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REVIEW ARTICLE

Advanced proteomics procedure as a detection tool for predictive screening in type 2 pre-Diabetes

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Abstract It has been suggested that a more precise selection of predictive biomarkers may prove useful in the early diagnosis of type 2 diabetes (T2D), even when glucose tolerance is normal. This is vital since many T2D cases may be preventable by avoiding those factors that trigger the disease process (primary prevention) or by use of therapy that modulates the disease process before the onset of clinical symptoms (secondary prevention) occurs. The selection of predictive markers must be carefully assessed and depends mainly on three important parameters: sensitivity, specificity and positive predictive value. Unfortunately, biomarkers with ideal specificity and sensitivity are difficult to find. One potential solution is to use the combinatorial power of different biomarkers, each of which alone may not offer satisfactory specificity and sensitivity. Recent technological advances in proteomics and bioinformatics offer a great opportunity for the discovery of different potential predictive markers. In this review, we described a cellular T2D model as an example with the intent of providing specific enrichment and new identification strategies, which might have the potential to improve predictive biomarker identification and to bring accuracy in disease diagnosis and classification, as well as therapeutic monitoring in the early phase of T2D.

Keywords Diabetes mellitus · Beta-cell failure · Glycotoxicity · Lipotoxicity · Predictive biomarker · Pancreatic polypeptide Y · Proteomics

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General introduction

Type 2 diabetes (T2D) is one of the fastest growing health concerns worldwide with a prevalence of approximately 5% in the United States (American Diabetes Association, 2002). Due to sedentary lifestyle, unhealthy nutrition and a prolonged lifespan, the prevalence in the developed and developing countries is still increasing. Health experts have warned of a global epidemic of diabetes caused by a rise in overweight and obesity. There are currently 120–140 million people worldwide with T2D, and if trends continue, this number is predicted to double in the next 25 years. In the Western world, around 90% of T2D cases are attributable to weight gain. Because of the severe health and cost implications of this disease, organizations such as the International Diabetes Federation (IDF) have called for increased efforts to prevent its development. The IDF estimates that 314 million people worldwide, or 8.2% of the global population, have impaired glucose tolerance (IGT), a state that often precedes T2D. In addition, T2D is a heavily under-diagnosed disease and is usually only discovered at a late stage, when the first complications occur. Detection in an early state would be highly desirable, as the progression of T2D can then be slowed down by simple, nonpharmacologic means, i.e. weight loss and exercise. The existing diagnostic tests, fasting plasma glucose (FPG), the oral glucose tolerance (OGT) test or the euglycemic hyperinsulinemic clamp are either relatively insensitive and inaccurate or so difficult to perform that they do not lend themselves to mass screening.

The identification of a reliable, accurate, cost-effective and non-invasive test for T2D that could be applied in the prediabetic state would present a sizeable opportunity for diagnostics and would be valuable as a monitoring marker for clinical studies in T2D. Although significant progress

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has been made in the treatment of T2D, new insulin sensitizing drugs such as the thiazolidinediones display significant side-effects and major health concerns still prevail for this class of compounds. Most importantly, there is no medication on the market that slows down the progressive beta-cell (β-cell) failure, a hallmark of late-stage T2D. The identification of pathways and factors involved in either insulin-resistance of skeletal muscle or β-cell failure could lead to new drug targets and the development of novel pharmaceuticals for the treatment of impaired glucose tolerance (IGT) and for the prevention of β-cell failure, respectively.

Beta-cell failure is explained by both a relative loss of β-cell mass as well as secretory defects including enhanced basal insulin secretion by the β-cells and a selective loss of sensitivity to insulin mainly in skeletal muscle but also in other organs. Beta-cell failure is the last step in the progression of type 2 diabetics towards dependence on external insulin. The loss of β-cell function is believed to be triggered by long-term exposure to enhanced levels of glucose and lipids (glycol- and lipotoxicity). However, the molecular mechanisms of this phenomenon are poorly understood. Although insulin sensitizers hold some promise, there is currently no proven treatment for T2D patients that would prevent progression towards β-cell failure. It would also be useful to identify better targets and/or predictive markers for β-cell failure or function that are more sensitive or more reliable than the targets/markers commonly used, such as proinsulin or its processing products insulin or C-peptide. This is vital since many T2D cases may be preventable by avoiding those factors that trigger the disease process (primary prevention) or by use of therapy that modulates the disease process before the onset of clinical symptoms (secondary prevention) occurs. For a better understanding of the molecular events leading to the loss of β-cell function and to define possible targets for a function-preserving treatment a cellular model for β-cell failure is thus required. Furthermore, there is currently no good test available to assess β-cell functionality. Such a test would be of great value in potential clinical trials for pharmaceuticals that preserve β-cell function. For this reason, several labs systematically analyzed β-cell failure to identify significant changes that occur after exposure to both palmitate and high glucose [1].

The rat insulinoma cell line INS-1 displays β-cell-like features making it a good *in vitro* model for this cell type. It has preserved many β-cell characteristics, including glucose-stimulated insulin secretion. Furthermore, study of these β-cells with decreased insulin secretion as compared to cells with normal function could lead to a better understanding of the biology of β-cell failure and could highlight possible targets for therapeutic intervention. Study of these cells may also lead to the identification of markers for β-cell failure or function that are more reliable or sensitive than the C-peptide.

In conclusion, important steps were made towards the identification of β-cell specific proteins that might serve in the future as predictive markers for β-cell function or failure. Furthermore, it would be an advantage to identify such markers that can be detected in plasma of humans. Follow-up experiments using animal models and human blood and plasma samples were designed to validate opportunities to pharmaceutically target β-cell failure and to generate novel medicines for T2D.

Current status of diabetes diagnostics

Blood glucose testing is the most commonly used method for the diagnosis and monitoring of diabetes mellitus. FPG levels and plasma glucose levels 2 h after the uptake of a 75 g dose of glucose are two possible methods to detect the disease. Two independently obtained positive test results are required to establish the diagnosis. As it is easier and less time-consuming to perform, the FPG test is recommended by the American Diabetes Association. However, FPG levels will only be increased, when an increased insulin secretion fails to compensate for insulin resistance, which will only be the case in late stages of T2D. The OGT test and the euglycemic hyperinsulinemic clamp [1] are two methodologies that have the potential to detect insulin resistance at an earlier stage, but are relatively time-consuming to perform and are not amenable to mass testing. Once the diagnosis is established, the efficacy of a treatment regimen can be monitored by measuring glycated hemoglobin (HbA1c) which reflects the plasma glucose levels over the last 4–6 weeks [2]. For an assessment of β-cell function, the measurement of the concentration of C-peptide is, at this time, the best method available [3, 4]. Measurement of this parameter is however not recommended for clinical practice as it suffers from a large variability [5].

Current biomarkers for diabetes

The classical risk factors for type 1 diabetes (T1D) are, apart from familiar predisposition, genetic markers, like certain HLA-DQ or CTLA4 genotypes associate with increased risk of developing T1D. The detection of auto antibodies to islet cell antigens (ICA), insulin, glutamic acid decarboxylase (GAD-65), or IA-2 protein tyrosine phosphatase marks the onset of the autoimmune disease and can be detected years before clinical symptoms of diabetes appear. While certain variants in genes encoding a β-cell specific ion-channel (SUR8/Kir6.2) are associated with transient or permanent neonatal diabetes and mutations in the ABCA1 gene have recently been associated with T2D (reviewed in [6]), obesity is still the greatest known risk

factor for T2D. Though familiar clustering of T2D implies the existence of genetic risk factors, these are still unknown.

While for T1D early diagnostic markers exist in the form of auto antibodies, T2D can so far only be diagnosed when the clinical symptoms appear at the later stages of the disease. Metabolic markers like hyperglycemia, insulin or C-peptide level, and HbA1c are used to diagnose diabetes and monitor the disease state in all types of diabetes. Since an early diagnosis and an early therapy hold the greatest promises to postpone full disease development and to delay secondary morbidities like cardiovascular diseases, reliable, sensitive markers for early T2D are a key concern.

Several novel biomarkers for T2D have been reported and reviewed recently [7]. Among the T2D marker candidates are markers of fatty liver routinely tested like alanine aminotransferase (ALT) and γ-glutamyl transferase (γ-GT), both well correlated with diabetes risk [8]. Low levels of sex homone binding globuline (SHBG), a hepatically secreted hormone negatively regulated by insulin, can indicate high insulin levels, insulin resistance and risk of T2D, a correlation that is stronger in women than in men [9]. Low SHBG has been reported to be predictive of gestational diabetes [10]. Inflammatory markers interleukin 6 (IL-6) and C-reactive protein (CRP) induced by IL-6 have also been associated with T2D [11], as has another acute phase marker ferritin [12]. Some studies suggested that CRP and IL-6 levels positively correlated with insulin resistance and with the risk of acquiring type 2 DM. However there is no correlation between plasma ferritin level and glycemic control or diabetic microangiopathic complications. Serum ferritin, initially a marker of cellular iron stores, is therefore not fully validated as a indicator of T2D risk [13]. Adiponectin, a hormone produced by adipocytes and involved in the regulation of fat and glucose metabolism, has an inverse correlation to diabetes, i.e. low adiponectin levels correlate with high T2D risk [14] and genetic variants of adiponectin have been associated with increased risk of T2D [15]. Adiponectin exerts insulin-sensitizing and anti-infamatory functions through two different seven-transmembrane receptors expressed predominantly in β-cells and skeletal muscles (ADIPOR1) or the liver (ADIPOR2) [16]. Leptin, another adipocyte hormone regulates appetite and has been associated with T2D but the significance is unclear, as leptin levels can be deregulated in leptin resistant obese patients and a positive and a negative association with T2D can be found [17]. Tissue plasminogen activator (tPA) and its inhibitor PAI-1 have also been linked to T2D [18–20]. Both of these proteins can be secreted form endothelial cells and high plasma levels usually reflect endothelial stress or inflammation. PAI-1 can also be released from adipocytes and hepatocytes and its plasma levels increase with adiposity, weakening the association with T2D. Like tPA and PAI-1 von Willebrand factor (vWF) is a maker of endothelial dysfunction associated with risk of T2D in the literature [21] and like the former it is induced by proinflammatory cytokines. The association of vWF with T2D is less robust and significance is lost after adjustment for obesity or inflammatory cytokines in some studies [20]. Other risk factors for T2D have been described, many of these are also associated with obesity or inflammation and thus might not be useful to discern an obese patient at risk to develop T2D form one not at risk. Furthermore, many risk factors have been analyzed only in small study groups. All risk factors identified so far are present in healthy populations as well though at a different levels. But it is not only T2D that will influence their expression level, age, sex, ethnicity might also play a role increasing the variability and reducing the specificity.

Current treatment scheme for T2D

In the early stages of the disease, weight loss and an increase in physical activity are often sufficient to restore insulin sensitivity (American Diabetes Association, 2002). If this regimen fails, non-insulinotropic agents such as acarbose or metformin (and glitazones) are the first-line therapeutics. When insulin levels drop, insulin secretagogues (sulfonylureas, repaglinide) need to be added to the therapeutic regimen. Eventually, when this regimen has become insufficient, insulin injection becomes necessary.

Scientific rationale for β-cell failure as a disease model for T2D

Beta-cell failure occurs at a late stage in the progression of T2D and leads to overt diabetes. The mechanisms by which β-cell function is impaired are largely unknown but have been shown to involve hyperglycemia as well as hyperlipidemia [22].

Interestingly, β-cell failure does not appear to be an inevitable consequence of hyperinsulinemia. Some animal models of hyperinsulinemia/diabetes show persistent lifelong hyperinsulinemia (e.g. ob/ob mice, fa/fa rats) while others show deteriorating β-cell function and apoptosis of β-cells (e.g. db/db mice, *Psammomys obesus*, and rhesus monkeys) [23]. The reasons for these differences are unclear and include genetic factors [24]. The situation in humans may be similar, as insulin resistance is an almost inevitable consequence of obesity but only some individuals have frank diabetes and progress into insulin dependence. The fact that β-cell failure is avoidable in some animal models encourages research towards the prevention of β-cell failure. *In vitro* studies have demonstrated a glucotoxic effect that was only partially reversible [23, 25, 26].

Lipotoxicity plays an equally important role [27, 28]. A profound effect on β-cell function is observed in models combining both factors [22]. Some scientists speculate that these two mechanisms are independent of each other and involve distinct pathways (Dr. M. Donath, Zurich, personal communication). Apoptosis plays a critical role in β-cell failure and T2D [29] and is induced by Fas-FasL interaction where the latter is constitutively expressed by β-cells and the former induced by cytokines and glucose (Dr. M. Donath, personal communication). FLIP blocks the cascade from Fas-FasL interaction ➔ caspase 8 ➔ 3 and finally apoptosis and even reverses the effect of high glucose, thus resulting in proliferation instead of apoptosis. This suggests that prevention of β-cell failure is feasible. The apoptotic effect of lipids, mediated by Bcl-2 and ceramide, is less well documented. Importantly, although apoptotic pathways have been explored extensively, the present review addresses functional β-cell failure outside apoptosis. A decrease in β-cell mass was observed in patients with advanced T2D but not in the early stages of the disease [30]. The metabolic disturbances of most patients will go unnoticed until frank hyperglycemia is detected. However, the vast majority of patients will remain in a stage of partial compensation before moving on to dependence on exogenous insulin. At this particular stage, compounds preventing β-cell failure could be applied, therefore the goals of our proteomics study are twofold: (i) The first line of experiments will be directed towards the identification and validation of a potential monitoring marker for clinical studies. Indeed, the current gold standard for the measurement of β-cell function, the measurement of the insulin C-peptide, is not sufficiently reliable for this purpose. To ensure its broad acceptance, a marker would have to be established well in advance of a clinical program aimed at demonstrating disease modifying (i.e., β-cell failure preventing) efficacy of a novel compound. (ii) Secondly, the changes in protein expression patterns should be studied in order to improve our understanding of the biological processes involved in β-cell failure, ultimately leading to the identification of pathways and novel targets for therapeutic intervention.

Methodology

Cellular model system for the identification of potential markers of β-cell failure

Isolated islets and insulin secreting cell lines become desensitized upon exposure to high levels of glucose and palmitate [22]. The rat insulinoma cell line INS-1 and the mouse cell line Min6 appear to be the best available model cell lines at this time [31, 32]. They have retained many characteristics of primary β-cells, including the susceptibility to the combined toxic effect of glucose and lipids.

Two classes of proteins could fulfill the criteria for a marker of β-cell failure: (i) Highly expressed β-cell specific proteins could become detectable in the bloodstream upon cell death, and (ii) the level of β-cell specific secreted proteins could significantly drop as a sign of impaired β-cell function. Ideally the latter would be constitutively secreted and not depend on glucose concentration as shown in the results of the OGTT. For this purpose, a catalogue of proteins expressed in β-cell lines was established to identify β-cell specific proteins that have the potential to become detectable in human blood and that could serve as a marker for β-cell failure. Beta-cell specific proteins identified by this strategy will have to be first validated in an animal model for β-cell failure, for example in blood samples collected from rat strains rapidly developing full-blown diabetes (e.g. ZDF rats), before human blood samples can be tested.

New enrichment and identification strategies of differentially regulated proteins in failing β-cells

Beta-cell failure can be mimicked in several cell culture models by adding a combination of fatty acids and increased concentrations of glucose to the culture medium as shown in [22, 28]. Comparison of expression patterns from untreated cells, cells treated with either a fatty acid or high glucose, and cells treated with both fatty acid and high glucose should highlight gene products whose regulation correlates with β-cell failure in a cell culture model. The results from this study directed the proteome comparison towards the compartments that appeared most affected based on the transcript analysis. In a second step these changes can be validated using animal models such as ZDF rats or db/db mice. Finally, findings from the primary β-cells must be further validated by using human plasma samples from different donor groups (e.g. patients with impaired glucose tolerance (IGT), impaired fasting glucose (IFG), type 2 diabetes (T2D), type 1 diabetes (T1D), and healthy donors).

Critical points of the β-cell failure model:

- A reliable, reproducible model of β-cell failure induced by gluco- and lipotoxicity after a reasonable exposure time must be established first.
- Triggering failure of the cultured cells to secrete insulin is a time-dependent process that must be carefully monitored (see Fig. 2). Establishing the appropriate monitoring readouts is therefore crucial to this project. Selecting the appropriate time point based on the measurement of the selected parameters is difficult and requires kinetic measurements.

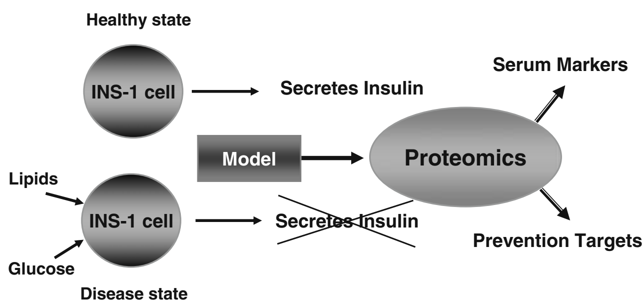

Fig. 1 *In vitro* model of β-cell failure: Concept of gluco/lipotoxicity

- Up-scaling of the cell culture is necessary for obtaining sufficient quantities of starting material for a proteome analysis (see Fig. 4). This may lead to variations. Careful monitoring by measurement of the parameters defined by the pilot experiments is therefore mandatory.
- Beta-cell specific proteins, even if highly abundant intracellularly, or secreted proteins may not be present in blood in detectable quantities. Therefore highly sensitive ELISA must be used to validate findings in human blood samples
- A fractionation scheme that efficiently enriches for secretory vesicles has to be established.
- The concentration of secreted proteins in the culture supernatant is likely to be very low. The accessibility of these proteins by mass spectrometric methods should be therefore assessed.

Methodology approach and cellular study design

Features of β-cell failure can be performed by chronic exposure of β-cells to a combination of high glucose/fatty acids (FAs), suggesting that hyperlipidemia as well as hyperglycemia may contribute to decompensation of β-cells. INS-1 cells pretreated for 30 h with a combination of 10 mM glucose and 0.5 mM palmitate can be used for these experiments. Establishment of β-cell culture conditions for proteomics analysis should be carried out in parallel (see Figs. 1 and 3). Screen INS-1 cell cultures under treatment with glucose/fatty acids and identification of different expressed protein levels between glyco/lipotoxicity and combination. Focus on secreted proteins in the supernatant as possible markers for β-cell failure/function. Enrichment and identification of secreted INS-1 peptides/proteins in the medium by heparin chromatography followed by LC-MS were applied.

General introduction and strategies for enrichment and purification of secreted proteins

In order to identify proteins secreted by INS-1 [31, 33] two methods can be applied: (i) Fractionation of the cells by differential sedimentation into subcellular compartments with subsequent identification of the proteins based on their peptide mass fingerprint using MALDI-TOF mass spectrometry, and (ii) enrichment of glycoproteins by heparin chromatography followed by one-dimensional SDS-PAGE and identification of proteins by analysis of the tryptic peptides resulting from protein digest by liquid chromatography coupled to tandem mass spectrometry resulting in identification based on protein sequence tags (see Fig. 4). The combination of these two purification strategies allowed us to increase the efficiency of protein identification in the cellular compartments as well as in the medium of cultured cells.

Enrichment of putative secreted proteins by heparin columns from the medium and identification by LC-MS

Visualization and identification of secreted proteins may facilitate the identification of novel drug targets and different biomarkers including predictive markers. However, not all proteins in organism are expressed in amounts sufficient for

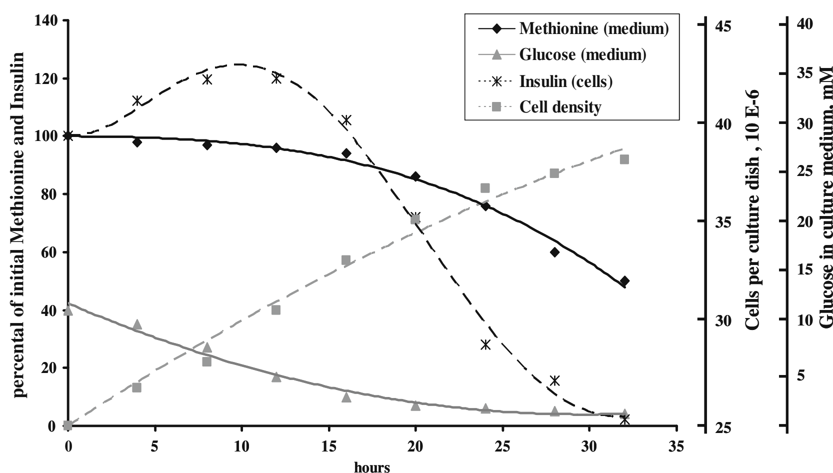

Fig. 2 Characterization and whole protein profiling of INS-1 cells: Model of β-cell failure induced by gluco- and lipotoxicity after 35 h exposure time

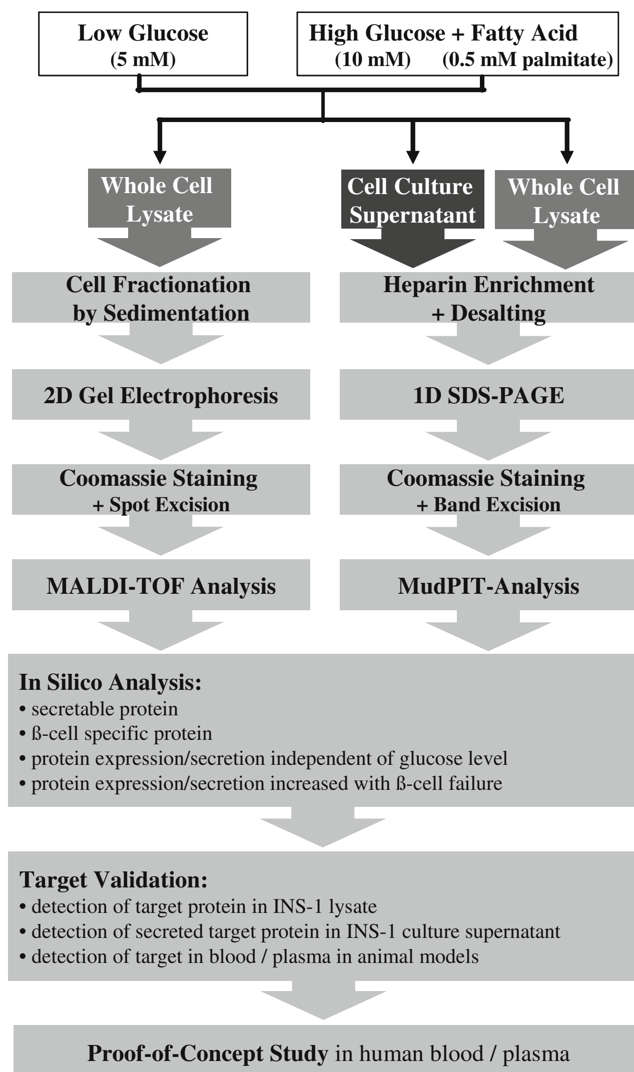

Fig. 3 Flowchart of proteomics approach to identify markers of β-cell failure using mass spectrometry (MudPIT=Multi protein identification technology and MALDI=matrix assisted laser desorption/ionisation mass spectrometry) in combination with *in silico* analysis

detection by two-dimensional or one dimensional polyacrylamide gel electrophoresis. In order to visualize and identify low-copy gene products with signaling function, enrichment of low-abundance cytosolic proteins by applying heparin chromatography were chosen because heparin has a high protein binding capacity and can discriminate and enrich proteins with minor differences in their pI-values and glycosylation patterns [34, 35]. As most secreted proteins are glycosylated, [36] heparin sepharose and/or lectin columns are versatile tools for the enrichment of many classes of glycosylated proteins such as proteins with signaling functions, growth factors, coagulation factors and steroid receptors [37, 38]. The ligand in a heparin sepharose column is a naturally occurring sulfated glycosaminoglycan, which is extracted from the native proteoglycan of porcine intestinal mucosa. Heparin consists of alternating units of uronic acid and D-glucosamine, most of which are substituted with one or two sulphate groups. Immobilized heparin has two main modes of interaction with proteins: It can operate as an affinity ligand; e.g., in its interaction with coagulation factors and it can also function as a high capacity cation exchanger because of its anionic sulphate groups, leading thus to an additional enrichment of positively charged proteins.

To enrich for secreted rat insulinoma proteins with signaling function heparin chromatography was applied prior to proteomic analysis. The column was operated with a syringe instead of a liquid chromatography pump. Elution was performed by increasing the ionic strength with 2 M NaCl. Separation of the eluted proteins was carried out by one-dimensional (1D-) polyacrylamide gel electrophoresis (PAGE) and the proteins were identified by multidimensional protein identification technology (MudPIT) tandem mass spectrometry [39, 40] in combination with *in silico* analysis (see Figs. 4 and 5).

Moreover, heparin chromatography may be useful in the depletion of albumin from body fluids, such as plasma and cerebrospinal fluid, in which it represents more than 50% of total proteins [34, 41]. For example, serum albumin, which is represented by a strong band in the starting material (see Fig. 6), was completely recovered in the flow-through fraction. Specific removal of albumin, as well as of other high abundance proteins allowed the visualization and identification of minor components of the samples, whose levels may change in certain disorders [42]. In addition to the easier design of protein purification steps, use of selected chromatography steps prior to the LC-MS/MS, can significantly facilitate the analysis of complex protein mixtures. Methodological sample preparation and purification, as well as systematically identification by MudPIT and MALDI are published in detail [43, 44].

Identification of putative secreted proteins by *in silico* analysis

Secreted proteins are characterized by a signal peptide sequence that helps in threading the protein's N terminus through a membrane before it is cleaved off by a signal peptidase. Signal peptides are only loosely defined, often with a positively charged polar section, followed by a hydrophobic stretch, and a short pattern around the cleavage site.

The web tool that are used in order to identified or predicted signal peptide from SwissProt, was an developed software tool based on a set of specialized, manually curated Hidden Markov Models (HMMs) that attempt to recognize the (sparse) sequence features common to signal peptides or anchors, respectively [43]. As these sequence signals cannot be reliably predicted, the "signal" and "anchor" scores that any input sequence is assigned are

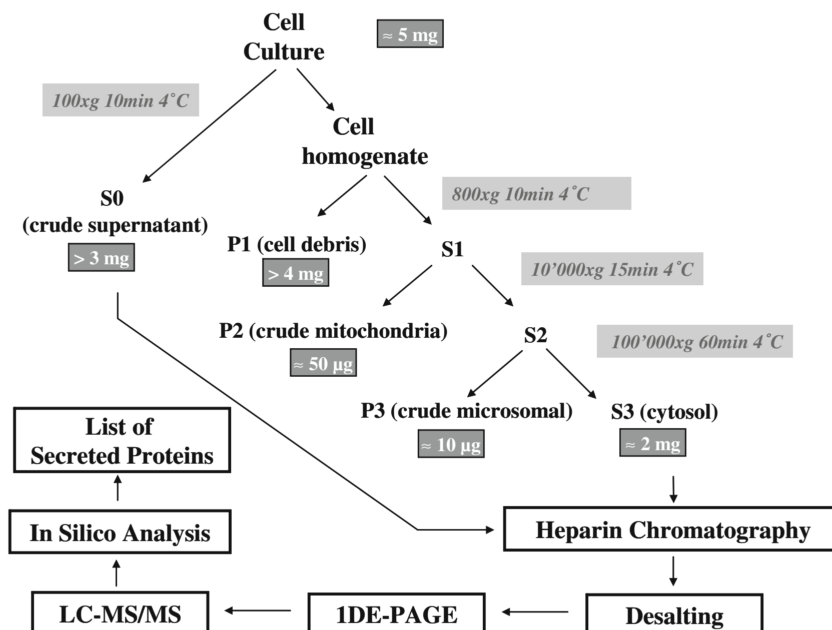

Fig. 4 Scheme of centrifugal prefractionation of rat INS-1 proteins. Different centrifugal force leads to enrichment of cellular components such as mitochondria, microsomal and cytosolic proteins. The cytosolic fraction was subjected to further fractionation by heparin chromatography followed by separation on a 10% homogenous polyacrylamide gel and protein identification by LC-MS/MS

fed into a Support Vector Machine (SVM) in a second analysis step. The SVM was trained on a set of *bona fide* examples for both classes. On this training set, the SVM obtained the following results on three training sets (signal—anchor—neither):

signal 96% sensitivity and 96% specificity
anchor 87% sensitivity and 98% specificity
neither 96% sensitivity and 97% specificity

These results are as good as the best claims in the literature (note, however, that most tools only look at the signal peptides and do not attempt to predict membrane anchors).

For the default setting of this web interface, a list is produced that simply classifies each input sequence in either of the three categories “Signal”, “Anchor”, or “Normal” (the latter indicating that neither signal peptide nor membrane anchor are predicted).

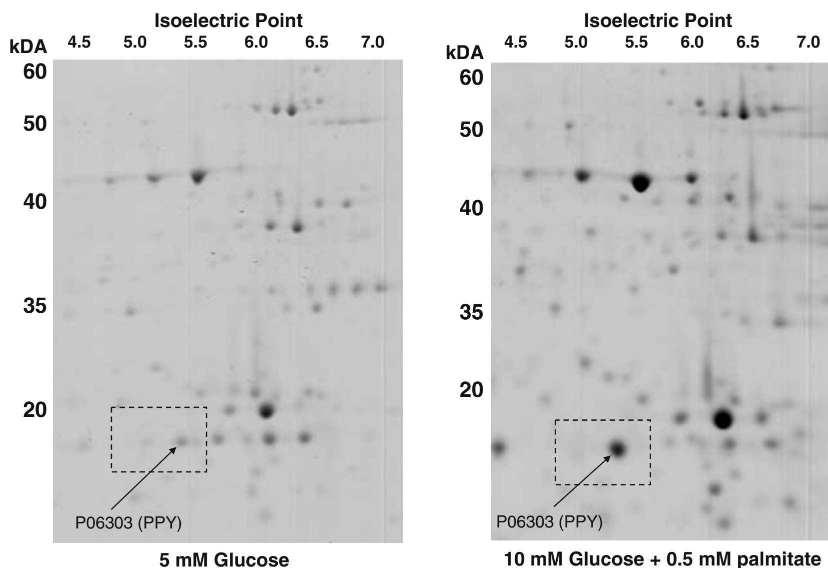

Fig. 5 2D-PAGE images of differential protein expression patterns in INS-1 cells incubated under lipo/glucotoxic conditions (10 mM glucose and 0.5 palmitate) *versus* control (5 mM glucose). The selected spot of protein (P06303) was identified by MALDI-TOF as pancreatic polypeptide Y [43]

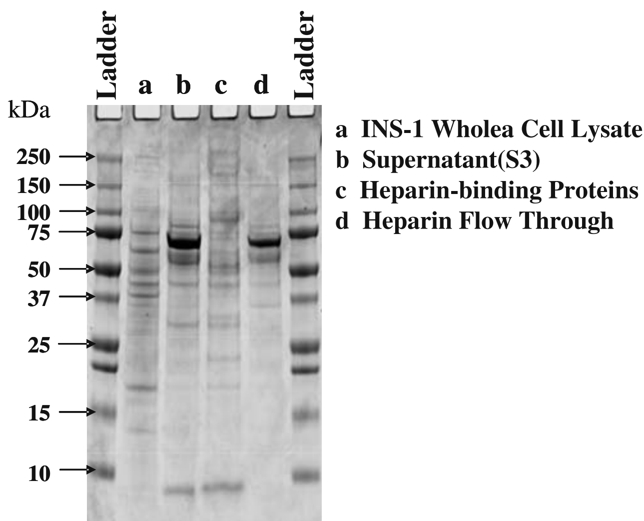

Fig. 6 1DE gel analysis of fractions eluted from heparin chromatography. Fraction enriched with cytosolic rat INS-1 proteins was prepared as stated in [43]. Gels were stained with colloidal Coomassie blue and protein bands were identified by MudPIT [43]

For the "verbose" mode, there is a much more detailed table that has, for every input sequence, the following columns:

seqID, Classification—these are the same as in the default output discussed above.

Confidence—this qualifier can take the values "Unique", "Ambiguous", or "No Class", depending on the SVM results. The SVM determines how well the sequence scores fit its model of either of the three classes. In the "Unique" case, the scores match only one of the three classes, to which the sequence is then assigned. In the "Ambiguous" case, the sequence scores match more than one class—the sequence is assigned to that class into which it fits best. In the "No class" case, the scores do not match the expectations for any of the three classes—the sequence is assigned to that class for which the discrepancy is smallest.

D (Anchor), D (Normal), D (Signal)—these numbers specify the "distances" to a line separating the three classes (to use an intuitive metaphor). For a "Unique" classification, only one of these "distances" is positive (indicating that the sequence is "on the right side of the fence"), whereas the other two are negative ("on the wrong side of the fence" for the two other classes). The larger the numbers, the clearer the classification. For an "Ambiguous" classification, more than one of the D's is positive, for a "No class" classification, all three D's are negative.

SW-classification, SW-note—finally, sequences from the SwissProt database already come with an annotation regarding signal peptides and membrane anchors. For them, we show the SwissProt classifications and any additional comments on them for reference and comparison. Sequences from the HUMANGP, MOUSEGP, and RATGP databases have "none" for both fields.

The **HUMANGP, MOUSEGP,** and **RATGP** databases have been completely annotated with this prediction algorithm, the results are displayed in the **XR SIGNAL/ANCHOR** annotation lines and listed in Table 1.

Visualization and identification of putative secreted proteins by MudPIT analysis enriched by heparin chromatography

Heparin binding fractions of cell culture supernatants were analyzed by LC-MS and MALDI-MS and the identification of the putative markers were reported [43]. Five secreted proteins were further confirmed: Chromogranin b, Pancreatic Polypeptide (PPY), Phospholipase A2, Fibronectin and Vinculin were clearly shown to be secreted by INS-1. The amount of secretion into the medium correlated strongly to the insulin content of the INS-1 β-cells. Based on the organ specify, PPY seemed to be one of the most interesting candidates and was selected for further validation by ELISA. PPY is a secretory glycoprotein with a widespread distribution mostly expressed in endocrine pancreas. These data were underlined by additional OGTT studies which corroborate the findings and show clearly, that PPY is secreted indepented from the glucose level in blood (see Fig. 8). Furthermore it has been suggested that PPY has an autocrine inhibitory effect on iAPP and insulin secretion by different intra- and extracellular mechanism [45]. Ten proteins were selected for further OGTT evaluation. Evaluation/validation by immunoassays in a well-characterized patient population was performed only with PPY. Pancreatic Polypeptide levels are higher in diabetics than controls. A more profound analysis has to demonstrate that the levels correlate with β-cell failure and not other diabetes-related phenomena (see Fig. 7). A priority application was made for all candidates before a full validation was done.

Detection of PPY in human plasma—Proof-of-Concept

Clinical utility of the novel marker PPY was assessed by measuring its levels in 10 diabetic patients depending on injections of exogenous insulin and comparing the levels with those measured in 10 patients with demonstrated normal β-cell function [43].

The OGTT has fallen into disfavor as a tool to examine the mechanism responsible for impaired glucose metabolism in T2D subjects. The rate of glucose absorption varies considerable from one subject to another and cannot be quantified easily. Consequently, the rise in plasma glucose

Table 1 List of identified secreted INS-1 proteins enriched by heparin chromatography [43]

Fraction	SW.-Nr.	Access. Nr.	Status	Full Protein Name	kDa
Sup.	S106_RAT	P05964	Sec.	Calcyclin (Prolactin receptor associated protein).	10.1
Hep.	KV5C_MOUSE	P01635	Sec.	Ig kappa chain V-V region K2 precursor (Fragment).	12.6
Sup.	TL19_MOUSE	Q9CQU0	Sec.	Thioredoxin-like protein p19 precursor	19.0
Sup.	CYPC_MOUSE	P30412	Sec.	Peptidyl-prolyl cis-trans isomerase C (EC 5.2.1.8)	22.8
Hep.	CYPB_RAT	P24368	Sec.	Peptidyl-prolyl cis-trans isomerase B prec. (EC 5.2.1.8)	23.0
Hep.	TIM2_RAT	P30121	Sec.	Metalloproteinase inhibitor 2 precursor (TIMP-2)	24.3
Hep.; Sup.	TRY1_RAT	P00762	Sec.	Trypsin I, anionic precursor (EC 3.4.21.4)	25.9
Hep.; Sup.	TRY2_MOUSE	P07146	Sec.	Trypsin II, anionic precursor (EC 3.4.21.4)	26.2
Hep.	PAHO_RAT	P06303	Sec.	Pancreatic prohormone prec. (Pancreatic polypeptide)	27.1
Hep.; Sup.	IBP4_RAT	P21744	Sec.	Insulin-like growth factor binding protein 4 (IGFBP-4)	27.7
Hep.	ER29_RAT	P52555; P80749	Sec.	Endoplasmic reticulum protein ERp29 prec. (ERp31).	28.6
Sup.	PDX4_MOUSE	O08807;	Sec.	Peroxiredoxin 4 (EC 1.11.1.-) (Prx-IV)	31.0
Hep.; Sup.	SPRC_RAT	P16975; O08953	Sec.	SPARC precursor	34.4
Hep.	FSL1_RAT	Q62632	Sec.	Follistatin-related protein 1 prec. (Follistatin-like 1).	34.6
Hep.; Sup.	OSTP_RAT	P08721	Sec.	Osteopontin precursor (Secreted phosphoprotein 1)	34.9
Hep.; Sup.	DRNG_MOUSE	O55070	Sec.	Deoxyribonuclease gamma precursor (EC 3.1.21.-)	35.7
Sup.	CALU_RAT	O35783	Sec.	Calumenin precursor (Crocalbin)	37.0
Sup.	CATM_MOUSE	Q9JL96; Q91Z75	Sec.	Cathepsin M precursor (EC 3.4.22.-).	37.4
Sup.	CATB_RAT	P00787;	Sec.	Cathepsin B precursor (EC 3.4.22.1) (Cathepsin B1)	37.4
Hep.	CATL_RAT	P07154	Sec.	Cathepsin L prec. (EC 3.4.22.15)	37.6
Hep.	IF35_MOUSE	Q9DCH4	Sec.	Eukaryotic translation initiation factor 3 subunit 5	38.0
Sup.	RCN1_MOUSE	Q05186	Sec.	Reticulocalbin 1 precursor.	38.1
Sup.	DKK3_MOUSE	Q9QUN9	Sec.	Dickkopf related protein-3 precursor (Dkk-3)	38.4
Hep.	PGS2_RAT	Q01129	Sec.	Decorin precursor (Bone proteoglycan II)	39.8
Sup.	CB45_RAT	Q91ZS3	Sec.	45 kDa calcium-binding protein precursor (Cab45)	42.0
Hep.	SELP_RAT	P25236	Sec.	Selenoprotein P precursor (SeP).	42.6
Hep.	PAI1_RAT	P20961	Sec.	Plasminogen activator inhibitor-1 precursor (PAI-1)	45.0
Sup.	T11B_RAT	O08727	Sec.	Tumor necrosis factor recept. superfamily member11B	46.2
Hep.; Sup.	PEDF_MOUSE	P97298	Sec.	Pigment epithelium-derived factor precursor (PEDF)	46.2
Hep.	HS47_RAT	P29457	Sec.	47 kDa heat shock protein prec.	46.5
Sup.	MFGM_RAT	P70490	Sec.	Lactadherin precursor (Milk fat globule-EGF factor 8)	47.4
Hep.; Sup.	CRTC_RAT	P18418; P10452	Sec.	Calreticulin precursor (CRP55) (Calregulin) (HACBP)	48.0
Hep.	LGMN_RAT	Q9R0J8	Sec.	Legumain precursor (EC 3.4.22.34)	49.4
Sup.	NCB2_RAT	Q9JI85	Sec.	Nucleobindin 2 precursor (DNA-binding protein NEFA).	50.1
Hep.	PCO1_RAT	O08628	Sec.	Procollagen C-proteinase enhancer protein precursor	50.2
Sup.	VAS1_RAT	O54715	Sec.	Vacuolar ATP synthase subunit S1 prec. (EC 3.6.3.14)	51.1
Hep.	HRA1_MOUSE	Q9R118	Sec.	Serine protease HTRA1 precursor (EC 3.4.21.-).	51.2
Hep.	CLUS_RAT	P05371	Sec.	Clusterin precursor (Sulfated glycoprotein 2) (SGP-2)	51.3
Hep.; Sup.	SRPX_RAT	Q63769	Sec.	Sushi repeat-containing protein SRPX prec.	51.5
Hep.	ANT3_MOUSE	P32261	Sec.	Antithrombin-III precursor (ATIII).	52.0
Hep.	LIPL_RAT	Q06000	Sec.	Lipoprotein lipase precursor (EC 3.1.1.34) (LPL).	53.0
Hep.	SG3_MOUSE	P47867	Sec.	Secretogranin III precursor (SgIII).	53.3
Hep.	NCB1_RAT	Q63083; P97623	Sec.	Nucleobindin 1 precursor (CALNUC)	53.5
Hep.	ARSA_MOUSE	P50428	Sec.	Arylsulfatase A precursor (EC 3.1.6.8) (ASA)	53.7
Hep.; Sup.	PRTP_MOUSE	P16675	Sec.	Lysosomal protective protein precursor (EC 3.4.16.5)	53.8
Hep.; Sup.	PLTP_MOUSE	P55065; Q99L70	Sec.	Phospholipid transfer protein precursor	54.4
Sup.	DPP2_RAT	Q9EPB1	Sec.	Dipeptidyl-peptidase II precursor (EC 3.4.14.2)	55.1
Hep.	CPS1_MOUSE	P03940	Sec.	Cytochrome P450 XXIA1 (EC 1.14.99.10)	55.3
Sup.	PDA3_RAT	P11598	Sec.	Protein disulfide isomerase A3 precursor (EC 5.3.4.1)	56.6

Table 1 (continued)

Fraction	SW.-Nr.	Access. Nr.	Status	Full Protein Name	kDa
Sup.	PDI_RAT	P04785; P13700	Sec.	Protein disulfide isomerase precursor (PDI)	56.9
Hep.	MM19_MOUSE	Q9JHI0	Sec.	Matrix metalloproteinase-19 precursor (EC 3.4.24.-)	59.1
Hep.; Sup.	HEXA_MOUSE	P29416; Q64246	Sec.	Beta-hexosaminidase alpha chain prec. (EC 3.2.1.52)	60.6
Sup.	TPP1_RAT	Q9EQV6	Sec.	Tripeptidyl-peptidase I precursor (EC 3.4.14.9) (TPP-I)	61.3
Hep.	GPC1_RAT	P35053	Sec.	Glypican-1 precursor (HSPG M12).	61.7
Sup.	ALBU_RAT	P02770; P11382	Sec.	Serum albumin precursor	68.7
Sup.	ASM_MOUSE	Q04519	Sec.	Sphingomyelin phosphodiesterase prec. (EC 3.1.4.12)	69.9
Hep.	SPL1_RAT	P24054	Sec.	SPARC-like protein 1 prec. (Matrix glycoprotein Sc1).	70.6
Hep.; Sup.	KNG_RAT	P08934; P08933	Sec.	Kininogen precursor	70.9
Sup.	IDUA_MOUSE	P48441	Sec.	Alpha-L-iduronidase precursor (EC 3.2.1.76).	71.1
Hep.	GR78_RAT	P06761	Sec.	78 kDa glucose-regulated protein precursor (GRP 78)	72.3
Hep.	PDA4_RAT	P38659	Sec.	Protein disulfide isomerase A4 precursor (EC 5.3.4.1)	72.8
Hep.; Sup.	BGLR_MOUSE	P12265; Q61601	Sec.	Beta-glucuronidase precursor (EC 3.2.1.31).	74.2
Hep.	FBL1_MOUSE	Q08879; Q08878	Sec.	Fibulin-1 prec. (Basement-membrane protein 90)	78.0
Hep.	SM3B_MOUSE	Q62177	Sec.	Semaphorin 3B precursor (Semaphorin A)	82.8
Sup.	PLO1_RAT	Q63321	Sec.	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	83.6
Hep.	LOL3_MOUSE	Q9Z175; Q9JJ39	Sec.	Lysyl oxidase homolog 3 precursor (EC 1.4.3.-)	83.6
Hep.; Sup.	AD10_MOUSE	O35598	Sec.	ADAM 10 precursor (EC 3.4.24.-)	83.9
Sup.	MEPA_MOUSE	P28825	Sec.	Meprin A alpha-subunit precursor (EC 3.4.24.18)	84.1
Hep.	PLO3_MOUSE	Q9R0E1	Sec.	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	84.9
Hep.; Sup.	A4_RAT	P08592	Sec.	Alzheimer's disease amyloid A4 protein homolog	86.6
Sup.	APP2_RAT	P15943	Sec.	Amyloid-like protein 2 precursor	86.8
Hep.	ENPL_MOUSE	P08113; P11427	Sec.	Endoplasmin prec. (Endoplasmic reticulum protein 99)	92.4
Sup.	DAG1_MOUSE	Q62165; Q61094	Sec.	Dystroglycan precursor	96.8
Hep.	FPRP_RAT	Q62786	Sec.	Prostaglandin F2 receptor negative regulator precursor	98.7
Hep.	NAH5_RAT	Q9Z0X2	Sec.	Sodium/hydrogen exchanger 5 Na(+)/H(+) exchanger	99.0
Hep.; Sup.	DSC3_MOUSE	P55850; O55110	Sec.	Desmocollin 3 precursor.	100.6
Hep.	TSP3_MOUSE	Q05895	Sec.	Thrombospondin 3 precursor.	103.9
Hep.; Sup.	PEX6_RAT	P54777; O55097	Sec.	Peroxisome assembly factor-2 (PAF-2)	104.4
Hep.	ITH2_MOUSE	Q61703	Sec.	Inter-alpha-trypsin inhibitor heavy chain H2 precursor	105.9
Hep.	CA16_MOUSE	Q04857	Sec.	Collagen alpha 1(VI) chain precursor.	108.4
Hep.	AMD_RAT	P14925; P70710	Sec.	Peptidyl-glycine alpha-amidating monooxygenase	108.6
Sup.	CLS1_MOUSE	Q9EPL2	Sec.	Calsyntenin-1 precursor.	108.8
Sup.	OXRP_RAT	Q63617	Sec.	150 kDa oxygen-regulated protein precursor (Orp150)	111.2
Hep.	M2B1_MOUSE	O09159; O55037	Sec.	Lysosomal alpha-mannosidase precursor EC 3.2.1.2	114.5
Sup.	M2B2_MOUSE	O54782	Sec.	Epididymis-specific alpha-mannosidase (EC 3.2.1.24)	115.6
Hep.; Sup.	TSP1_MOUSE	P35441	Sec.	Thrombospondin 1 precursor.	129.6
Hep.; Sup.	LMG2_MOUSE	Q61092	Sec.	Laminin gamma-2 chain precursor	130.1
Hep.	DIA1_MOUSE	O08808	Sec.	Diaphanous protein homolog 1	139.3
Sup.	RGSC_RAT	O08774; O88383	Sec.	Regulator of G-protein signaling 12 (RGS12).	150.4
Hep.; Sup.	A2MG_RAT	P06238	Sec.	Alpha-2-macroglobulin precursor (Alpha-2-M).	163.6
Sup.	A1I3_RAT	P14046	Sec.	Alpha-1-inhibitor III precursor.	163.7
Hep.; Sup.	LMG3_MOUSE	Q9R0B6	Sec.	Laminin gamma-3 chain precursor	172.2
Hep.	LMG1_MOUSE	P02468	Sec.	Laminin gamma-1 chain precursor (Laminin B2 chain).	177.2
Sup.	CO3_RAT	P01026	Sec.	Complement C3 precursor	186.3
Sup.	LTB1_RAT	Q00918	Sec.	Latent transforming growth factor beta binding prot. 1	186.5
Hep.	LMB1_MOUSE	P02469	Sec.	Laminin beta-1 chain precursor (Laminin B1 chain).	196.8
Hep.	LMA4_MOUSE	P97927; O88785	Sec.	Laminin alpha-4 chain precursor.	201.7
Hep.	PGG2_RAT	Q00657	Sec.	Chondroitin sulfate proteoglycan NG2 precursor	251.8

Table 1 (continued)

Fraction	SW.-Nr.	Access. Nr.	Status	Full Protein Name	kDa
Sup.	FINC_RAT	P04937	Sec.	Fibronectin precursor (FN).	272.3
Hep.	PGBM_MOUSE	Q05793	Sec.	Basement memb.-spec. heparan sulfate proteoglycan	398.0
Hep.; Sup.	LMA5_MOUSE	Q61001	Sec.	Laminin alpha-5 chain precursor.	403.7
Hep.; Sup.	LRP2_RAT	P98158	Sec.	Low-density lipoprotein receptor-related protein 2	518.9

concentration differed markedly from one subject to another and is constantly changing. This presents an ever-fluctuating glycemic stimulus that, when combined with intrinsic differences in β-cell function from one individual to another, leads to large variations in the plasma insulin profile between subjects as shown in [45]. Because the two primary variables of interest—plasma glucose and plasma insulin concentration—are changing simultaneously, it is difficult to draw any conclusion about insulin secretion or insulin sensitivity. Lastly, it is difficult to quantitate changes in hepatic glucose production after glucose ingestion because the rate of entry of glucose into circulation is unknown. In order to investigate the influence of the plasma glucose concentration on the PPY level, an OGTT with four healthy subjects were performed and all have revealed very similar results. Our investigations indicate that early insulin secretion, absolute insulin concentration at 30 min, the incremental insulin concentration (ΔI) at 30 min, or the incremental insulin concentration factored by the incremental glucose concentration ($\Delta I/\Delta G$) at 30 min had less predictive value for the β-cell function than the determination of PPY. PPY absolute concentration did not vary so much between the healthy individuals and it was shown that the secretion rate to be independent of the plasma glucose and insulin concentration. Furthermore, the PPY plasma concentration between the healthy individuals was very constant and only significantly elevated in T2D and IGT+IFG individuals as shown in Fig. 7 and proved to be a better predictor of progression to T2D than OGTT. Since the plasma glucose concentration is the primary determinant of insulin secretion, it is obvious that fasting insulin and glucose concentration alone can not be used to predict the progression of normal-glucose-tolerant subjects to T2D. It is anticipated that our results and methods are able to establish a framework for the identification of additional marker candidates that need to be examined and provide a sound scientific basis for the identification and more precise definition of predictive biomarkers in T2D.

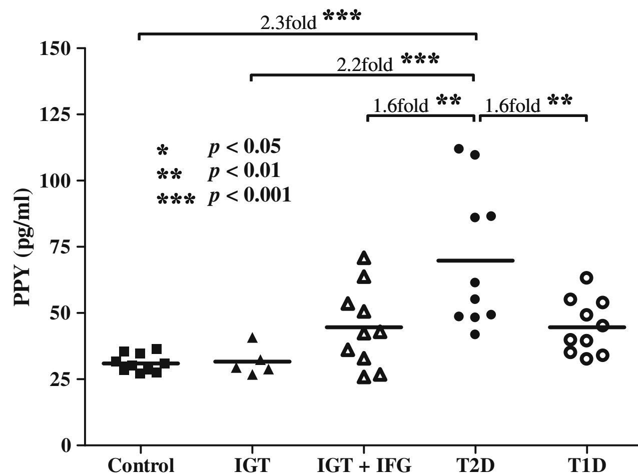

Fig. 7 Primary results: Validation of PPY in human blood / plasma samples in five different healthy donors by ELISA [43]

Summary

Individuals with T2D are characterized by abnormalities in insulin action and insulin secretion. However, despite intensive investigation, the proteins/peptides or genes responsible for the insulin resistance and impaired insulin secretion remain undefined. The candidate-proteins/genes approach has failed to identify any specific proteins or genes or combination of proteins/genes that can account for even a minority of adult cases of T2D. Although a number of laboratories have initiated genome- and proteome-wide

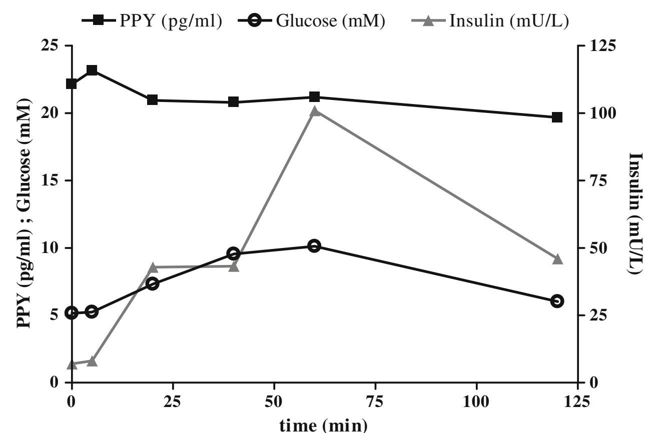

Fig. 8 Means of plasma glucose, insulin and PPY concentration during a 100 g OGTT in 4 healthy normal weight individuals. The results indicate that early insulin secretion has a less predictive value for the β-cell function than the determination of PPY levels. The absolute PPY concentration varies much less between healthy individuals and the results show that the secretion rate of PPY from the endocrine pancreas is independent of the plasma glucose and insulin concentration [43]

search to identify potential susceptibility targets for T2D, consistent and reproducible linkage to predictive markers has yet to merge. It has been suggested that a more precise definition of the diabetic phenotype may prove useful in delineating diabetogenetic genes or gene products. This review should provide an inside into new analytical strategy for the specific enrichment and identification of secreted and pancreas specific proteins/peptides, which are responsible for abnormalities of insulin secretion in T2D and which may serve as predictive markers for β-cell function.

All together the analysis in [43] resulted in the identification of 391 different gene products derived from the INS cells. One hundred eighty-eight proteins bound to the heparin matrix, 101 of which were identified as secreted proteins with signaling functions and are listed in Table 1. Forty-four of the enriched proteins had not been detected by 2-DE without previous enrichment by combination of subcellular prefractionation and heparin chromatography. Heparin chromatography specifically enriched several enzymes that had not been identified before [46–49], like peptidyl-glycine alpha-amidating monooxygenase, sodium/hydrogen exchanger 5, etc. The identified proteins can be divided into five classes:

- Hormones and related molecules (amylin, pancreatic polypeptide, neuroendocrine convertase)
- Protease inhibitors (e.g. cystatin)
- Secretory vesicle proteins (e.g. chromogranin, secretogranin)
- Cell adhesion or extracellular matrix proteins (e.g. protocadherin)
- Secreted enzymes (e.g. phospholipase)

From all identified secreted proteins only pancreatic polypeptide Y (PPY) was identified as pancreas specific, fulfilling the criteria of a predictive marker and hence was selected for further validation [43].

PPY (pancreatic polypeptide Y), a 78-amino acid peptide is synthesized in pancreatic islets of Langerhans and acts as a regulator of pancreatic and gastrointestinal functions. The peptide is structurally similar to another pancreatic hormone family consisting of neuropeptide Y (NPY) b, and peptide YY (PYY) which are closely related to each other. The propeptide is enzymatically cleaved to yield the mature active peptide with amidated C-terminal ends while receptor binding and activation functions reside in the N- and C-termini respectively. PPY occurs in neurons, intestinal endocrine cells, and the pancreas and exists as monomer and dimer. The physiological role for the icosapeptide has not yet been elucidated. PPY was identified as specifically secreted by the endocrine pancreas and it is mainly produced by β-and f-cells [47]. Its plasma concentration has been used as a marker of parasympathetic activity. Recent work in rodents suggests that there is both sympathetic and parasympathetic innervation of white adipose tissue and that parasympathetic activity is anabolic resulting in lipid accumulation [48]. It was examined whether in humans increased PP levels are associated with increased β-cell dysfunctions, and thereby early β-cell failure in T2D. Together with amylin, the peptide has been shown to be the precursor for the amyloid deposits frequently observed in patients with T2D [50–53]. At very high doses PPY inhibits insulin secretion by the perfused rat pancreas *in vitro* [54] and pancreatic PPY and amylin deposits have been shown to precede the appearance of glucose intolerance in spontaneously diabetic monkeys [55]. In the course of the study published in [43], the authors were able to demonstrate that elevated plasma PPY levels can be observed in IGT and IFG subjects, in glucose-intolerance first degree relatives of type 2 diabetic patients (see Fig. 7), and in animal models of diabetes. Although pancreatic and plasma PPY levels were significantly elevated, hyperglycemia and hyperinsulinemia were not observed (see Fig. 8). In summary, based on the findings/observation, the evidence that PPY is responsible for some of the defects in pancreatic islets is very strong and it seems that PPY plays an important role in impaired β-cell function in T2D.

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