and its relation to insulin expression.
Results We report here that pentose phosphate pathway (PPP) metabolites accumulate remarkably during chronic but not acute glucose treatment, indicating altered processing of glucose through the pentose phosphate pathway. Subsequent functional studies in INS-1E cells and human islets revealed that a disturbance in this pathway contributes to decreases in insulin gene expression and a lack of glucose-stimulated insulin secretion. These effects were found to depend on the activation of extracellular-regulated-kinase (ERK1/2). Long-term inhibition of 6-phosphogluconic acid dehydrogenase resulted in accumulation of PPP metabolites, induced ERK1/2 activation independently of high glucose and impaired beta cell function. In turn, inhibition of ERK1/2 overstimulation during chronic glucose exposure partly inhibited metabolite accumulation and restored beta cell function.
Conclusions/interpretation Based on unbiased metabolite analyses, the data presented here provide novel targets, namely the inhibition of PPP metabolite accumulation

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towards the therapeutic goal to preserve and potentially improve beta cell function in diabetes.

Keywords Beta cell · Diabetes · Extracellular-regulated protein kinase · Glucotoxicity · Metabolite profiling · Pentose phosphate pathway

Abbreviations

6-AN	6-Aminonicotinamide
BETA2	Beta cell E-box transactivator 2
ERK	Extracellular signal-regulated protein kinase
MAFA	v-Maf musculoaponeurotic fibrosarcoma oncogene homolog A (avian)
PC	Principal component
PCA	Principal component analysis
PDX-1	Pancreatic and duodenal homeobox 1
6PGD	6-Phosphogluconate dehydrogenase
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
si	Small interfering
TCA	Tricarboxylic acid
TOF	Time of flight

Introduction

The current epidemic increase in the incidence of type 2 diabetes mellitus is a major challenge of global health. Impaired beta cell function and survival are major components in this development, but also in the progressive deterioration of type 2 diabetes. Glucose is a key modulator of beta cell function and survival that regulates an array of cellular signalling networks. Chronically elevated glucose leads to beta cell desensitisation and glucotoxicity, affecting beta cell function on both secretory and expression levels [1, 2]. Hyperglycaemia-induced beta cell dysfunction is associated with the downregulation of key islet genes and transcription factors (e.g. insulin, *PDX-1* [also known as *PDX1*], *MAFA*) concomitant with a marked upregulation of several suppressed factors (e.g. lactate dehydrogenase A, hexokinase, IL-1β, reactive oxygen species [ROS]) [3–6]. While short-term glucose stimulation promotes insulin transcription, chronic exposure to high glucose impairs insulin gene expression [7]. The precise mechanism behind this phenomenon remains to be elucidated despite eminent clinical relevance in patients with type 2 diabetes, who regularly experience a progressive deterioration of glucose control and beta cell function.

Besides the genome, transcriptome and proteome of a tissue, a metabolic profile can give an instantaneous snapshot of cellular physiology as metabolites are intermediates or endproducts of metabolic events and various metabolites have been reported to act as second messengers modifying functionality of the cell. To identify potential novel mediators of insulin gene suppression via long-term high glucose treatment, an unbiased time course analysis of metabolites was performed in rat pancreatic INS-1E beta cells. We report here that pentose phosphate pathway (PPP) metabolites accumulate remarkably during chronic but not acute glucose treatment. Functional studies in INS-1E cells and human islets confirm that a disturbance in the PPP contributes to beta cell dysfunction, acting through ERK activation.

Methods

Islet isolation and cell culture Human islets were isolated from eight donors at the University of Illinois at Chicago as previously described by Oberholzer et al. [8]. After 24 h preculture, islets were exposed to CMRL-1066 culture medium containing 5.5, 11.1 or 33.3 mmol/l glucose with or without the addition of 10 μmol/l PD98059 (Calbiochem, La Jolla, CA, USA), 1 μmol/l UO126 (Calbiochem), 500 μmol/l 6-aminonicotinamide (6-AN; Sigma, Munich, Germany), 200 μmol/l diazoxide (Sigma, Munich, Germany) or 50 nmol/l somatostatin (Bachem, Bubendorf, Switzerland) for 72 h. INS-1E cells were kindly provided by C. B. Wollheim, Geneva, Switzerland. Cells were pre-cultured in RPMI 1640 medium containing 3 mmol/l glucose for 24 h [9] before exposure to 3 mmol/l and 16 mmol/l glucose± inhibitors (PD98059 or 6-AN) for the indicated time periods. [$^{12}C_6$]glucose was replaced by uniformly isotope-labelled [$^{13}C_6$]glucose (Sigma) to confirm origination of metabolites from glucose metabolism.

RNA interference Small interfering (si)RNA–Lipofectamine2000 complexes were prepared according to the manufacturer's instructions (Lipofectamine2000 obtained from Invitrogen, Karlsruhe, Germany, see electronic supplementary material [ESM] for details).

Metabolite extraction and GC-time of flight (TOF)-MS measurements Metabolite profiling was performed on a Leco Pegasus 3 TOF mass spectrometer (Leco, St Joseph, MI, USA) equipped with a direct thermal desorption injector (ATAS GL) coupled to an HP 5890 gas chromatograph and a dual-arm autosampler with automatic derivatisation and liner exchange, chromatograms were processed using Leco ChromaTOF software (version 3.25) and principal component analysis (PCA) was performed with MATLAB 7.0 (Mathworks, Ismaning, Germany; see ESM for details).

RNA expression analysis Total RNA of isolated islets was extracted after overnight culture as described previously by

Maedler et al. [5]. For quantitative analysis, we used the LightCycler Quantitative PCR System (Roche, Mannheim, Germany) with a commercial kit (LightCycler FastStart DNA Master plus SYBR Green I; Roche). Comparison of the target data with α-tubulin and β-actin showed similar results. Total RNA from INS-1E cells was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. One-step real-time PCR was carried out using the 7900 HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with SYBR Green Mastermix (Qiagen, Hilden, Germany) or TaqMan primer/probes (*6PGD* [also known as *PGD*], β-actin; Applied Biosystems). For the primers, see ESM.

Western blot analysis Islets were lysed as described by Ardestani et al. [10]. Membranes were incubated with rabbit anti-phospho-44/42 MAP-kinase (Thr202/Tyr204), anti-6-phosphogluconate dehydrogenase (6PGD) (Abcam, Cambridge, UK) and anti-actin antibodies (Cell Signaling, Beverly, MA, USA) overnight at 4°C followed by incubation with horseradish-peroxidase-linked anti-rabbit IgG (Jackson, Newmarket, UK).

Glucose-stimulated insulin secretion For acute insulin release in response to glucose, islets were washed for 30 min in KRB containing 2.8 mmol/l glucose and 0.5% BSA. KRB was then replaced by KRB with 2.8 mmol/l glucose (basal) or by KRB with 16.7 mmol/l glucose (stimulated) for 60 min. Supernatant fractions were analysed by human insulin ELISA (Alpco, Salem, SH, USA).

Results

Reduced insulin and transcription factor gene expression due to chronic high glucose Insulin mRNA levels were examined in response to elevated glucose levels in INS-1E cells and human isolated islets over time periods of 48 and 96 h, respectively. While insulin mRNA was unchanged in INS-1E (Fig. 1a) cells over a time period of 8 h and human islets at 1 h (Fig. 1b), chronic exposure to high glucose reduced insulin transcription levels after 24 and 48 h by 55% and 64%, respectively, compared with low glucose in INS-1E cells (Fig. 1a) and by 43% and 39% (Fig. 1b, $p<0.01$) in human islets after 72 and 96 h, respectively. These differences were mirrored by significantly reduced mRNA levels of the transcription factors v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (avian) (*Mafa*), beta cell E-box transactivator 2 (*Beta2* [also known as *Neurod1*]) and pancreatic and duodenal homeobox 1 (*Pdx-1*) in INS-1E cells after 48 h (Fig. 1c, $p<0.05$) and of *MAFA* and *PDX-1*, which are key regulators of insulin transcription, in human islets after 96 h (Fig. 1d, $p<0.05$).

Accumulation of metabolites of the PPP during chronic glucose exposure contributes to reduced insulin expression To identify potential mediators of insulin gene suppression via long-term high glucose treatment, an unbiased time course analysis of metabolites was performed in INS-1E cells, during which 73 metabolites were uniquely identified (ESM Table 1). PCA was performed on the data obtained to illustrate the disparity between both the time points and the applied glucose concentrations on the metabolite level

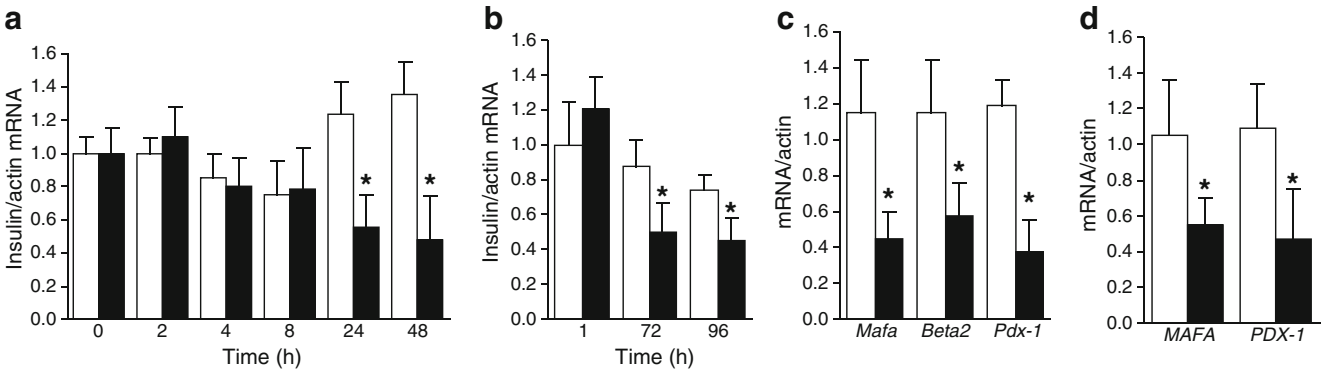

Fig. 1 The duration of exposure to high glucose levels is most critical for maintaining insulin gene expression. INS-1E cells were exposed to 3 mmol/l and 16 mmol/l glucose during 48 h (**a**, **c**) and human islets to 5.5 and 22.2 mmol/l glucose during 96 h (**b**, **d**) of culture. Quantitative RT-PCR analysis of insulin (**a**, **b**) and of the transcription factors *Mafa*, *Beta2* and *Pdx-1* after 48 h (**c**) or *MAFA* and *PDX-1* after 72 h (**d**). Mean expression levels are normalised to control (low glucose) and to actin and tubulin with the same result. Data shown are means±SE from three different experiments (INS-1E and islets) from three different islet donors. *$p<0.05$ high glucose compared with low glucose, same time point. **a**, **c** White bars, 3 mmol/l glucose; black bars, 16 mmol/l glucose. **b**, **d** White bars, 5.5 mmol/l glucose; black bars, 22.2 mmol/l glucose

(Fig. 2a). Principal component (PC)1 alone accounted for 91.4% of the total variance contained within the dataset and was able to resolve both time series and glucose treatments. This therefore renders PC2, which only accounts for 6.2%, and all subsequent components of only relatively minor relevance. In Fig. 2a, high glucose treated cells can already be discriminated from low glucose counterparts 5 min after treatment. Prolonged exposure to high glucose caused clear differentiation compared with early time points, thus demonstrating the differential impact of acute and chronic glucose exposure on the beta cell. Only those metabolites that are differentially regulated are shown in Table 1. These data are consistent with the accumulation of glucose metabolic intermediates in several pathways, as observed under chronic high glucose conditions in INS-1 832/13 cells [11]. The most important metabolites driving the separation from acute to

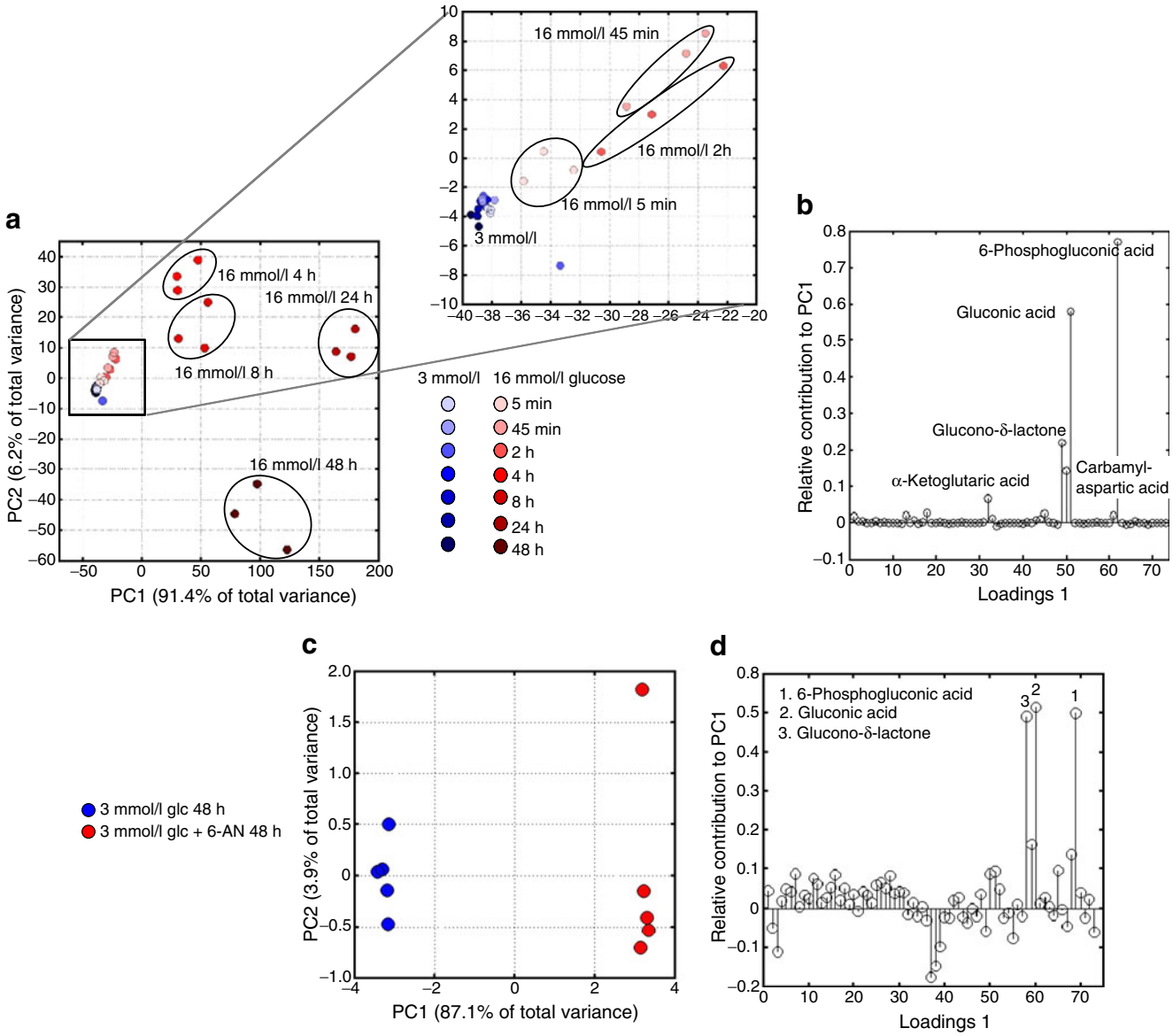

Fig. 2 Metabolites of the PPP drive differences in the metabolic intracellular patterns of INS-1E cells acutely and chronically treated with low (3 mmol/l, blue circles) and high (16 mmol/l red circles) glucose. PCA score plots (**a**) illustrate separation of samples according to culture conditions and exposure time. Each data point represents the entire metabolite profile of a sample. Loading plot (**b**) describes the contribution of each metabolite to PC1. The PPP metabolites glucono-δ-lactone, 6-phosphogluconic acid and gluconic acid, the pyrimidine pathway intermediate carbamyl-aspartic acid and the TCA cycle intermediate α-ketoglutaric acid were the most dominant factors driving the observed separation. Colour intensity of circles relates to duration of exposure, with lightest dots corresponding to 5 min and darkest to 48 h. **c** PCA score plot illustrates separation of INS-1E treated with 3 mmol/l glucose (glc) (blue) and 3 mmol/l glucose+ 100 μmol/l 6-AN (red) for 48 h (data were log-transformed prior to PCA). Loading plot (**d**) describes the contribution of each metabolite to PC1. The PPP metabolites glucono-δ-lactone, 6-phosphogluconic acid and gluconic acid were driving the observed separation. Representative plots are shown from three (**a**, **b**) and five (**c**, **d**) independent experiments

Table 1 Identified metabolites differentially regulated in INS-1E under high vs. low glucose conditions after short-term (45 min) and chronic incubation (48 h). Presented are the relative metabolite median fold changes between 3 mmol/l and 16 mmol/l glucose (n=3 in each condition) from two independent time course experiments. Origination of measured metabolites from glucose metabolism was determined by [13C] glucose labeling and those metabolites successfully traced are marked with "a".

Metabolite	Median fold change at 16 vs. 3 mmol/l glucose	
	Incubation time	
	45 min	48 h
Glucose[a]	+4.1	+5.0
Glycolysis		
Glucose 6-phosphate[a]	+3.9	+10.9
Fructose 1,6-bisphosphate[a]	+2.7	+2.5
Glycerate 3-phosphate[a]	+3.7	+3.1
Phospho(enol)pyruvic acid[a]	+2.7	+3.0
Pyruvic acid[a]	+3.6	+30.1
Lactic acid[a]	+2.5	+7.3
Krebs cycle		
Citric acid[a]	+2.0	+9.9
α-Ketoglutaric acid[a]	–	+28.6
Succinic acid[a]	+2.3	+13.5
Fumaric acid[a]	+2.5	+4.8
Malic acid[a]	+4.4	+22.7
Polyol synthesis		
Sorbitol[a]	–	+2.0
Fructose[a]	–	+2.1
PPP		
Gluconic acid-δ-lactone[a]	+3.4	+91.8
Gluconic acid[a]	–	+249.3
6-Phosphogluconic acid[a]	+10.7	+330.1
Ribose 5-phosphate[a]	–	+2.0
Glycerol synthesis		
Glycerol[a]	–	+4.8
DNA/RNA synthesis		
Carbamyl-aspartic acid[a]	–	+305.8
GABA synthesis		
γ-Aminobutyric acid[a]	–	+2.0
Polyamine synthesis		
Ornithine	–	−2.0
Putrescine	–	+3.9

chronic glucose were gluconic acid, glucono-δ-lactone and 6-phosphogluconic acid (Fig. 2b), metabolites of the PPP, carbamyl-aspartic acid from pyrimidine synthesis and α-ketoglutaric acid from the tricarboxylic acid (TCA) cycle.

We further investigated whether inhibition of the PPP following 6-phosphogluconic acid formation has an effect on insulin gene transcription and secretion. The addition of 6-AN, a potent inhibitor of 6-phosphogluconic acid dehydrogenase [12, 13], to 3 mmol/l glucose-treated cells for 48 h also promoted accumulation of those PPP metabolites, thus mediating the metabolic state of the beta cells under high glucose conditions and also demonstrating the specificity of the chemical inhibitor (Fig. 2c,d).

PCA was performed on data obtained from INS-1E cells cultured with 3 mmol/l glucose in the presence and absence of 100 μmol/l 6-AN. Indeed, low glucose treated samples could be clearly differentiated from those treated concomitantly with 6-AN (Fig. 2d). The PC loadings (Fig. 2d) demonstrate that the PPP metabolites 6-phosphogluconic acid, glucono-δ-lactone and gluconic acid drove the separation. In addition, the results of the metabolite analysis indicate metabolism of glucose through the PPP even under low glucose conditions. The origination of the PPP metabolites from glucose metabolism, especially gluconic acid, which has been seldom reported in mammalian cells [14, 15], was demonstrated by [$^{13}C_6$]glucose tracer experiments (ESM Figs 1–4).

Analysis of insulin mRNA levels in INS-1E cells (Fig. 3a) and in human islets (Fig. 3b) revealed that the inhibition of 6PGD and the accompanied rise in PPP metabolites indeed led to a significant decrease of insulin gene expression compared with low glucose alone, similarly to exposure to chronic high glucose. When human islets were exposed over 3 days to elevated glucose or 6-AN, glucose-stimulated insulin secretion was abolished. The reduction in glucose-stimulated insulin secretion was dose dependent (Fig. 3c).

A second set of experiments tested whether genetic inhibition of *6PGD* using siRNA interference would have similar effects. Human islets plated on extracellular matrix were transfected with 100 nmol/l siRNA to *6PGD* (a set of four different siRNA sequences was used) for 4 days at 5.5 or 22.2 mmol/l glucose, which resulted in a 53% reduction of *6PGD* mRNA (Fig. 3f). Similar to 6-AN exposure, glucose-stimulated insulin secretion was impaired at 5.5 mmol/l glucose and further impaired at 22.2 mmol/l glucose (Fig. 3d,e). Chronic glucose exposure (22.2 mmol/l) inhibited glucose-stimulated insulin secretion (77% reduction of stimulatory index vs 5.5 mmol/l glucose). Stimulatory index was 25% reduced by *6PGD* siRNA vs scramble siRNA at 5.5 mmol/l glucose (p<0.05, Fig. 3d) and further impaired at 22.2 mmol/l glucose (30% reduced vs scramble siRNA at 22.2 mmol/l glucose, p<0.05, Fig. 3e).

In contrast, chronic glucose exposure up to 96 h did not change expression levels of *6PGD*. Glucose-induced regulation of *6PGD* mRNA or 6PGD protein level was not detected in either INS-1E cells (ESM Fig. 5a,b) or human islets (ESM Fig. 5c–e), while insulin mRNA was reduced in the same experiments (not shown).

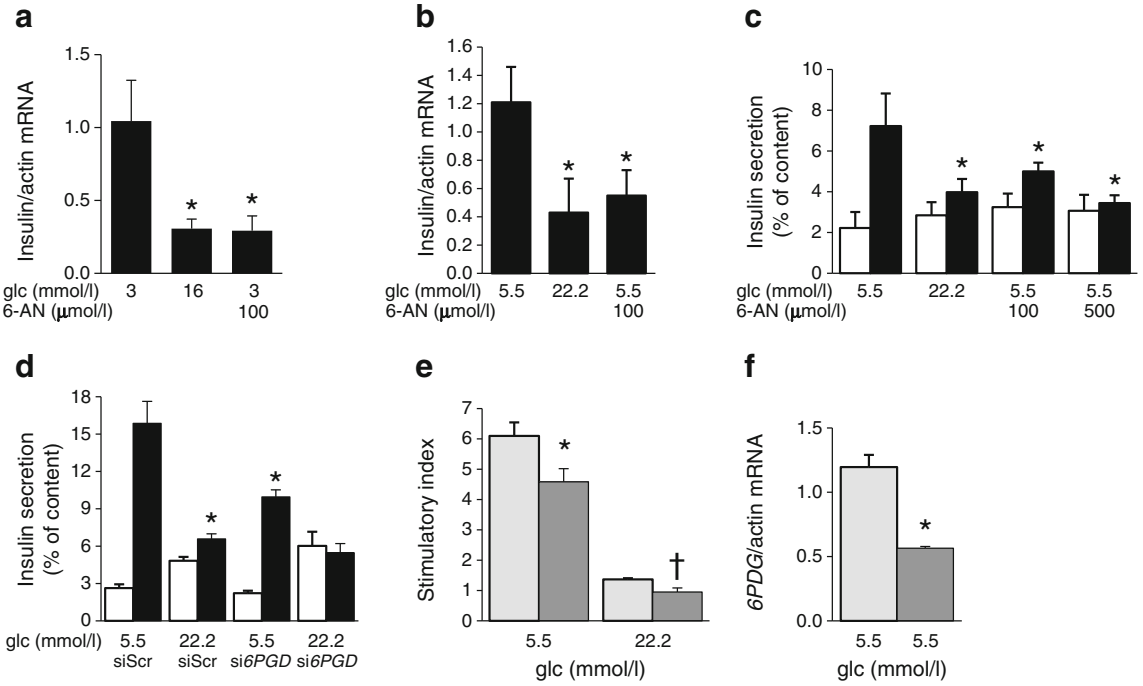

Fig. 3 **a**, **b** Insulin mRNA levels significantly decreased during high glucose or 6-AN treatment. INS-1E cells were exposed to 6-AN for 48 h (**a**) and human islets for 72 h (**b**). mRNA levels are expressed relative to β-actin. Glucose-stimulated insulin secretion was performed in islets after 72 h pre-culture with 5.5 or 22.2 mmol/l glucose or 5.5 mmol/l glucose in the presence of 100 or 500 μmol/l 6-AN. Basal and stimulated insulin denote the amount of insulin secreted during 1 h at 2.8 or 16.7 mmol/l glucose (basal [white bars] and stimulated [black bars], respectively) and expressed as % of insulin content. **d**–**f** Glucose-stimulated insulin secretion is impaired in 6PGD-depleted human islets. Glucose-stimulated insulin secretion was performed in islets 96 h after transfection with 100 nmol/l si*6PGD* at 5.5 or 22.2 mmol/l glucose. **d** Basal and stimulated insulin denote the amount of insulin secreted during 1 h at 2.8 or 16.7 mmol/l glucose (basal [white bars] and stimulated [black bars], respectively). **e** Stimulatory index is given as the ratio of stimulated to basal insulin. **f** mRNA levels are expressed relative to β-actin. **e**, **f** Light grey bars, scramble siRNA; dark grey bars, si*6PGD*. Data shown are means±SE from three different experiments (islets and INS-1E) from three different islet isolations. *$p<0.05$ vs 5.5 mmol/l glucose; †$p<0.05$ vs 22.2 mmol/l glucose. siScr, scramble siRNA

Exposure to high glucose and inhibition of the PPP activate ERK1/2 Glucose-dependent regulation of insulin transcription is dependent on extracellular signal-regulated protein kinases (ERK1/2). Previously, we have demonstrated that ERK1/2 kinase is activated by glucose [16, 17] and that this activation requires glucose metabolism [17, 18]. In agreement with this, short-term incubation (up to 1 h) of INS-1E cells (ESM Fig. 6a) and human islets (Fig. 4a) with high glucose concentrations led to a robust ERK1/2 phosphorylation, which declined between 2 and 12 h after initial glucose exposure. Extended exposure to high glucose levels (24 to 72 h) again led to significant ERK1/2 activation (Fig. 4a, ESM Fig. 6a).

We speculated that this late ERK1/2 activation may be due to accumulation of metabolites from the PPP as a result of impaired glucose catabolism through the pentose phosphate shunt and that subsequently, insulin expression through ERK1/2 activation may be affected. Indeed, inhibition of 6PGD by 6-AN at low glucose concentrations promoted ERK1/2 phosphorylation to a similar extent as long-term high glucose treatment alone in INS-1 cells (ESM Fig. 6b,c) and in human islets (Fig. 4b,c). ERK1/2 phosphorylation was potentiated after 72 h culture of human islets with 6-AN (Fig. 4b,c).

ERK1/2 inhibition attenuates accumulation of PPP metabolites and improves beta cell function To test whether the PPP metabolite accumulation induced by chronic exposure to glucose is mediated by prolonged ERK activation, we determined PPP metabolites under ERK inhibition. The simultaneous cultivation of INS-1E cells with 16 mmol/l glucose and the ERK inhibitor PD98059 attenuated the accumulation of glucono-δ-lactone (Fig. 5a), gluconic acid (Fig. 5b) and 6-phosphogluconic acid (Fig. 5c). In comparison with 16 mmol/l glucose treatment, metabolite levels were reduced by 55%, 62% and 66%, respectively, in the presence of PD98059.

Subsequently, we tested whether ERK1/2 inhibition under chronic glucose exposure may also lead to normalisation of insulin transcription and beta cell function. INS-1E cells and human islets were cultured for 48 h in low and high glucose in the presence or absence of PD98059 or

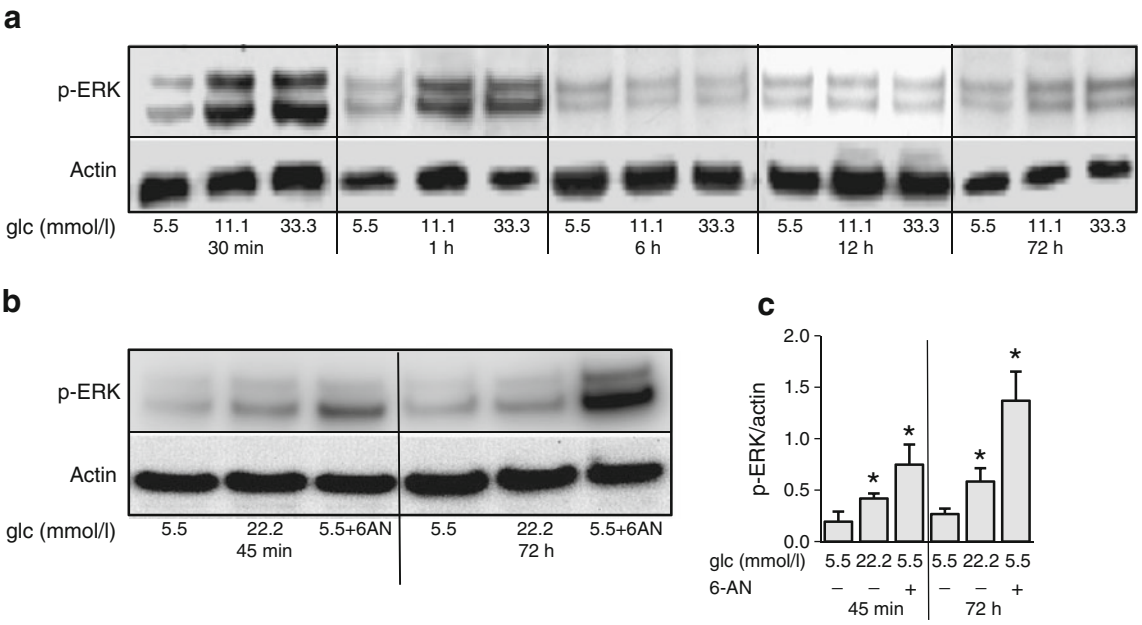

Fig. 4 Increased ERK1/2 activity during acute and chronic high glucose (glc) treatment and after PPP inhibition at low extracellular glucose concentrations. Western blot analyses of p-ERK1/2 of human islets (**a**) exposed to 5.5, 11.1 and 33.3 mmol/l glucose during 72 h culture and (**b**) cultured at 5.5 mmol/l glucose+500 μmol/l 6-AN for 72 h. Representative blots are shown from three organ donors. Densitometry analyses of bands normalised to actin (**c**), data are mean±SE; *$p<0.05$ treated vs control condition (5.5 mmol/l glucose) at the same exposure time (INS-1E results, see ESM)

U0126, specific chemical inhibitors of ERK1/2 [19, 20]. We observed that ERK1/2 inhibition was sufficient to prevent the insulin gene suppression induced by high glucose in both INS-1E cells (Fig. 5d) and human islets (Fig. 5e). This was paralleled by the restoration of *MAFA* and *PDX-1* expression in human islets (Fig. 5e; *BETA2* not tested) and a partial restoration of *Mafa*, *Beta2* and *Pdx-1* in INS-1E cells (Fig. 5d).

To determine whether chronically elevated glucose levels alter insulin secretion in parallel to changes in insulin transcription, human islets were maintained at low and elevated glucose levels for 3 days with or without ERK1/2 inhibitor PD98059 and exposed to an additional short-term high glucose challenge (Fig. 5f,g). While insulin secretion stimulated by acute glucose was enhanced in islets pre-cultured at low glucose, islets chronically exposed to high glucose (11.1 or 33.3 mmol/l) failed to secrete insulin upon additional glucose challenge (Fig. 5f). Western blot analysis confirmed that loss of glucose-stimulated insulin secretion in islets pre-treated with high glucose was paralleled by blunted ERK1/2 phosphorylation (Fig. 5f, blot). Co-incubation with the ERK1/2 inhibitor PD98059 during the 3 day culture period followed by glucose stimulation without the inhibitor significantly improved glucose-induced insulin secretion together with ERK1/2 phosphorylation (Fig. 5g). Similar results were obtained by using U0126 (not shown). Also, protection from beta cell overwork during the glucose incubation by co-culture with diazoxide, a K_{ATP}-channel opener that inhibited insulin secretion during long-term glucose exposure, restored acute glucose-stimulated insulin secretion together with ERK activation (Fig. 5h). These effects were independent from insulin secretion, as demonstrated by parallel incubation of human islets with somatostatin during chronic and acute glucose exposure (Fig. 5i). Despite unchanged insulin levels, glucose alone was able to induce ERK activation (Fig. 5i, blot). As glucose desensitisation is a temporary state that can be reversed upon restoration of normoglycaemia, we postulated that in parallel to glucose sensing, islets would also regain the ability to respond to glucose by activating ERK1/2. To test this hypothesis, islets chronically incubated at 5.5, 11.1 or 33.3 mmol/l glucose were cultured for another 2 days at 5.5 mmol/l glucose to allow recovery from chronic hyperglycaemia before acute glucose stimulation (Fig. 5j). The recovery period restored acute enhanced insulin secretion and ERK1/2 phosphorylation in islets previously treated with 11.1 mmol/l glucose for 3 days. In contrast, at chronic culture with 33.3 mmol/l glucose, normoglycaemia failed to reverse the effects of glucose and neither insulin secretion (Fig. 5j, graph) nor ERK1/2 phosphorylation (Fig. 5j, blot) was re-induced in those islets in response to acute glucose challenge. These data demonstrate that in addition to culture time, glucose concentration also plays a crucial role in the ability of beta cells to recover from hyperglycaemia-induced impaired beta cell function.

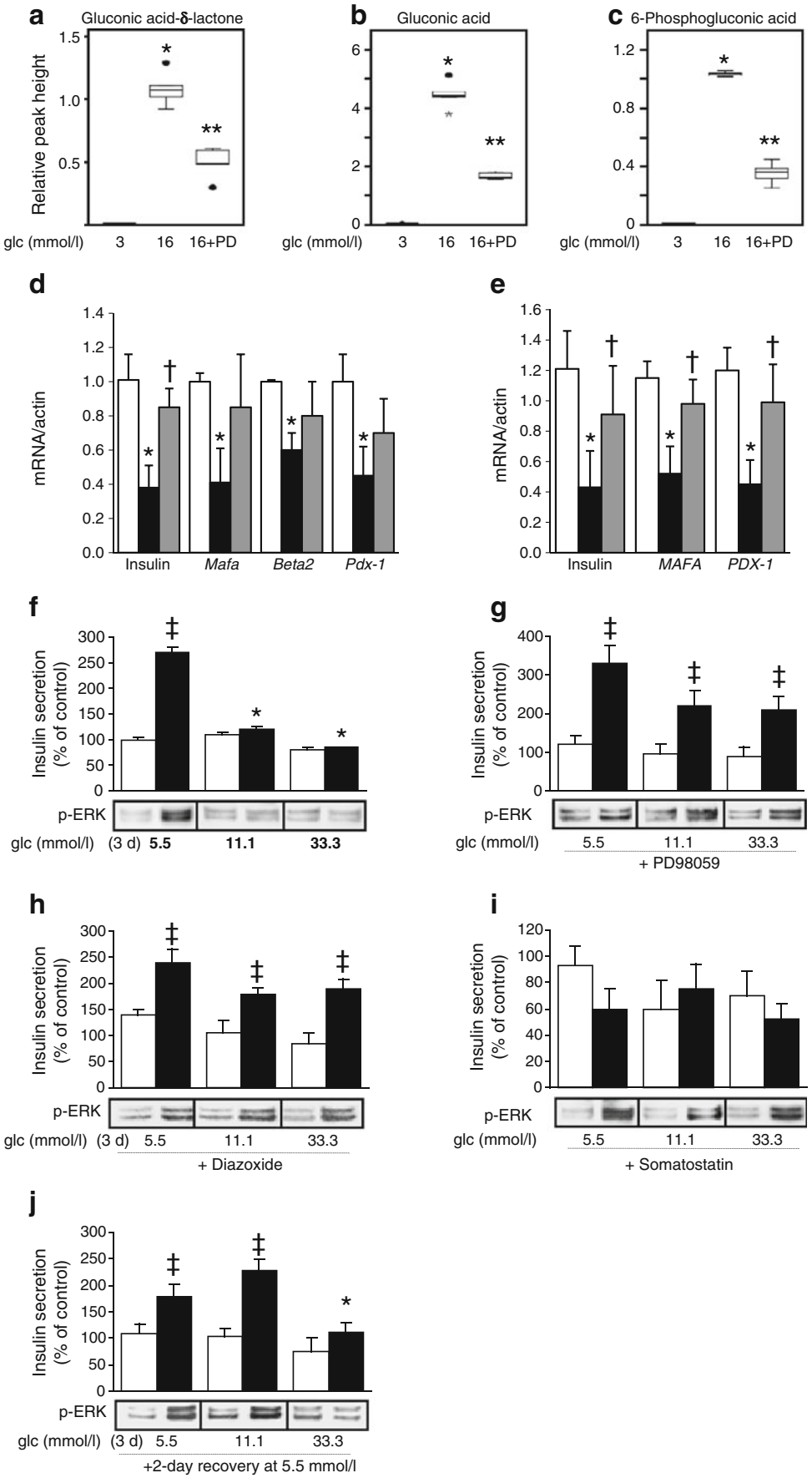

Fig. 5 ERK1/2 inhibition alters PPP metabolite levels and improves beta cell function. **a–c** INS-1E cells were cultured at 3 mmol/l, 16 mmol/l and 16 mmol/l glucose (glc)+ PD98059 (PD) for 48 h. Intracellular metabolites gluconic acid-δ-lactone (**a**), gluconic acid (**b**) and 6-phosphogluconic acid (**c**) were measured with GC-TOF-MS. **d**,**e** Insulin mRNA and mRNA of islet transcription factors from INS-1E cultured for 48 h at 3 mmol/l glucose (white bars), 16 mmol/l glucose (black bars) and 16 mmol/l glucose+ PD98059 (grey bars) (**d**) and from isolated human islets cultured for 72 h at 5.5 mmol/l (white bars) or 22.2 mmol/l (black bars) glucose or 22.2 mmol/l glucose+PD98059 (grey bars) (**e**). Quantitative RT-PCR analysis shows mean expression levels normalised to control (low glucose) and to actin and tubulin with the same result. (**f–j**) Following a chronic 3 day culture of human islets at 5.5, 11.1 or 33.3 mmol/l glucose with (**g–i**) or without (**f**, control) additional treatment, glucose-stimulated insulin secretion and ERK phosphorylation in human islets were assessed after additional culture for 1 h at either 5.5 or 33.3 mmol/l glucose. During the 3 day culture period, human islets were treated with 10 μmol/l PD98059 (ERK-inhibitor) (**g**), 200 μmol/l diazoxide (K_{ATP}-opener) (**h**) or 50 nmol/l somatostatin (inhibition of insulin secretion) (**i**). Somatostatin was also included during the acute glucose stimulation (**i**). In (**j**), islets were treated after the 3 day culture period for another 2 days at 5.5 mmol/l (recovery period) and then stimulated. Data shown are means±SE from five (**a**; **f–j**) or three (**d**,**e**) different experiments (INS-1E and islets) from five or three different islet donors. *$p<0.05$ high glucose compared with low glucose; †$p<0.05$ treated vs untreated at same glucose concentration; ‡$p<0.05$ stimulated vs basal insulin secretion. **f–h** White bars, basal; black bars, stimulated; **g** black bars, stimulated + somatostatin

Discussion

We report that metabolites from the PPP accumulate in pancreatic beta cells during chronic exposure to high glucose and suggest that impaired processing of glucose through the PPP is involved in insulin gene suppression in an ERK1/2-dependent fashion.

Chronically elevated glucose impairs beta cell function and insulin expression [1, 2]. This indicates glucose metabolism in the beta cell and suggests that specific glucose-derived intracellular metabolites may act as second messengers linking glucose metabolism and insulin expression and secretion. Using an unbiased approach we aimed to identify intracellular metabolites affecting beta cell function. A mass spectroscopic screen of 73 metabolites was performed where three PPP metabolites were found to account for the vast majority of the variance in the data following acute and chronic increases in glucose and suggests that those PPP metabolites may mediate glucose-induced impairment of the beta cell.

The unbiased analysis demonstrates association, but does not per se demonstrate causality. Such a mechanistic link is provided by experiments using the 6-phosphogluconic acid dehydrogenase inhibitor 6-AN, which caused an increase in PPP metabolites and a decrease in insulin gene transcription, mimicking chronic high glucose treatment. Those data support that the accumulation of PPP metabolites alone is sufficient to impair beta cell function.

Physiologically, the contribution of the PPP in glucose utilisation is very limited. After acute stimulation, the beta cell mainly metabolises glucose through glycolysis, and only a small portion enters the pentose phosphate shunt [21]. Almost 100% of the total glucose used is for oxidative CO_2 production [22]. Increased NADPH production by the PPP itself and through the Krebs cycle pyruvate/malate shuttle [23] would inhibit glucose-6-phosphate dehydrogenase, the ‘switch-on’ enzyme of the PPP. The PPP correlates with neither CO_2 formation nor with insulin release [22]. Glucose use through the PPP under pathophysiological conditions, such as glucotoxicity, may however be altered and should be investigated in more detail. We hypothesise that during glucotoxicity, an increased demand for NADPH and in turn consumption by NADPH-requiring processes may explain why a portion of glucose is still metabolised in the PPP and metabolites accumulate with a negative effect on insulin secretion. As a result of chronic high glucose exposure and hyperglycaemia NADPH-requiring metabolic pathways such as sorbitol synthesis [24] and fatty acid synthesis are activated. Increased levels of sorbitol have been measured in the present study and an increase in lipogenesis and fatty acid synthase mRNA levels has been reported [25]. Other NADPH-requiring pathways, including biosynthesis and catabolism of proteins, cholesterol and pyrimidine synthesis could be increased and increased carbamyl-aspartic has been reported in the present study. Even though still hypothetical, an increased demand for NADPH during glucotoxicity may explain why a portion of glucose is still metabolised in the PPP. The detected accumulation of the PPP metabolites may, however, not necessarily be a result of increased glucose flux through the PPP, but rather due to relative insufficient 6-PGD enzyme activity apparent during long-term elevated glucose. Our data show that *6-PGD* deletion results in impaired beta cell function, a finding in favour of such speculation; however, in contrast, the protein and mRNA levels of the enzyme remain unchanged following chronic high glucose exposure, thereby indicating that enzyme synthesis is not impaired. We suggest that the enzyme activity of the enzyme is negatively regulated, but by which factors remains to be investigated.

Besides regulation of insulin secretion by the TCA cycle [26], the concept that the pentose phosphate shunt plays a role in glucose-induced insulin secretion was established over 40 years ago [27–30], but its involvement during the switch from glucose-induced insulin secretion to impaired function has not been identified. Ammon et al. showed that 6-AN at a comparable concentration as used in this study reduces insulin mRNA in isolated rat islets when injected i. p. before isolation, but 24 h incubation of islets in vitro has no effect on insulin mRNA [30]. In our study, robust changes in insulin mRNA could only be detected after long-term stimulation; reduction in insulin mRNA levels in human islets were shown only after 72 h. In line with this, 6-AN also reduced insulin mRNA after 48 h, indicating that only extended metabolite accumulation affects insulin mRNA levels. Increased chronic 6-AN effects were also observed at the level of ERK activation. After 72 h exposure of human islets to 6-AN, ERK was further increased when compared with an acute exposure of 45 min. The mechanisms of this glucose switch may therefore be that acute-glucose-induced ERK activation and insulin secretion occurs partially through PPP metabolism but during glucotoxicity, massive accumulation of PPP metabolites result in prolonged ERK activity; this, in turn, enhances metabolite accumulation and finally affects insulin mRNA production.

ERK1/2 is a common pathway of acute glucose signalling in the beta cell, which directly links to insulin secretion [31], but prolonged ERK1/2 activation fosters impairment in beta cell function and beta cell apoptosis [16]. The addition of ERK1/2 inhibitors (PD98059 or U0126) during chronic high-glucose incubation prevented the observed decrease in insulin transcription and attenuated accumulation of metabolites of the PPP. The two inhibitors of ERK1/2 had similar effects on glucose-stimulated insulin secretion. Inhibitory effects independent of the MAP kinase pathway were reported in the past.

Both PD98059 and U0126 affect the cellular AMP/ATP ratio, which could lead to an activation of AMPK and a complete reset of the metabolic state of the cell after exposure to these inhibitors [32]. Glutathione levels in rat hepatocytes are elevated by PD98059 independent of the inhibition of MEK, the inactive form of mitogen-activated protein kinase (MAPK) [33]. We cannot exclude that such indirect effects modulated the measured metabolites. But, as ERK inhibition by PD98059 reduced PPP accumulation and vice versa—6-AN induced PPP accumulation and activated ERK—we can assume a specific ERK inhibition in this experimental model.

Because of physiological effects of ERK and its important role in the beta cell for glucose-stimulated insulin secretion, inhibition of ERK as a generalised therapy for diabetes is not desirable. In contrast, specific activators of PPP enzymes, e.g. activators of the 6PGD, could be novel targets for therapy. Such a possibility is currently under investigation in our laboratory.

Prolonged ERK1/2 activation induced a resistance to glucose-induced ERK1/2 activation and thus prevented further ERK1/2 activation as well as insulin secretion in response to glucose. As PPP metabolites also accumulate independently of glucose, it can be speculated that ERK1/2 activation may be triggered by: the metabolites themselves; interference of these metabolites with other metabolic pathways; or an abnormal cytosolic redox state. Different PPP metabolites are potent inhibitors of phosphoglucose isomerase [34–36]. The inhibition of phosphoglucose isomerase would consequently prevent glucose turnover in glycolysis. Impediment of glycolysis would explain why stimulatory glucose concentrations in islets after prolonged exposure to high glucose remain ineffective to mobilise insulin release. Contrary to this, inhibition of the ERK1/2 pathway attenuates accumulation of PPP metabolites, suggesting that ERK1/2 itself may be involved in the regulation and the pentose phosphate shunt (see ESM Fig. 7).

Whether glucotoxicity occurs after those metabolites simply cross a certain concentration threshold or after being elevated for a prolonged period remains to be determined. As beta cell failure results from interplay of many different factors, it is possible that such accumulation is induced secondary to other known mediators of glucotoxicity, such as post-translational modifications, oxidative damage, endoplasmic reticulum stress, which particularly contribute to the observed effects on insulin expression or secretion. At present, little is known about ERK1/2 phosphorylation by intracellular molecules in pancreatic beta cells. In other cell types intracellular ROS generation inhibits ERK1/2-directed phosphatase activity [37] resulting in sustained ERK1/2 activation. An impairment of the oxidative portion of the PPP might be crucial, as the PPP provides a mature portion of the cells´ NADPH at a very early stage of glucose metabolism. NADPH provides reducing equivalents for the regeneration of reduced glutathione, which serves a protective role against ROS formation. Exposure of cells to chronic high glucose results in increased ROS production; glutathione peroxidase plays a crucial role in protection against oxidative stress [38] and is present at low levels in the beta cell even under non-glucotoxic conditions [39]. PPP metabolite accumulation would suggest that NADPH is abundant. NADPH inhibits glucose-6-phosphate dehydrogenase [28]. Further conversion of glucose to gluconolactone would then be inhibited and not result in increased levels of the PPP metabolites. This explains why an increased glucose concentration during acute stimulation inhibits processing of glucose through the PPP [22].

It is unquestionable that the analysis presented here does not uncover all existing links between glucose metabolism and beta cell dysfunction. Many well-established signals in insulin secretion, specifically those contributing to the cellular redox state such as ATP/ADP, NADP/NADHP and NAD/NADH, were not studied, despite substantial evidence that they contribute to the regulation of beta cell function [11]. Our data do not exclude the contribution of such additional pathways, but strongly suggest that the isolated accumulation of PPP metabolites is sufficient to induce beta cell dysfunction.

In conclusion, our data demonstrate that PPP metabolites accumulate during long-term exposure to high glucose concentrations and may act as secondary messengers activating ERK1/2 and contribute to the inhibition of beta cell function. The insights presented here may provide novel perspectives in the therapeutic goal to preserve and potentially improve the beta cell.

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