Modulation of Neuronal Pentraxin 1 Expression in Rat Pancreatic β-Cells Submitted to Chronic Glucotoxic Stress

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Insulin secretory granules are β-cell vesicles dedicated to insulin processing, storage, and release. The secretion of insulin secretory granule content in response to an acute increase of glucose concentration is a highly regulated process allowing normal glycemic homeostasis. Type 2 diabetes is a metabolic disease characterized by chronic hyperglycemia. The consequent prolonged glucose exposure is known to exert deleterious effects on the function of various organs, notably impairment of insulin secretion by pancreatic β-cells and induction of apoptosis. It has also been described as modifying gene and protein expression in β-cells. Therefore, we hypothesized that a modulation of insulin secretory granule protein expression induced by chronic hyperglycemia may partially explain β-cell dysfunction. To identify the potential early molecular mechanisms underlying β-cell dysfunction during chronic hyperglycemia, we performed SILAC and mass spectrometry experiments to monitor changes in the insulin secretory granule proteome from INS-1E rat insulinoma β-cells cultivated either with 11 or 30 mM of glucose for 24 h. Fourteen proteins were found to be differentially expressed between these two conditions, and several of these proteins were not described before to be present in β-cells. Among them, neuronal pentraxin 1 was only described in neurons so far. Here we investigated its expression and intracellular localization in INS-1E cells. Furthermore, its overexpression in glucotoxic conditions was confirmed at the mRNA and protein levels. According to its role in hypoxia-ischemia-induced apoptosis described in neurons, this suggests that neuronal pentraxin 1 might be a new β-cell mediator in the AKT/GSK3 apoptotic pathway. In conclusion, the modification of specific β-cell pathways such as apoptosis and oxidative stress may partially explain the impairment of insulin secretion and β-cell failure, observed after prolonged exposure to high glucose concentrations. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.018051, 244–254, 2012.

Type 2 diabetes (T2D) is a multifactorial disease that results from insulin resistance of the target tissues (adipose tissue, skeletal muscle, and liver) and decreased insulin secretion by the pancreatic β-cells. It is, however, still unclear which event is the primary defect in the development of T2D. These two defects lead to chronic hyperglycemia, a main characteristic of T2D. However chronic hyperglycemia is not involved in the initiation of T2D but is rather implicated in the worsening of the pathology. Notably, in recent years, the notion of “glucotoxicity” has emerged to describe the toxic effects of glucose (2–5). Glucotoxicity exerts deleterious effects on β-cells, leading to the increase of apoptosis and therefore the decrease of β-cells mass observed in T2D pathology (6–8). Excess of glucose was shown to initiate various apoptosis-related mechanisms, including mitochondrial dysfunction causing production of ROS, endoplasmic reticulum stress, an increased level of intracellular calcium, and modulation of IRS/Pi3K/AKT signaling (9–11). Pi3K/AKT signaling appears to be important for β-cells growth (12, 13), and GSK3, as a downstream element in this pathway, has been proposed as a possible target for β-cell protection (11).

Insulin secretory granules (ISGs) are organelles specialized in insulin processing and storage in the pancreatic β-cells. Their content is released by exocytosis in response to an acute increase of blood glucose, other nutrients, as well as hormonal and neuronal stimulation. The recent establishment of the proteome of ISG allowed identification of novel players potentially involved in ISG biogenesis, trafficking, and exocytosis, such as Rab37, VAMP8, and several lysosomal proteins (14, 15). A better understanding of ISG composition and function led to the consideration of ISG as a pivotal organelle of β-cells function, because it is now thought to be directly or indirectly related to various signaling pathways from exocytosis to proliferation/apoptosis (16–18).

Several studies have been undertaken to monitor the modifications of the ISG proteome induced by chronic hyperglycemia. Altered expression of several ISG proteins was shown

1 The abbreviations used are: T2D, type 2 diabetes; ISG, insulin secretory granules; NP1, neuronal pentraxin 1; SILAC, stable isotope labeling by amino acids in cell culture; GSK3, glycogen synthase kinase 3.
to affect insulin secretion (19–21). Furthermore, the expression of β-cell exocytotic proteins is modified not only after chronic hyperglycemia in vitro (3) but also in isolated islets (22, 23) and from diabetic organ donors (24), the latter suggesting the consequence of altered gene expression after hyperglycemia in vivo.

To elucidate novel mechanisms affected by prolonged glucose exposure, we performed a comparative proteomic analysis on ISGs after stimulation with either medium or high glucose concentrations. The SILAC strategy was applied to INS-1E rat β-cells, and ISGs were purified after 24 h of growing with different glucose concentrations. This analysis revealed 14 proteins significantly affected by chronic high glucose concentration. Neuronal pentraxin 1 (NP1), a new β-cell protein whose expression was modulated by glucose stimulation, was further investigated. So far, NP1 was described to be exclusively localized within the brain (25, 26). The present study demonstrates that its expression is modulated by chronic high glucose concentrations in insulin-secreting cells and that NP1 in INS-1E cells is linked to the AKT/GSK3 pathway.

**Experimental Procedures**

**Cell Culture and Stable Isotopic Amino Acid Incorporation**—Rat insulinoma INS-1E cells were grown in a RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C and under 5% CO2 and humidified atmosphere. SILAC experiments were conducted as described by Couté et al. (4). Briefly, normal RPMI 1640 medium (Sigma-Aldrich) depleted in arginine, leucine, and lysine was supplemented with leucine (25 mg/L; Sigma), lysine (25 mg/L; Sigma), and arginine (100 mg/L; Sigma) for the “light” medium, and with 13C6-Leu, 13C6-N, N Lys (Cambridge Isotope Laboratories), and arginine in the same concentrations for “heavy” medium. Amino acid incorporation was done for 4 weeks. Glucose stimulation was performed for the last 24 h, using light and heavy RPMI media supplemented with 2% fetal bovine serum, and either 11 mM glucose or 30 mM glucose, or 11 mM glucose and 0.5% (v/v) SDS, 10% (v/v) glycerol, 0.1 M dithioerythritol, and traces of bromphenol blue and heated 5 min at 95 °C, before separation on a 12.5% polyacrylamide gel (27). The proteins were run for 1.5 cm, and the gel was stained with Coomassie Blue R-250 (Merek).

**Mass Spectrometry Analysis**—SILAC-PAGE lanes (n = 4) were cut into six identical slices, and the proteins contained in each slice were digested in gel by trypsin. Briefly, the samples were reduced in 10 mM dithioerythritol and alkylated in 55 mM iodoacetamide, and the gel pieces were dried. The proteins were then digested overnight at 37 °C using 6.25 ng/μL of trypsin (Promega). Tryptic peptides were extracted from gel slices, dried, and resuspended in an appropriate amount of 5% ACN, 0.1% formic acid (FA). NanoLC-MS/MS analyses were performed on a NanoAcuity system (Waters) coupled to a LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Electron). Peptides were trapped on a home-made precolumn (0.1 × 20 mm filled with 5 μm beads of 200 Å Magic C18 AQ, Michrom) and separated on a home-made column (0.75 × 150 mm filled with 5 μm beads of 100 Å Magic C18 AQ, Michrom). The analytical separation was run for 65 min using a gradient of 99.9% H2O with 0.1% formic acid (solvent A) and 99.9% CH3CN with 0.1% formic acid (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nl/min. For MS survey scans, the Orbitrap resolution was set to 60,000, and the ion population was set to 5 E+5 with a m/z window from 400 to 2000. Eight precursor ions were selected for collision-induced dissociation (normalized collision energy set to 35%, ion population set to 7 E + 3 and isolation width of 2 m/z). Two injections were performed for each band to obtain technical replicates.

**Data Analysis**—Acquired raw files were processed with MaxQuant v1.0.13.13 as described by Cox et al. (28). Briefly, Quant module of MaxQuant was used to generate peaklists, with the following parameters: Orbitrap/FT Ultra; SILAC doublets with heavy labels Leu6 and Lys8 and a maximum of three labeled amino acids. Generated results were then submitted to Mascot search engine v2.2 (Matrix Science, London, UK) using parameters defined in Quant: variable modifications were set as oxidation (Met) and acetylation (protein N terminus), and carbamidomethyl (Cys) was chosen as fixed modification. Rat International Protein Index v3.52 (40,168 entries, comprising 262 contaminant entries) was selected as the database, trypsin (no proline restriction) was selected as the enzyme, and two missed cleavages were allowed; MS and MS/MS tolerance were respectively set to 10 ppm and 0.5 Da. The acquired .dat files were then submitted to the Identify module in MaxQuant for the quantification step, using the following parameters: peptide and protein false discovery rates were set to 1%, maximum posterior error probability was set to 1, minimum of peptides and unique peptides was set to 2, and minimum of peptide length was set to 6 amino acids. For quantification, default parameters were used. An experimental design template was created to invert ratios of half of the experiments and to separate the different biological (n = 4) and technical (n = 2) replicates.

**Western Blot**—Proteins were separated by SDS-PAGE and transferred on nitrocellulose membrane. For total extracts from cells grown with medium and high glucose concentrations (n = 4), antibodies raised toward NP1 (monoclonal, dilution 1/1000; BD Transduction Laboratories) or β-actin (monoclonal, dilution 1/20,000; Sigma) were used. For experiments on different ISG populations, VAMP4 (rabbit polyclonal antibody; Sigma), β-granin (rabbit polyclonal antibody; Eurogentec), and NP1 antibodies were chosen. Primary antibodies were then put in contact with the membrane for one night at 4 °C before washings and
incubation with horseradish peroxidase-coupled secondary antibodies for 45 min (Dako). After revelation by chemiluminescence, bands of interest were quantified using ImageQuant (GE Healthcare).

Immunohistochemistry—Four-micrometer-thick pancreas sections were deparaffinized in xylol and rehydrated in descending ethanol series. NP1 epitopes were recovered by heating slides 10 min in micro-waves at 98 °C in a buffer containing 0.01M citrate (pH 6). No pretreatment was required for the insulin immunohistochemistry. Mouse monoclonal anti-NP1 antibody (BD Transduction Laboratories) was used at 2.5 μg/ml in diluent from Dako. Mouse monoclonal anti-insulin antibody (Sigma) was diluted at 1/1000 in the same diluent. After blocking endogenous peroxidases, primary antibodies were incubated 1 h at room temperature. For NP1, the amplification Chem-Mate kit (Dako) was used according to the manufacturer’s instructions for the staining reaction using the Diaminobenzidine (DAB) substrate chromogen. For insulin, secondary horseradish peroxidase-labeled antibody from the envision kit (Dako) was incubated 30 min at room temperature before being washed in kit buffer. DAB staining was performed with the chromogen solution (Dako) for 5 min.

Quantitative RT-PCR—Total RNA extraction was performed using a RNeasy kit (Qiagen) according to the manufacturer’s instructions. Design of primers for real time PCR was carried out with the software Primer Express (Applied Biosystems). The primers were as follows: NP1: 3’-GCAAACTTTGCAATCCCTCAA-5’ (forward) and 3’-TGTTGGTTTGCGTCGGAAGAT-5’ (reverse).

First strand cDNA synthesis was carried out with 500 ng of RNA using random hexamers and Superscript II reverse transcriptase following the manufacturer’s instructions (Invitrogen). Quantitative RT-PCR (n = 3) was carried out in optical 384-well plates and labeled using the SYBR green master mix (Applied Biosystems), and the fluorescence was quantified with a Prism 7900 HT sequence detection system (Applied Biosystems). The results were normalized against the rat β-tubulin, EEF1A1, and RPS9 genes.

RESULTS

ISG Proteome—Because β-cell failure is one of the major characteristics of type 2 diabetes mainly through a defect of insulin secretion, we focused our work on ISGs and analyzed their proteome changes under chronic high glucose exposure. We previously defined the conditions that mimic early glucotoxicity by testing viability, apoptosis, insulin secretion, and content (4). Glucotoxicity-induced modifications were therefore assessed using the SILAC strategy on INS-1E rat clonal β-cells grown either with 11 mM of glucose (medium concentration) or with 30 mM of glucose (high concentration) for 24 h before harvesting. For SILAC experiments, we used two different isotopic amino acids: 13C6-leucine and 13C615N2-lysine, because it is well known that arginine could be converted into proline in certain cell lines, inducing a bias in the quantification (29). This is indeed the case for INS-1E cells (data not shown). The choice of these two amino acids for SILAC labeling increases the probability for a tryptic peptide to contain at least one isotopic amino acid, because it is well known that arginine could be converted into proline in certain cell lines, inducing a bias in the quantification (29). This is indeed the case for INS-1E cells (data not shown).

Fig. 1. Experimental workflow for SILAC labeling, ISGs purification, and sample preparation for MS analysis. Two injections per band were performed, resulting in a technical duplicate.
Four biological replicates were prepared combining heavy and light cells corresponding to medium and high glucose exposures. MS/MS analysis of the gel slices allowed identification of 1208 proteins with at least two unique peptides and a false discovery rate below 1%. A broad classification of the identified proteins was performed using Gene Ontology (see supplemental Table 1) and sorted them into five groups: intravesicular, membrane, mitochondrial, other, and unknown proteins (Fig. 2). The first group contained 120 proteins (10% of total proteins), including 59 hydrolases (49% of the group), such as neuroendocrine convertase 1 and 2, and 41 secreted proteins (34% of the intravesicular proteins), such as insulin or secretogranins 1, 2, and 3. The second group holds 451 membrane proteins, representing 36% of the total identified proteins. It comprises 88 proteins from endoplasmic reticulum/Golgi and more interestingly 22 Rab or Ras-related proteins, 7 syntaxins, and 3 VAMPs. The third group covers potential mitochondrial proteins (143 proteins, 12% of the identified proteins). The fourth group, named “other,” includes 182 proteins known to be localized in other cell parts or compartments. The last group contains 312 proteins and includes proteins that have no Gene Ontology correspondence.

Quantitative Analysis of ISGs Proteome Modifications under Chronic High Glucose Exposure—The MS results allowed quantifying 1154 proteins for which total ratio distribution was centered on 1 after normalization (Fig. 3 and supplemental Table 1). Significant differentially expressed proteins were filtered out using the following criteria: significance $B < 0.0005$, ratio $> 2$, and protein appearing in three of four biological replicates (Table I). Of the 14 proteins found to be differentially expressed, 4 were up-regulated in the high glucose condition, and 10 were down-regulated. Most of the proteins found differentially expressed are poorly described, having unknown or imprecise localization and function. For instance, the serine peptidase inhibitor b6a (Serpinb6a), phenazine biosynthesis-like domain-containing protein (Pbld), and prenylcysteine oxidase (Pcyox1) are proteins that are not attributed to any reliable pathway. However, other proteins are linked to interesting pathways, such as prolyl endopeptidase (Prep), thioredoxin reductase 1 (Txnrd1), and annexin A6 (Anxa6), which were found down-regulated, or junction plakoglobin (Jup) and NP1, found to be up-regulated (Table I). NP1 is a neuronal protein, which has a potential implication in apoptotic events linked to hypoxia-ischemia in neurons (30, 31), and might therefore be an interesting target for the understanding of $\beta$-cell failure in glucotoxic conditions.

Expression and Intracellular Localization of NP1 in INS-1E Cells and in Rat Islets—NP1 was hitherto exclusively described in neurons, and we substantiated its expression by mass spectrometry in clonal $\beta$-cell ISGs enriched fractions.
Western blot analyses confirmed its expression in INS-1E cells (Fig. 4). To gain insight into the intracellular localization of NP1 in INS-1E cells, we analyzed its distribution in a further fractionation step of ISG preparation used to separate mature ISGs from immature ISGs and contaminants. As shown in Fig. 5, NP1 is localized at the bottom of this gradient, in the same fractions as insulin and \( \beta \)-granin, indicating that NP1 is clearly present in ISGs and that it may be enriched in mature ISGs. Finally, we performed immunohistochemistry on pancreas sections and demonstrated the co-localization of insulin and NP1 in \( \beta \)-cells of rat pancreatic islets (Fig. 6).

**Modulation of NP1 Expression in INS-1E Cells Submitted to Glucotoxic Stress** — SILAC analyses revealed that NP1 expression was up-regulated 2.5-fold in cells submitted to 24 h of 30 mM glucose compared with cells incubated for the same time with 11 mM glucose. Western blot analysis confirmed this overexpression at the protein level (Fig. 4). A time course experiment was performed to monitor changes in NP1 levels in INS-1E cells exposed to high glucose exposure from 2 to 72 h. We could observe a significant increase of the protein starting at 12 h, confirming that NP1 overexpression represents an early event of chronic glucotoxicity (Fig. 7A).

To establish a possible correlation between the protein and mRNA expression levels in response to chronic glucose stimulation, quantitative RT-PCR experiments were performed \((n = 3)\) for neuronal pentraxin 1. We found a significant increase \((p < 0.05)\) of NP1 mRNA levels commencing already at 2 h of high glucose stimulation of INS-1E cells compared with culture in 11 mM glucose (Fig. 7B).

A **Link between AKT/GSK3 Pathway and NP1 Expression** — Considering the role attributed to NP1 in apoptosis in neurons, the chemical compound CT99021 was used in INS-1E cells to decrease GSK3 activity (11) and to probe for its impact on NP1 expression in both medium and high glucose conditions. Western blots revealed that a decrease of GSK3 activity with 2.5 \( \mu M \) of CT99021 for 24 h leads to a significant decrease of NP1 levels, demonstrating a link between GSK3 activity and NP1 expression in INS-1E cells. Interestingly, this experiment points out that the treatment with GSK3 activity

### TABLE I

| Protein names                                                                 | Gene names | Posterior error probability | Unique peptides | Ratio count | Ratio heavy/light normalized |
|-------------------------------------------------------------------------------|------------|-----------------------------|-----------------|-------------|-------------------------------|
| Acylamino acid-releasing enzyme                                               | Apeh       | 1.14E-108                   | 9               | 30          | 0.27                          |
| Cellular retinoic acid-binding protein 1                                      | Crabp1     | 7.81E-06                    | 3               | 4           | 4.39                          |
| S-Adenosylmethionine synthetase isoform type-2                                | Mat2a      | 5.04E-40                    | 3               | 15          | 0.22                          |
| Junction plakoglobin                                                          | Jup        | 8.10E-63                    | 5               | 7           | 4.24                          |
| Prolyl endopeptidase                                                          | Prep       | 2.02E-08                    | 3               | 5           | 0.29                          |
| Serine (or cysteine) peptidase inhibitor, clade B, member 6a                  | Serpinb6a  | 8.99E-46                    | 6               | 27          | 0.43                          |
| 23-kDa protein                                                                |            | 3.02E-78                    | 6               | 52          | 2.91                          |
| Neural F box protein NFB42                                                    | Fbxo2      | 1.34E-59                    | 3               | 20          | 0.34                          |
| Phenazine biosynthesis-like domain-containing protein                          | Pblld      | 5.29E-51                    | 11              | 23          | 0.46                          |
| Creatine kinase, mitochondrial 1                                              | Ckmt1      | 2.34E-137                   | 11              | 157         | 0.47                          |
| Annexin A6                                                                    | Anxa6      | 5.17E-206                   | 18              | 78          | 0.47                          |
| Thioredoxin reductase 1                                                        | Txnrd1     | 4.41E-81                    | 7               | 29          | 0.49                          |
| Prelycysteine oxidase                                                          | Pcsox1     | 9.10E-218                   | 13              | 128         | 0.5                           |
| Neuronal pentraxin-1                                                          | Nptx1      | 5.1823E-44                  | 3               | 7           | 2.55                          |

2 D. Schwartz, Y. Brunner, Y. Couté, M. Foti, C. B. Wollheim, and J.-C. Sanchez, manuscript in preparation.

![Fig. 4. Validation of NP1 overexpression in cells submitted to glucotoxicity.](graph.png)

![Graph showing NP1 and Actin expression in INS-1E cells under different glucose conditions.](graph.png)

**Fig. 4. Validation of NP1 overexpression in cells submitted to glucotoxicity.** Western blot analyses of neuronal pentraxin 1 were conducted on INS-1E cell lysates incubated for 24 h with either 11 or 30 mM glucose. Quantification of actin level was used for normalization. The bars correspond to the mean of band volumes (three biological replicates) with the standard deviation. *, significant \( p \) value < 0.05 (Mann-Whitney).
inhibitor induces a reduction of NP1 overexpression previously observed with 30 mM of glucose incubation for 24 h, recovering the same NP1 levels as observed with 11 mM of glucose (Fig. 8).

**DISCUSSION**

Chronic hyperglycemia is one of the hallmarks of diabetes and is implicated in the development of diverse diabetic complications, such as retinopathy, nephropathy, and neuropathy. Moreover, chronic hyperglycemia is also known to participate in the worsening of diabetes by aggravating the impairment of insulin secretion by the /H9252/-cells (32–34). It is assumed that the modulation of expression of key /H9252/-cell proteins in response to chronic hyperglycemia may in part explain the decline of /H9252/-cell functions (22, 24, 35). We focused our study on the effects of prolonged high glucose exposure in INS-1E cells. In a previous work, to adjust experimental conditions, we monitored viability, apoptosis, insulin secretion, and insulin content under low, medium, and high glucose concentrations and demonstrated the absence of any detectable effect of the SILAC strategy on the transcriptome and proteome of INS-1E /H9252/-cells (4). We also described that incubation with 5 mM of glucose induced a decrease of cell viability and extended the observations made by Dubois et al. (32) by showing that growing INS-1E cells with 30 mM of glucose during only 24 h significantly decreased glucose-induced insulin secretion. In the present work, to gain further insight on the effects of glucotoxicity on /H9252/-cells at the molecular level, we used the SILAC strategy to investigate the modifications of the ISG proteome on INS-1E cells submitted or not to glucotoxic conditions.

**An Update of the ISG Proteome**—Several studies were conducted to establish the ISG proteome, using different purification techniques. The first ones identified 50 proteins using affinity purification (15) and 130 proteins after a biochemical fractionation procedure using two gradient centrifugation steps (14). However, there is a modest agreement between the two studies (15 proteins), and it was noted that major ISGs components were missing (18). In the present
Modification of ISG Proteome by Prolonged High Glucose Exposure of β-Cells—The principal aim of this study was to identify some early molecular mechanisms of β-cell failure. For this, we monitored changes in the ISG proteome of cells exposed to chronic high glucose concentration compared with control cells. The actual need was to extend the quantitative analysis and then to focus on low abundant ISG proteins to find new culprits in β-cell dysfunction. This led to the identification of 14 proteins, of which the expression was significantly modulated by glucotoxic conditions (Table I) by using strict criteria (two unique peptides/protein, significance B value below 0.0005 and consistent results between replicate experiments). This apparently small number of modulated proteins is in agreement with our previous results on total cell extracts (4). It can be explained by two main factors: 1) very early events of the glucotoxicity process have been studied in this work, and 2) the effects of a high glucose (30 mM) were not compared with those of low and nonstimulatory glucose concentrations (e.g. 5 mM) as is classically the case, but rather to those of a “medium” glucose concentration (11 mM) because we reported previously that 5 mM glucose induced a rapid decrease of INS-1E cell viability (4).

Among the proteins found to be differentially expressed, CRABP-1 was already shown to exhibit a differential expression level between control and INS-1E cells submitted to high glucose concentration (4). This was also the case for other fatty acid metabolism-related proteins, giving predominance to a link between glucotoxicity and fatty acid metabolism. Modulation of the expression of fatty acid metabolism-related proteins is linked with several deleterious processes in β-cells, such as changes in cell membrane composition and cellular trafficking (42). Prolyl endopeptidase (Prep), which was found to be down-regulated (ratio = 0.3) is involved in the maturation and degradation of peptide hormones or neuropeptides. Annexin A6, a calcium-binding protein, was found to be 2-fold down-regulated in the present study. This vesicular protein is involved in several processes such as calcium homeostasis, membrane organization, and exocytosis (43, 44). These different processes are essential for proper β-cell function and insulin secretion. Therefore the modulation of annexin A6 levels by high glucose concentration might lead to defective ISGs processing and secretion. JUP is a junctional plaque protein and participates in the β-catenin phosphorylation cascade (45). Other proteins, such as thioredoxin reductase 1, and neuronal pentraxin 1 are linked to apoptosis. Interestingly, thioredoxin reductase 1 has been shown to negatively regulate hypoxia-induced apoptosis, whereas NP1 participates to the induction of apoptosis in neurons (46–48). Thioredoxin reductase 1, for instance, has anti-apoptotic properties by counterbalancing oxidative stress in case of hypoxia. It is of interest in this context that chronic high glucose, by activation of the transcription factor ChREBP and its target TXNIP, has been demonstrated to increase β-cell apoptosis by inhibiting the function of thioredoxin, which is

work, the coverage of the ISG proteome was considerably extended to more than 1000 proteins, notably thanks to the dramatic gain of sensitivity of the state of the art technical platforms. Accordingly, it allowed identifying dozens of new ISG proteins. For instance, we found aminopeptidase B, which is a marker of the secretory pathway in PC12 cells (36). Moreover, granulin-1, a secreted glycoprotein, was identified for the first time in ISGs. Interestingly, granulin precursor, progranulin, was shown to be localized in chromogranin A-containing vesicles in neurons (37). Other interesting examples are the insulin-degrading enzyme and the Niemann-Pick disease type C2 protein, which are secreted proteins, already described as localized at different intracellular localizations (38–40).

Considering the need for ISGs to contain all the machinery for insulin processing and release and for granule budding, trafficking, and fusion but also considering their role in different regulatory functions, we now can assume that the ISG proteome in INS-1E cells should contain at least 350 different proteins. Furthermore, because organelles are dynamic compartments and because it was shown that 39% of organelar proteins have multiple localizations (41), this number is probably underestimated. By identifying 1208 different protein, this work allowed the discovery of most of the ISG proteins. However, this number also suggests that potential contaminants inherent to every proteomic analysis based on subcellular fractionation strategies were also found. Bona fide resident proteins of ISGs can be in part discriminated from contaminants by an optimization of the purification workflow. However, this could also induce the loss of proteins weakly associated to ISG. Therefore, classifying some of the identified proteins as contaminants or ISG proteins still represents a real challenge.
under the control of thioredoxin reductase \( (49, 50) \). These down-regulations of negative regulators of apoptosis coupled to the up-regulation of positive regulators of this phenomenon indicate that the induction of the cell death program provoked by chronic hyperglycemia is a very early event. Furthermore, the finding that NP1 is enriched in ISGs of INS-1E cells submitted to glucotoxicity allows extrapolating a link between ISGs and propagation of proapoptotic signals.

**Fig. 9. Model for NP1 level modulation by glucotoxicity in β-cells.** 

A, GSK3 is an intermediate in both insulin/IGF1 and Wnt pathways. On the one hand, GSK3 inactivation by Akt-mediated phosphorylation leads to the activation of glycogen synthase for glucose storage. It induces also anti-apoptotic signaling and the maintenance of β-cell function notably through the action of IPF1/PDX1. On the other hand, GSK3 inactivation by the Wnt pathway induces an accumulation of β-catenin and therefore activates tumorogenesis. In β-cell, NP1 expression might be related to GSK3 activity. B, increased level of glucose might induce Akt deactivation by a decrease of its phosphorylation state. This loss of function would lead to a decrease of GSK3 phosphorylation state, corresponding to an increase of its kinase activity. This has an impact on several genes and especially induces an up-regulation of the NP1 level, potentially participating in induction of cell apoptosis.
NP1 as a Target of Glucotoxicity—NP1 was exclusively described in neurons so far. We already identified NP1 in ISGs of INS-1E β-cells using a proteomic approach (14). In the present work, we validated the expression of NP1 in rat pancreatic tissue and showed its co-localization with insulin. Furthermore, we described the early up-regulation of its expression following high glucose exposure (from 2 h at the mRNA level and 12 h at the protein level). The difference in the time frame between up-regulation of mRNA and protein expressions might be due to a delay between the translation and the transcription rates of NP1 mRNA. Our results indicated that NP1 up-regulation is not a consequence of short glucose stimulation but really an early molecular event linked to prolonged high glucose exposure of β-cells.

Concerning its biological role, NP1 has been described as a mediator for hypoxia-ischemia and potassium deprivation-induced apoptosis in neurons (30, 31, 47, 48). In β-cells, it is well known that long term chronic hyperglycemia induces apoptosis (51), leading to a decrease of the pancreatic β-cell mass. However, after 24 h of high glucose exposure, no signal of apoptosis was detectable by deoxynucleotidyltransferase-mediated dUTP nick end labeling assay. Therefore, NP1 may represent a very early actor in the induction of a cell death program provoked by chronic hyperglycemia. At the molecular level, it was described that NP1 is probably a mediator of neuronal death through the AKT/GSK3 pathways (30). GSK3 is an intermediary protein involved in different pathways, mainly related to cell maintenance and apoptosis. Activation of GSK3α/β activity influences transcription of many genes (52). Its activity is tightly regulated notably through Akt-mediated phosphorylation. Interestingly, it was shown that GSK3 deactivation, notably through activation of the insulin or insulin-like growth factor pathways, leads to glycogen synthase and IPF1/PDX1 activation, important for glucose storage and maintenance of β-cell function (53), as well as to β-catenin accumulation inducing tumorigenesis. Recent findings reported more details about NP1 activation in hypoxic-ischemic conditions in neurons, linking it to a decrease of Akt phosphorylation and consequently to a decrease in GSK3α/β phosphorylation state, increasing its kinase activity (48).

This neuronal model of NP1 up-regulation in case of apoptosis induction might be a cornerstone to understand the role of NP1 in glucotoxicity-induced apoptosis in β-cells. Through the use of an inhibitor, we clearly demonstrated that there is a link between GSK3 activity and NP1 expression in β-cells. We made the hypothesis that NP1 might be a mediator of β-cell apoptosis through a similar pathway than in neurons (Fig. 9). High glucose exposure might induce a decrease of Akt phosphorylation state leading to its deactivation. This would result in an increase of GSK3 kinase activity, in turn leading to the indirect regulation of the transcription of several genes, such as NP1. GSK3 is already a target for β-cell mass preservation in the context of type 2 diabetes. Indeed, it was shown to be linked to β-cell apoptosis when its activity increases (11, 54). GSK3 being a key player in several pathways notably involved in tumorigenesis (the Wnt pathways for instance), its inhibition by chemical inhibitors counteracting Akt deactivation, could lead to deleterious effects on the organism. Therefore, the discovery of NP1 as a potential mediator in the glucotoxicity-mediated apoptosis of β-cells, downstream of GSK3, is promising. NP1 may therefore represent a novel target for β-cell mass preservation in the context of type 2 diabetes.

Conclusions—Glucotoxicity is known to induce severe perturbations in the pancreatic β-cells, leading to impaired β-cell function and apoptosis. Modifications of the expression of key β-cell proteins by chronic hyperglycemia are certainly involved in this process. In the present study, using a combination of SILAC and subcellular fractionation, we demonstrated that growing INS-1E β-cells for 24 h with a high glucose concentration altered the expression of several ISG proteins known to be involved in different processes such as fatty acid metabolism, trafficking, or apoptosis. Furthermore, the co-localization with ISG of one of these proteins, NP1, was shown for the first time, as well as its presence in rat pancreatic islets where it co-localizes with insulin. Glucotoxicity-induced modulation of NP1 suggests that chronic high glucose concentrations potentially induce very early proapoptotic signaling linked to GSK3 activity. Functional studies questioning the involvement of NP1 in the impaired insulin secretion observed during INS-1E-cell submitted to glucotoxic conditions are now warranted.

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