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Proteomics in diabetes research

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Summary

Both type 1 and type 2 diabetes mellitus are heterogeneous diseases with alterations in many genes and their products. Not all transcriptional alterations lead to protein changes, which makes it very important to, in conjunction with mRNA expression studies, also address changes in cellular protein levels. Various proteomic techniques are available for measuring many protein changes simultaneously. Many proteomic studies have been performed in the context diabetes research, with the aims of both describing the healthy tissue and to unravel the complex pathophysiology behind the disease. In addition, effects on proteins induced by different treatments have also been investigated using proteomic approaches. In this paper the field of diabetes proteomics today will be reviewed. Findings from proteomic studies investigating pancreatic islets and β -cells as well as serum, fat, skeletal muscle and liver are described.

Introduction

Blood glucose homeostasis describes the balance of glucose ingestion and hepatic glucose production on one side, and peripheral glucose uptake and utilisation on the other side. This equilibrium is maintained by complex interplay of several organs, many glucose-elevating hormones like glucagon, cortisol, growth hormone and catecholamines, and one glucose-lowering hormone, insulin (figure 1). Diabetes mellitus is an abundant metabolic disorder characterized by deranged glucose homeostasis due to an absolute or relative lack of insulin (Donath and Halban 2004). The disease is divided into two main forms type 1 and type 2 diabetes mellitus. In type 1 diabetes mellitus (T1DM), β -cells are destroyed by an autoimmune attack. Macrophages and lymphocytes invade the islets of Langerhans causing a state termed insulinitis (Jörens, et al. 2005). These immune cells produce and release pro-inflammatory cytokines like interleukin- 1β (IL- 1β), interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) to which the pancreatic β -cells react by increasing the levels of iNOS and thus enhanced nitric oxide (NO) production that eventually this triggers apoptotic pathways in the β -cell (Eizirik and Mandrup-Poulsen 2001). Gradual depletion of pancreatic β -cell mass through apoptosis leads to the observed 70-80 % loss of β -cells at time of diagnosis (Gillespie 2006; Klöppel, et al. 1985).

From the United Kingdom Prospective Diabetes Study (UKPDS), it is clear that there is a progressive deterioration of β -cell function over time also in type 2 diabetes mellitus (T2DM) (Group 1998a, b). Recently, these rates have been quantified (Szoke, et al. 2008). Both first and second phase of glucose stimulated insulin secretion (GSIS) decrease in a linear fashion with a yearly rate of 2.2 % and 1.4 % respectively in people with impaired glucose tolerance.

In individuals with normal glucose tolerance the rate was slower (0.7 % per year for both phases) (Szoke et al. 2008). Since human pancreatic β -cell mass can not be studied over time it is difficult to determine whether the reduced secretory capacity is due to reduced β -cells mass or a lower functionality. Several post-mortem examinations of human pancreata from T2DM patients have however showed a reduction in β -cell mass as a result of enhanced apoptosis (Butler, et al. 2003; Maedler, et al. 2003), which indicates that a reduction in β -cell mass occurs in T2DM. Such a conclusion is supported by results showing that the activities of apoptosis associated proteins caspase-3 and caspase-8 are elevated in human β -cells from T2DM patients (Marchetti, et al. 2004). The precise nature of pancreatic β -cell apoptosis in T2DM is not known. There is evidence that both glucose and saturated fatty acids can induce apoptosis (Diakogiannaki, et al. 2007; Donath, et al. 2005; El-Assaad, et al. 2003; Maedler et al. 2003).

Both T1DM and T2DM are complex diseases with altered expression of many genes and their products (Gloyn 2003; Maier and Wicker 2005). It is therefore important to monitor the expressional variations of many proteins simultaneously. For this purpose microarrays have proven very informative. The mRNA array technique benefits from its inherent specificity and immediate identification of differentially expressed genes. In addition, it is possible to answer specific questions by designing mRNA arrays to measure changes in specific genes involved in the same signalling pathway. However, the technology fails to detect post-translational modifications of proteins, which might well be crucial for protein activity (Ohtsubo, et al. 2005). Generally there is a good correlation between the levels of a single mRNA and the corresponding protein (Orntoft, et al. 2002), although this is not always the case. For example, dexamethasone does not affect GLUT2 mRNA levels but decreases GLUT2 protein levels through enhanced degradation in pancreatic rat islets (Gremlich, et al. 1997). More extensive

correlation studies support the notion that the relationship between mRNA and protein levels is weak (Nie, et al. 2007). In an analysis of 19 proteins from human liver Anderson et al (Anderson and Seilhamer 1997) obtained a positive correlation with a r-value of 0.48. A comparison of mRNA and protein expression of metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinase 1 in prostate tumours did not show any significant relationship (Lichtinghagen, et al. 2002). Similar results were found in a study of lung adenocarcinomas (Chen, et al. 2002). Studies in yeast (Gygi, et al. 1999) showed that while the mRNA levels for some genes were the same, their corresponding protein levels varied more than 20-fold. The authors concluded that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. Therefore, techniques that measure protein levels directly are not only a complement to transcriptome analysis but are rather mandatory for full understanding of cellular function. The aim of proteomics is the simultaneous measurement of levels of large numbers of proteins obtained from complex biological samples. These data sets are subsequently analysed to elucidate molecular mechanisms in health and disease. In this paper we will describe and summarise the proteomic efforts that have been made to study diabetes. However, we have restricted ourselves not to include the field of diabetic complications, which are reviewed by others (Merchant and Klein 2007; Quin, et al. 2007; Thongboonkerd and Malasit 2005). Neither have we included discussions on the techniques used for proteomic studies but the interested reader may look at references (Cho 2007; D'Hertog, et al. 2006; Gorg, et al. 2000; Keshamouni, et al. 2006; Ortsäter, et al. 2007).

Pancreas and islets of Langerhans

The pancreatic gland is a mixed organ mainly composed of exocrine cells responsible for the synthesis and secretion of digestive enzymes. In addition there are the islets of Langerhans which are endocrine micro-organs scattered throughout the gland. The islets of Langerhans only account for 2 % of the total pancreatic mass (Slack 1995) and are composed of several types of hormone producing cells: β -cells (secreting insulin), α -cells (secreting glucagon), δ -cells (secreting somatostatin) and PP-cells (secreting pancreatic polypeptide). Within the islets there are also endothelial cells and neurons. From a protein profiling perspective the presence of several cell types in the islet can be a potential problem when it comes to assigning changes in protein levels in, for example islets preparations, to a change in a particular cell phenotype. On the other hand, the different cell types within the islets display extensive cross talk through paracrine signalling (Gromada, et al. 2007; Leung, et al. 2006; Singh, et al. 2007) and therefore protein changes in any cell type can be relevant for the overall islet function.

Insulin producing β -cells are the most abundant cells within the islets accounting for 50-90 % of all islet cells, depending on species and strain (Cabrera, et al. 2006). The β -cell is a highly specialised cell whose function is to produce, store and release the hormone insulin upon a blood glucose elevation. For this purpose the cell must be equipped with mechanisms to sense the circulating glucose concentration and to adjust both the production and secretion rates of insulin. For sensing purposes the β -cell relies on metabolism of glucose which produces second messengers to enhance exocytosis. Incretin hormones, other nutrients as well as neural signals can all provide additional signals to either enhance or attenuate glucose induced insulin secretion. In the human genome there is one insulin gene located on chromosome 11 whereas there are two copies in the murine genome located on chromosome 19 (Ins1) and 7

(Ins2). The gene products of the two genes are identical and deletion of one or other does not produce a diabetic phenotype (Leroux, et al. 2001). Biosynthesis of insulin starts with transcription of the insulin gene forming proinsulin mRNA which accounts for 20 % of the total mRNA production in the pancreatic β -cell (Van Lommel, et al. 2006). Estimates suggest that there are about 40.000 proinsulin mRNA copies in a β -cell (Tillmar, et al. 2002). From the proinsulin mRNA preproinsulin protein is produced. The production of preproinsulin accounts for 20 % of total protein production in glucose stimulated pancreatic islets (Permutt and Kipnis 1972) and can be as high as 50 % in purified β -cells (Schuit, et al. 1988). Apart from stimulating the production of insulin, glucose enhances the production of chaperones, convertases and transport proteins thus supporting the release of insulin as will be described below. In fact glucose can cause a 5-fold induction of total protein production in pancreatic islets (Vander Mierde, et al. 2007).

Here we will describe the proteomic studies that have addressed changes in the islet and β -cell proteome. However, first the normal pancreatic islet and β -cell will be described from a proteomic perspective.

Protein profiling of pancreatic islets and β -cells

Protein profiling efforts aim to characterise which proteins that are expressed and can be detected in a certain tissue type e.g. pancreatic islets (Ortsäter and Bergsten 2006). Often these studies result in tissue protein reference maps that may be useful as reference tools in research. The idea is that the maps could serve as protein identification tools for other researchers. In order to be useful as such a tool all experimental procedures must adhere to same protocol and as these detailed protocols are by today lacking the use of protein maps for protein identification are limited. The first islet proteome reference map was constructed by

Sanchez et al. (Sanchez, et al. 2001) who presented a 2-dimensional gel electrophoresis (2DGE) map of proteins extracted from C57Bl/6J mouse islets. In that study 63 spots were identified that corresponded to 44 unique proteins. Nicolls et al. (Nicolls, et al. 2003) isolated proteins from islets obtained from BALB/c mice and analysed these proteins by 2DGE and MALDI-TOF MS. From the gels they could excise about 150 spots and identify 87 different proteins. Of the 44 C57Bl/6 islet proteins described earlier, 50 % were also found in this study. Sanchez et al. did not, in their report, categorise the proteins with regard to function or cellular localisation. We have here used the Swiss-Prot database (<http://www.expasy.org/sprot/>) to obtain such categorisation and compared the Sanchez et al. protein categorisation with the categorisation presented by Nicolls et al. When it comes to cellular localisation both studies provided similar results. Most proteins fell into the cytosolic class followed by mitochondria and endoplasmic reticulum (ER) (figure 2A and B). In both studies a large part of the proteins have either metabolic or chaperone functions (figure 3A and B). This indicates both that these functions are important for islet cells, but also that proteins in these categories are abundantly expressed in islets. The latter conclusion is based on the fact that the 2DGE technique preferentially detects high-abundant proteins (Gygi, et al. 2000). This is important when considering that only 5 and 2 % of the identified proteins were classified with signalling functions in the Nicolls et al. and Sanchez et al. studies respectively. Signalling proteins are often short lived and normally only present a low levels. Hence, it is likely that these types of proteins are not detected in a general proteomic screening. In our categorisation of the Sanchez et al. data a large part of the proteins (19 %, figure 3A) were classified as catalytic enzyme whereas Nicolls et al. lacked this category in their classification. Among the literature describing proteomic analysis of pancreatic islet tissue there is no consensus in how to classify proteins. To classify proteins according to function and cellular localisation the Swiss-Prot database and the ontology databases through European

bioinformatics Institute (<http://www.ebi.ac.uk/>) can be used. These databases represent two freely available alternatives. However, not all proteins in these databases are annotated with function and/or cellular localisation and some proteins may have several functions and cellular localisations. Other alternatives for classification will be presented below.

While Sanchez et al. and Nicolls et al. used mouse pancreatic islets Ahmed et al. (Ahmed, et al. 2005) profiled human pancreatic islets with 2DGE and MALDI-TOF MS. They found 130 spots corresponding to 66 different proteins. Both when comparing the list of identified proteins and the classification charts (figure 2 and 3) the results obtained with human islets are similar to those obtained with mouse islets. In conclusion, despite the fact that the islets were from different species and mouse strains, the resulting protein profiles were similar. For instance it can be noted that all three studies identified the ER chaperones glucose-regulated protein (GRP)78 and protein disulphide isomerase (PDI).

It is evident from the number of identified proteins in these studies that only a very small part of the islet proteome is detected. In an attempt to increase coverage Metz et al. (Metz, et al. 2006) employed 2D LC/MS/MS for their characterisation of human pancreatic islets. Over 29.000 peptides were detected and identified corresponding to 3365 identified proteins which makes this study the most comprehensive islet proteomic study until date regarding number of identified proteins. For classification they used the Ingenuity Pathways Application (http://www.ingenuity.com/products/pathways_knowledge.html) in which identified proteins are placed in their functional pathways e.g. JAK/Stat or integrin signalling pathway.

It is also possible to increase proteome coverage by performing subcellular fractionations prior to proteomic analysis. For such initiatives to be successful they are dependent on

stringent control of organelle purity in the analysed fraction. Brunner et al. (Brunner, et al. 2007) performed a proteomic analysis of insulin secretory granules isolated from the rat clonal cell line INS-1E. First, a two step enrichment of insulin secretory granules was performed by centrifugation in a Nycodenz gradient and then in a Percoll solution. The resulting fraction was positive for insulin and betagranin, markers of insulin secretory granules, but negative for calreticulin which was used as a marker for ER resident proteins. When analysing this preparation 130 different proteins were identified, a number equal or even greater than the numbers obtained when analysing total cell lysates. Among these identified proteins the vast majority was either intravesicular proteins (57 proteins or 44%) or membrane proteins (59 proteins or 45%). Several of the proteins classified as intravesicular proteins were proteins known to be secreted by the pancreatic β -cell, e.g. insulin, chromogranin A, betagranin and secretogranin but there were also several proteins with hydrolase activity which is normally associated with lysosomes. Although it cannot be fully excluded that these proteins represent comigration of lysosomes and secretory granules in the fractionation procedure it may very well represent a physiological phenomena in that insulin is degraded by a process known as crinophagy where secretory granules are fused with lysosomes (Sandberg and Borg 2006). Alternatively, these results could support the so called “sorting by retention” hypothesis in which immature secretory granules can develop into either mature secretory granules or into lysosomes (Arvan and Castle 1998). In the membrane proteins class Brunner et al. identified several members of the Rab family of proteins that regulate steps in the vesicular trafficking process and members of the v-SNARE protein family.

Proteomic analysis of glucose induced changes in pancreatic islet and β -cells

Glucose is an insulin secretagogue that by itself can induce sustained insulin secretion. Metabolism of the carbohydrate through glycolysis, the Krebs cycle and subsequently the electron transport chain leads to an increase in the ATP/ADP ratio that will close K_{ATP} channels in the cell membrane leading to membrane depolarisation, calcium influx and exocytosis of insulin granules (Rutter 2001). The enzyme pyruvate carboxylase is abundantly expressed in pancreatic β -cells (Schuit, et al. 1997). Therefore as much as 40 % of all pyruvate that enters the mitochondria is converted to oxaloacetate (Khan, et al. 1996). Thus glucose also feeds intermediates into the Krebs cycle. In linkage with its metabolism glucose may also generate reactive oxygen species (ROS) as observed both in cell experiments (Sakai, et al. 2003) and *in vivo* (Tang, et al. 2007). Hence, prolonged exposure to supra physiological levels of glucose impairs β -cells function (Eizirik, et al. 1992; Marshak, et al. 1999; Song, et al. 2003) and can induce apoptosis (Buteau, et al. 2004; Maedler et al. 2003; Wang, et al. 2005), a phenomena named glucotoxicity.

The effect on glucose on changes in protein profiles have been investigated both in pancreatic islets and in clonal β -cells. Indeed, when rat islets were exposed to different glucose concentrations and subsequently islet proteins separated using this approach, the protein levels of numerous proteins were altered (Collins, et al. 1992; Collins, et al. 1990). The proteins responding to elevated glucose concentrations, “glucospondins”, were not identified, however. In a study by Guest et al. (Guest, et al. 1991) 2DGE analysis combined with fluorography and densitometric quantification was used to examine the effects of glucose on the biosynthesis of rat pancreatic islet proteins. A rise in glucose from 2.8 to 16.7 mM caused a 10-20 fold stimulation in the synthesis of 10 out of 260 detected islet proteins, as judged by

incorporation of [35S] methionine during a 20 min incubation. The synthetic rates of the majority of the remaining proteins were stimulated by 2-4-fold. By using subcellular fractionation it could further be shown that the majority of protein whose translation was stimulated by glucose was localised to secretory granules. The results presented by Guest et al. are in line with those later obtained by Ahmed and Bergsten (Ahmed and Bergsten 2005) who compared freshly isolated mouse islets isolated from C57Bl/6J with those cultured at 11 mM glucose for 24 hours. When analysed with 2DGE cultured islets displayed increased levels of molecular chaperones such as PDI A6, GRP58 and GRP94 which can be taken as an indication of increased demand on the cellular protein synthesis capacity. On the other hand, another form of PDI (PDIA1) and GRP78 were found to be lower in the cultured islets. Since the comparison is made between freshly isolated islets and cultured islets it is possible that the results are influenced by other factors than glucose. Furthermore, the results showed that proteins supporting granule movement and maturation, such as actin, tubulin beta 5, tubulin alpha, keratin type II cytoskeletal 8, prohormone convertase 2 and ATPase, H⁺ transporting, lysosomal 70 Mr, V1 subunit A, were up-regulated in cultured islets. Exposure to high glucose levels generates oxidative stress both in cell experiments (Sakai et al. 2003) and *in vivo* (Tang et al. 2007). This aspect of glucose stimulation was indicated in the proteomic study by Ahmed and Bergsten where cultured islets showed elevated levels of antioxidant proteins and heat shock proteins like cytoplasmic superoxide dismutase, peroxiredoxin 2 and 6, heat shock protein cognate 74 and heat shock protein 40 Mr. Overall these data suggest that glucose increases the capacity of the β -cells to produce and secrete insulin and that this response can occur alongside increased β -cell mass during adaptation to insulin resistance. Besides such adaptation, islets exposed to glucose also increase their protection against oxidative stress.

Fernandez et al. (Fernandez, et al. 2008) performed a somewhat similar study where the analysed both metabolomic and proteomic changes in INS-1 832/13 cells exposed to either 2.8 or 16.7 mM glucose for 48 hours. In a phenotypic characterisation, cells cultured at 16.7 mM displayed characteristics of glucocotoxicity with reduced K_{ATP} -channel dependent and K_{ATP} -channel independent insulin secretion as well as reduced insulin content. Metabolic profiling was conducted by gas chromatography coupled with mass spectrometry. Cells cultured at 16.7 mM glucose contained more Krebs cycle intermediates (fumarate, citrate and malate) and more ribose-5-phosphate from the pentose phosphate shunt. Nine amino acids were also detected. Of these alanine and hydroxyproline were increased in the high glucose group while glutamine and serine were decreased. Proteomic analysis was done by 2DGE and about 800 spots were detected on the analytical gels of which 305 spots were found to be significantly different when comparing gels from cells cultured at 2.8 mM glucose versus 16.7 mM glucose. Of these 305 spots the authors identified 75 unique proteins. Generally, there was little correlation between proteins and metabolite levels. The authors view this as an indication that substrate availability and allosteric enzyme regulation is more important in determining metabolite levels than the actual enzyme concentration. From the list of identified proteins it can be seen that the same protein was identified in several different spots. This is common in proteomic studies using 2DGE and represents post-translational modifications which change the protein's isoelectric point or mass or both. For example, PDI A3 precursor (Swiss-Prot accession number P11598) was identified from spot 1602 and spot 1379. Spot 1602 intensity was higher in gels produced from high glucose exposed cells while spot 1379 intensity was lower in gels produced from such cells. Such results indicate that glucose might not necessarily change the expression level of a protein but rather change its characteristics by for instance glycosylation. The use of specific dyes to detect glycosylated proteins (Wu, et al. 2005) could shed light on this matter. In the 16.7 mM glucose exposed cells, which displayed

a loss-of-function phenotype the ER chaperone GRP78 was lowered. This observation is in agreement with the findings obtained by Dowling et al. (Dowling, et al. 2006) in their proteomic screening of glucose responsive and glucose non-responsive MIN6 cells. Cells from this mouse clonal cell line with a high passage number (passage 40) do not increase their insulin secretion in response to glucose as well as cells with a low passage number (passage 18). These non-responsive cells had decreased protein levels of endoplasmic reticulum protein 29, GRP78, GRP94, PDI carbonyl reductase 3, peroxidoxin 4 and cytoplasmic superoxide dismutase. Thus, these cells do not have the same capacity to fold, modify or secrete proteins nor to protect themselves from oxidative stress.

Proteomic studies of pancreas and islets in animal models of obesity and T2DM

Insulin resistance puts pressure on the pancreatic β -cell which responds by increasing its insulin secretory output in order to maintain normoglycaemia. The Zucker fatty (ZF) rat and the Zucker diabetic fatty (ZDF) rat are animal models of obesity and T2DM respectively (Clark, et al. 1983). The ZDF rat was developed by successive inbreeding of the most glucose intolerant ZF rats. The ZF rat remains normoglycaemic in the face of increasing obesity due to their ability to increase their β -cell mass and maintain a constant insulin secretory capacity despite a reduction in insulin sensitivity (Topp, et al. 2007). These data indicate that β -cell proliferation and/or β -cell neogenesis from undifferentiated progenitor cells can occur if β -cell mass needs to be increased. Increased proliferation capacity as a consequence of diet induced obesity or insulin resistance was also indicated in a study by Qiu et al. (Qiu, et al. 2005). They performed 2DGE analysis on whole pancreas preparations from high fat diet induced diabetic mice and found increased levels of regenerating islet-derived 1 and 2 protein

(REG1 and REG2) in the early stages of T2DM development. In the late stage they also observed reduced levels glutathione peroxidase which can be taken as a sign of reduced oxidative stress protection. Since the preparation was made from whole pancreas and islets only constitute about 2 % of organ mass it cannot be taken for granted that these changes can be attributed to changes in β -cells or islets. In an attempt to elucidate mechanisms of obesity-induced changes in the islet proteome, islet maps from C57BL/6J and C57BL/6J^{lep/lep} mice were compared (Sanchez, et al. 2002). A mutation in the gene encoding the hormone leptin in C57BL/6J mice produces the obese C57BL/6J^{lep/lep} or *ob/ob* mouse strain (Zhang, et al. 1994). This mouse strain is obese and shows marked insulin resistance (Halaas, et al. 1995). To compensate the insulin resistance with enhanced secretion the C57BL/6J^{lep/lep} mouse develops β -cell hypertrophy (Baetens, et al. 1978). Comparison between 2DGE islet maps from C57BL/6J^{lep/lep} and lean litter mates revealed different expression levels of nine proteins of which several were involved in actin cytoskeleton organisation (Sanchez et al. 2002). Changes in actin cytoskeleton organisation occur during insulin granule movement and exocytosis (Li, et al. 1994; Orci, et al. 1972). Treatment of C57BL/6J^{lep/lep} with the insulin sensitising agent rosiglitazone restored these alterations.

A β -cell hypertrophy is also seen in the MKR mouse, an insulin resistance model (Lu, et al. 2008). The MKR mouse harbours a dominant-negative insulin-like growth factor-1 receptor mutation specifically targeted to skeletal muscle (Fernandez, et al. 2001), thus they have no primary β -cell defect. These mice are hyperinsulinemic already at 2 weeks of age and impaired glucose tolerance is evident from the age of 5 weeks. In a very recent study Lu et al. (Lu et al. 2008) compared the islet protein from 10-weeks old MKR mice and wild-type controls using the iTRAQ technology (Keshamouni et al. 2006). Of 590 unique protein identified, 1159 proteins were differentially expressed. In support of an increased demand for

insulin synthesis the results showed that MKR islets contained more molecular chaperones (GRP78 and GRP94), protein belonging to the disulfide isomerase family and the peptidyl prolyl isomerase family. In addition proteins involved in endoplasmic reticulum associated protein degradation (Kincaid and Cooper 2007) were also up-regulated in islets from the MKR mouse. Such results indicate induction of the unfolded protein response (Wu and Kaufman 2006) and enhanced protein folding in these islets. Protein disulfide isomerases catalyse the formation of disulfide bonds and after catalysis these enzymes is regenerated in process that includes the formation of ROS (Malhotra and Kaufman 2007). Thus, an enhanced protein folding activity can produce cytotoxic substances. It would be interesting to see if β -cell cytotoxicity occurs at later stages in the MKR mice. In contrast to the up-regulation of proteins involved in protein biosynthesis, folding and degradation, proteins involved in insulin processing and exocytosis was observed in MKR islets. In addition, MKR islet had lower levels of proteins involved in glucose metabolism such as the glucose transporter GLUT-2, pyruvate carboxylase and members of the mitochondrial respiratory chain. Altogether these finding provide an explanation for attenuated glucose-induced insulin secretion in MKR islets.

Proteomic studies of pancreas from pancreatectomy models

The partial pancreatectomy (Px) model is a widely used model to study pancreatic regeneration (Bonner-Weir, et al. 1983). In studies by Yang et al. (Yang, et al. 2006) and Shin et al. (Shin, et al. 2005) changes in the protein expression in the regenerating rat pancreas on the third day after 90 % Px (Yang et al. 2006) or on the second day after 60 % Px (Shin et al. 2005), as compared with sham operated rats were analysed with 2DGE followed by mass

spectrometry. Unfortunately, there was very little correlation between the detected proteins in the two studies. A priori one would expect to find elevated levels of growth factors in the preparations from Px rats but as pointed out by Shin et al. the expected concentration of such growth factors in these preparations are below the detection limit of available gel staining methods. Nevertheless, both studies reported proteins classified as glucose, lipid and amino acid metabolism, protein synthesis, chaperone, signal transduction and apoptosis to be affected by Px.

Proteomic analysis of pancreatic islets and β -cells after cytokine exposure

Cytokines produced from inflammatory cells during the development of T1DM are mediators of cytotoxic signals that induce apoptosis in pancreatic β -cells (Eizirik and Mandrup-Poulsen 2001). To gain insights into the effects of cytokine exposure several proteomic studies have been performed where insulin producing cell lines or isolated pancreatic islets are exposed to one or several cytokines *in vitro*. D'Hertog et al. (D'Hertog, et al. 2007) have performed a comprehensive time-resolved proteomic study on INS-1E cells treated with either IL-1 β or IFN- γ or a combination of the two cytokines for 1, 4 and 24 hours. Changes in protein levels were then analysed by 2D differential in-gel electrophoresis (2D-DIGE). First it could be noted that most protein changes occurred only after 24 hours and when IL-1 β and IFN- γ were combined. Altogether 92 different proteins were identified as being cytokine regulated. In addition the study revealed that many proteins (like GRP78 and oxygen-regulated protein 150) were regulated by post-translational modifications. The data on GRP78 supports the idea that cytokines can induce multiple phosphorylations on this chaperone. To characterise the identified proteins they were placed in interaction networks. These networks are constructed

on the basis that mutations in the genes for any of the network protein members give rise to comparable phenotypes (Lage, et al. 2007). Of the 92 different proteins identified 42 could be placed in a single interaction network. Interestingly GRP78 was placed in the centre of one of the identified networks, indeed GRP78 is a master regulator of the unfolded protein response (Wu and Kaufman 2006). This kind of analysis adds the informative value of these kinds of studies.

Studies on the effect of IL-1 β on pancreatic islet protein expression have also been conducted (Andersen, et al. 1997. Much of this work has been extensively covered in previous reviews {D'Hertog, 2006 #1009; Larsen, et al. 2001; Sparre, et al. 2003; Sparre, et al. 2002; Sparre, et al. 2005) and will only be briefly summarised here. By using 2DGE the effect of IL-1 β on the cellular level of about 2,200 proteins was investigated in rat islets (Andersen et al. 1997).

Whereas the cytokine up-regulated 52 spots, 47 were down-regulated and 6 was the result of *de novo* synthesis (Andersen et al. 1997). By preventing IL-1 β induced NO formation IL-1 β caused changes in 23 of the altogether 105 original spots affected by IL-1 β (John, et al. 2000).

It was concluded that IL-1 β induces effects on islet protein expression that are both NO dependent and NO independent. When identification of these IL-1 β related islet proteins was attempted, identities were obtained for 57 different proteins (Larsen et al. 2001). The identified proteins were assigned to broad classes according to their known or putative function. The largest group contained 25 proteins, which were involved in protein synthesis chaperoning and protein folding, thus a similar to glucose-induced changes in protein expression (Ahmed and Bergsten 2005). In support of the dependency of IL-1 β effects on active protein synthesis, the protein synthesis inhibitor cycloheximide protected islets from IL-1 β induced destruction (Eizirik, et al. 1993). The effect of IL-1 β has also been investigated using islets isolated from the diabetes-prone BB rat. Protein expression changes induced by

IL-1 β caused similar changes in islet protein expression patterns of these BB rats when compared with control islets (Larsen et al. 2001; Sparre et al. 2002). The identified differentially expressed proteins represent diverse areas of cellular functions and it has been difficult to determine what changes may be primary.

Protein profiling of blood, serum and plasma

Diabetes is a disease where many organs are affected and since all tissues are in contact with blood, proteins secreted or leaking from the different tissues are reflected in the circulation. Many individual serum proteins have been shown to vary between healthy individuals and persons with insulin resistance and T2DM. Examples of such proteins are interleukin-6, resistin, leptin, adiponectin and visfatin (Berndt, et al. 2005; Considine, et al. 1996; Hotta, et al. 2000; Norata, et al. 2007; Vozarova, et al. 2001). Many of the circulating proteins have also been connected, in various ways, to the pathogenesis of the disease. Blood insulin concentration at different stages of diabetes is a good example of how a single protein in the blood directly reflects changes in β -cell physiology. The easy accessibility of blood makes it attractive to study changes also of other blood-borne proteins, which may be related to different aspects of the disease. During recent years the development of different methodologies capable of simultaneously measuring large numbers of proteins present in biological samples has accelerated and protein profiling of blood has been successfully used in context of many diseases, like breast and prostate cancer (Laronga, et al. 2003; Qu, et al. 2002). An important consideration when performing blood protein profiling is the overall composition of blood proteins, where a limited number of proteins, like albumin and

immunoglobulins, account for a large part of the total blood protein amount (Anderson and Anderson 2002; Echan, et al. 2005; Pieper, et al. 2003; Tirumalai, et al. 2003). To aid detection of medium and low abundant proteins, depletion of high abundant proteins by different methods has been utilised (Echan et al. 2005; Gong, et al. 2006). On the other hand, when pre-fractionations are used, the risk of losing potentially interesting proteins increases (Mehta, et al. 2003). One of the first studies using blood from patients with T2DM was done by Jiang et al., who searched for altered proteins in the red blood cell membranes in T2DM (Jiang, et al. 2003). In total, 27 membrane proteins were up-regulated and 15 down-regulated when comparing the red blood cell membranes of T2DM individuals to healthy controls. Two of the up-regulated proteins were identified as flotillin-1 and arginase, while one of the down-regulated proteins was identified as syntaxin 1C. The results led to another study where the interactions of flotillin-1 and arginase were investigated (Jiang, et al. 2006). It was found that soluble arginase located to the red blood cell membranes through interaction with membrane bound flotillin-1. This interaction in turn, was shown to increase the arginase activity, also measured in T2DM patients.

Another early study using a proteomic approach on human serum in the context of type 2 diabetes and insulin resistance was performed by Zhang et al. to mine low abundant proteins, using an approach where fractionated serum samples were analysed with surface enhanced laser desorption/ionisation time-of-flight spectrometry (SELDI TOF-MS) (Zhang, et al. 2004). When comparing serum from patients with T2DM or insulin resistance to control serum, haptoglobin was found to be elevated. Also, several other proteins involved in the inflammatory response, like α -2 macroglobulin, fibrinogen, complement C3 and C1 inhibitor, were altered when comparing serum from persons with insulin resistance with control serum. Many of the found proteins had been connected to diabetes in various ways. For instance, the

acute phase protein haptoglobin has been associated with glucose and lipid metabolism (Heliövaara, et al. 2005), while fibrinogen has been shown to be related to the development of T2DM (Festa, et al. 2002). Also haemoglobin (Hb) was increase in T2DM, which is not in line with the finding of a recent study, where both Hb α - and β -chains were found to be decreased in T2DM patients (Sundsten, et al. 2007). In the latter study, the plasma Hb was also measured by an ELISA which confirmed the observation. The overall aim of this study was to investigate variations in plasma protein levels in persons with T2DM and differences in early insulin response (EIR), and compare these protein levels with the levels in normal glucose tolerant (NGT) individuals. Plasma proteins of individuals in three groups with differences in EIR were profiled and nine proteins with different plasma concentrations were found. Most of these proteins showed different levels when comparing NGT to T2DM, irrespective of differences in EIR. Apolipoprotein H (ApoH) was decreased in plasma from T2DM individuals with high EIR compared to NGT individuals. The levels of ApoH in T2DM individuals with low EIR were not reduced compared to the levels found in NGT subjects. Since the glycaemic environment in the two T2DM groups was comparable, differences in EIR were suggested to reflect variations in the genetic background of the study subjects. No single protein in the present study could by itself explain the differences seen in EIR, reinforcing the polygenic nature of T2DM. The differences in plasma proteins were interpreted as manifestations of the disease state, rather than being causative. This conclusion was consistent with the results from another study where the aim was to determine the relative importance of the diabetic environment versus genetic background for the development of β -cell failure (Sundsten, et al. 2008b). Serum protein profiles of NGT and newly diagnosed T2DM individuals were compared and persons with differences both in β -cell function (estimated by HOMA- β) and family history of diabetes (FHD) were included. Altogether 13 proteins showed varying serum levels between the NGT and T2DM groups. Five proteins

were lowered and eight were elevated in serum from T2DM. In a second comparison, the individuals were categorised into persons with low β -cell function with FHD and persons with high β -cell function without FHD. Whereas three of the initially found proteins were rediscovered and interpreted to be a consequence of genetic factors, ten proteins were not rediscovered and interpreted to be unrelated to FHD, but rather varying because of the diabetic environment. Among these proteins, apolipoprotein CIII (ApoCIII), albumin and one yet unidentified protein could be classified as being changed because of different genetic backgrounds. On the other hand, 10 proteins for instance transthyretin (TTR) differed as a result of the diabetic environment. These studies performed on human serum and plasma identified proteins out of which many previously have been connected to diabetes. For instance increased levels of ApoCIII found in T2DM patients (Sundsten, et al. 2008a) have also been measured in serum from individuals with T1DM (Juntti-Berggren, et al. 2004) and are also considered a cardiovascular risk factor (Gervaise, et al. 2000). Transthyretin on the other hand was decreased in serum from individuals with T2DM in both studies (Sundsten et al. 2007; Sundsten et al. 2008b). The finding aligned well with results reporting lowering of TTR in streptozotocin-treated rats (Kim, et al. 2006). Reduced levels of TTR have also been associated with inflammatory conditions (Myron Johnson, et al. 2007) and type 1 diabetes (Itoh, et al. 1992). In patients with insulin resistance and diabetes several acute phase reactants like C-reactive protein, have been shown to be increased in serum (Festa et al. 2002). This has been interpreted to be a part of the low-grade inflammation present in these patients (Pickup 2004; Sjöholm and Nyström 2006), which could also explain both lowered TTR levels in T2DM serum. Lowering of albumin and ApoH are other characteristic changes in inflammation (Gabay and Kushner 1999; Mehdi, et al. 1991). Both these alterations could be explained by the low-grade inflammatory response, which has been suggested to be a part of the pathogenesis of T2DM (Pickup 2004). The observation has also been made in humans

(Castaneda, et al. 2000) where T2DM was associated with poor nutritional status. Since the individuals with T2DM were all newly diagnosed and did not report any kidney disorders, it seems unlikely that the observed decrease in serum albumin reflects enhanced urinary albumin excretion. In addition, albumin was not only lower when individuals with T2DM were compared with individuals with NGT, but also when NGT individuals with and without FHD were compared. Increased urinary excretion of albumin has, nevertheless, been observed in normoalbuminuric patients with T2DM (Narita, et al. 2006), which may contribute to explain the findings.

To get new insights into the molecular alterations implicated in diabetes, serum protein profiles of normal rats and rats with streptozotocin-induced diabetes have been analysed (Cho, et al. 2006). Eight proteins were found, all increased in diabetes. One of the increased proteins was identified as C-reactive protein. Even if the protein has previously been shown to be increased in T2DM, and also predicts the development of the disease in humans (Pradhan, et al. 2001), it is however not a strong acute phase reactant in rats (Schreiber, et al. 1989).

Effects on protein levels by different anti-diabetic agents from plants and fungi have been evaluated. The anti-hyperglycaemic effects of green tea on mouse serum proteins was investigated (Tsuneki, et al. 2004) using SELDI-TOF MS. Seven proteins were found to be more than three-fold decreased while seven were more than three-fold increased, when comparing mice with T2DM to control mice. To find the specific proteins, which were affected by green tea, the mice with T2DM were re-investigated after green tea administration, and compared with saline-treated control mice. When the two sets of results were compared, one protein specific for diabetes and sensitive for green tea was found. This un-identified protein with the mass 4212 Da was significantly reduced both in type 2 diabetes

and by the green tea administration. In another study, the anti-hyperglycaemic effect of fungal polysaccharide treatment on protein patterns monitored by 2DGE was evaluated in normal rats and rats with streptozotocin-induced diabetes (Kim et al. 2006). In total about 50 proteins were found to be differentially regulated and 20 spots were identified as diabetes-associated proteins. When the protein patterns were monitored over time, different patterns were found. Nine proteins (albumin, apolipoprotein A1, apolipoprotein E, haptoglobin, immunoglobulin kappa-chain, kallikrein binding protein, transthyretin monomer, transthyretin tetramer and vitronectin) were significantly changed during diabetes induction and were restored to healthy levels after treatment. Seven proteins (α 1-inhibitor III, apolipoprotein A-IV, ceruloplasmin, fetuin β , hemopexin, serine protease inhibitor and transferrin) changed upon diabetes induction, but were not restored by treatment. In addition, the plasma proteome effects of the Japanese traditional medicine Kampo have been performed (Kiga, et al. 2005). The plasma protein expression profiles of spontaneously diabetic rats with nephropathy were compared to normal rats and ten proteins were found to have different levels. Some of these peaks were altered by the kampo treatment, which was interpreted by the authors to be proteins with special association with the development of nephropathy.

Protein profiling of fat, muscle and liver

Insulin lowers the blood glucose by acting on three main target tissues, namely adipose cells, muscle and liver. First, the glucose uptake and utilization in muscle and adipose tissue is enhanced. In liver and muscle cells glycogen synthesis is enhanced, while break-down is suppressed, resulting in net storage of glycogen. Glucose release from liver is suppressed by inhibition of enzymes of the gluconeogenic pathway. The opposite reactions happen in the

fasting state, when blood glucose and insulin levels are low. Glucose production is then promoted by enhanced hepatic gluconeogenesis and glycogenolysis. At the same time, glycogen production and glucose-uptake in insulin-sensitive tissues is decreased, leading to elevation of the blood glucose level. When normal glucose homeostasis becomes impaired like in type 2 diabetes, it is therefore very important to not only study protein patterns in pancreatic β -cells or blood but also in these other tissues involved in the glucose homeostasis. Adipose tissue, muscle cells and hepatocytes are all in different ways involved in T2DM and insulin resistance. In order to detect markers of high fat diet and insulin resistance Schmid et al. performed an interesting 2DGE study (Schmid, et al. 2004). Muscle, white and brown adipose tissue and liver were taken from normal mice fed a high fat or a normal chow diet. Many differentially expressed proteins between obese and lean mice were detected, out of which more than half were found in brown adipose tissue. Several of these proteins were stress and redox proteins. In addition, significant changes in mitochondrial enzymes involved in the Krebs cycle and in the respiratory chain were detected in high fat fed animals. From the proteomic data the authors conclude that high fat fed animals increase their energy expenditure to defend against weight gain by regulating the brown adipose tissue proteome, which in turn leads to energy dissipation.

Fat mass is closely associated with T2DM and visceral obesity is correlated with insulin resistance (Wilding 2007). White adipose tissue plays a central role in storage and release of energy, but is also an active endocrine organ. Numerous bioactive molecules called adipokines, are produced and secreted from adipose tissue. It has been suggested that impairments of the production of the many adipokines participate in the pathophysiology of insulin resistance, type 2 diabetes (McPherson and Jones 2003) as well as cardiovascular disease (Goralski and Sinal 2007). One possible mechanism coupling visceral obesity and

metabolic disorders is the chronic, low-grade inflammation associated with visceral obesity, which finally leads to the development of T2DM (Esposito, et al. 2006).

Both in mouse and man white adipose tissue proteome has been characterised using 2DGE (Corton, et al. 2004; Lanne, et al. 2001; Sanchez et al. 2001). However, in unravelling the possible mechanisms linking the adipocyte to diabetes, it is important, to characterise the white adipose tissue secretome. The proteins secreted from human adipose tissue have recently been characterised using a proteomic approach combining SDS-PAGE, SELDI TOF-MS and LC-MS/MS (Alvarez-Llamas, et al. 2007). The result was almost 300 identified proteins out of which 70 were interpreted to be secreted from adipose tissue. In the study adipose tissue from normal individuals was used. In order to elucidate the role of adipose tissue secreted proteins in the context of diabetes, it would be valuable to also characterize fat tissue from diabetes patients and compare the differences of the secretomes.

A failure in adipocyte differentiation has been proposed to be a critical factor leading to type 2 diabetes (Danforth 2000; Weber, et al. 2000). When the human adipogenesis was characterised with 2DGE 175 individual proteins were identified out of which 40 were involved in adipogenesis (DeLany, et al. 2005). Proteins in different functional categories were found to be modulated during adipogenesis. Again, a large number of heat shock proteins were found and interpreted to be candidate proteins regarding the aetiology of T2DM.

Skeletal muscle contributes to the maintenance of glucose homeostasis by taking up and utilising glucose in an insulin-dependent manner. When this energy balance is compromised, muscles might also contribute to the pathogenesis of different metabolic diseases like obesity or T2DM. Hittel et al. characterised cytosolic skeletal muscle proteins in lean and obese individuals (Hittel, et al. 2005). The study identified increased levels of adenylate kinase,

glyceraldehyd-3-phosphate dehydrogenase and aldolase A in skeletal muscle of obese women when compared to lean women. Proteomics analysis of human muscle is an important approach for gaining insight into the biochemical basis for normal and pathophysiological conditions. Hojlund et al. (Hojlund, et al. 2008) have recently characterized the human skeletal muscle proteome from healthy subjects by combining 1DGE and HPLC electrospray ionization tandem mass spectrometry. Nearly a thousand different proteins were identified in healthy human muscle. Beside muscle contractile proteins, many of the found proteins were involved in glucose and lipid metabolic pathways. Mitochondrial proteins accounted for a fairly large percentage (22%) of all proteins identified. In addition also enzymes involved in cell signalling and calcium homeostasis pathways were found. In order to be able to investigate the pathophysiological mechanisms in the diabetic state, similar approaches characterizing individuals with diabetes are required.

Hepatic insulin resistance and lipoprotein overproduction are found in the insulin resistant states. Analysis of liver protein expression profiles connected to hepatic insulin resistance was performed by Morand et al. (Morand, et al. 2005) using an insulin resistant hamster model. In their search for mechanisms linking development of hepatic insulin resistance and overproduction of atherogenic lipoproteins the authors analysed the proteome of the liver ER. By 2DGE 34 differentially expressed proteins were identified. Hepatic ER proteins ER60, ERp46, ERp29, glutamate dehydrogenase and TAP1 were all decreased, while alpha-glucosidase, P-glycoprotein, fibrinogen, protein disulfide isomerase, GRP94 and apolipoprotein E were all increased in the insulin resistant animal. As the authors point out, also global proteomic analysis of the liver would be useful in the context of insulin resistance. This is also true for the other tissues involved in insulin resistance. On the other hand,

subproteome analysis can aid the detection of low-abundant proteins and also more focused questions can hopefully be answered.

To investigate the proteomic effect of PPAR treatments on liver cells, several studies have been performed. First, the effects of PPAR- α agonist were studied on *ob/ob* mouse liver cell proteins by Edvardsson et al. (Edvardsson, et al. 1999). The results showed 16 up-regulated liver proteins out of which 14 belonged to the peroxisomal fatty acid metabolism pathway. The conclusion was that the therapeutic effect of PPAR- α agonist was through an up-regulation of the peroxisomal fatty acid β -oxidation. While PPAR- α agonists are used to treat hypertriglyceridemia, the PPAR- γ agonists are used to treat insulin resistance. PPAR- γ agonists are known to act via activating a nuclear receptor, thus leading to increased insulin sensitivity. To identify new potential drug targets, the effect of rosiglitazone on different tissues was determined in C57BL/6J^{lep/lep} mice (Sanchez, et al. 2003). Initially 34 proteins were shown to be differently expressed between C57BL/6J^{lep/lep} mice and lean littermates. Eleven of these proteins were modulated by rosiglitazone treatment. In muscle cells lactose-binding lectin and fructose biphosphate aldolase 1 were restored to the lean level. In white adipose tissue an albumin fragment and carbonic anhydrase fragments were restored to lean levels. The most proteins found to be affected by the treatment were liver proteins. In another similar study the effects of PPAR- α and PPAR- γ agonists on the mouse liver proteome in *ob/ob* mice were compared (Edvardsson, et al. 2003). Many effects on proteins were similar between the two types of PPAR-activators, but also some differences were found.

Rosiglitazone was particularly effective in normalising levels of enzymes involved in the amino acid metabolism. In total five liver proteins were normalised by treatment with rosiglitazone. Many of the same proteins found to be differently expressed between lean and

ob/ob mice were identified in the two studies. However, the proteins normalised by treatment were not the same.

Conclusions

The aim of proteomics is the simultaneous measurement of large numbers of proteins in complex biological samples. These data sets are subsequently analysed to elucidate molecular mechanisms in health and disease. Generally the strategy is to first characterise sample proteins and subsequently connect changes in protein levels to a certain phenotype. In diabetes research many of the performed studies aim at characterising the protein expression pattern in various tissues connected to diabetes pathophysiology. Results of such studies are limited to lists of proteins which are either increased or decreased in the disease state. Sometimes found proteins are classified by sorting criteria such as function and cellular localisation. However, comparison between studies is hampered by the lack of consensus in protein classification strategies. Anyhow, a study design which places proteins in interaction networks is more informative and makes use of the power of a proteomic approach. There are many proteomic studies dealing with diabetes from various angles and many interesting results have been presented. Vast amount of proteins have been identified and discussed in the context of possible disease mechanisms, however only a handful of confirmatory follow-up studies are available to date. On the other hand, many of the proteomic techniques are per se time consuming and for some the techniques also the actual identification process is tedious. Several studies have also been published during recent years, which may indicate that confirmatory work is in progress. Characteristic feature of any omic study is the massive

number of data that are generated. Therefore, thoroughly worked out hypothesis, experimental planning, statistics and bioinformatics tools are crucial for a successful study.

In the field of proteomic research today, many techniques with different methodological pros and cons are available, and combining several techniques can sometimes be elucidative. It can sometimes be difficult to evaluate results from studies using differing techniques and protocols. Obviously with the same protocols more easily comparable results would be obtained. On the other hand, comparable results are obtained when using 2DGE even if islets from different species are used and the study protocols are not identical.

One apparent reflection when summarising the results from serum proteomics is that most of the studies do indeed identify high-abundant proteins like albumin, immunoglobulins and other acute phase proteins. Even when efforts are made to specifically favour low-abundant proteins, the high-abundant ones are detected (Zhang et al. 2004). Refined pre sample fractionations enriching low-abundant proteins might circumvent this problem.

In conclusion, proteomic techniques in diabetes research have yielded many exciting results both in regard to disease pathophysiology as well as effects of anti-diabetic drugs. In the future improved proteomic technology alongside better ways to present data will together with confirmatory follow up studies definitely help to unravel the enigma of diabetes.

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Figure legends

Figure 1

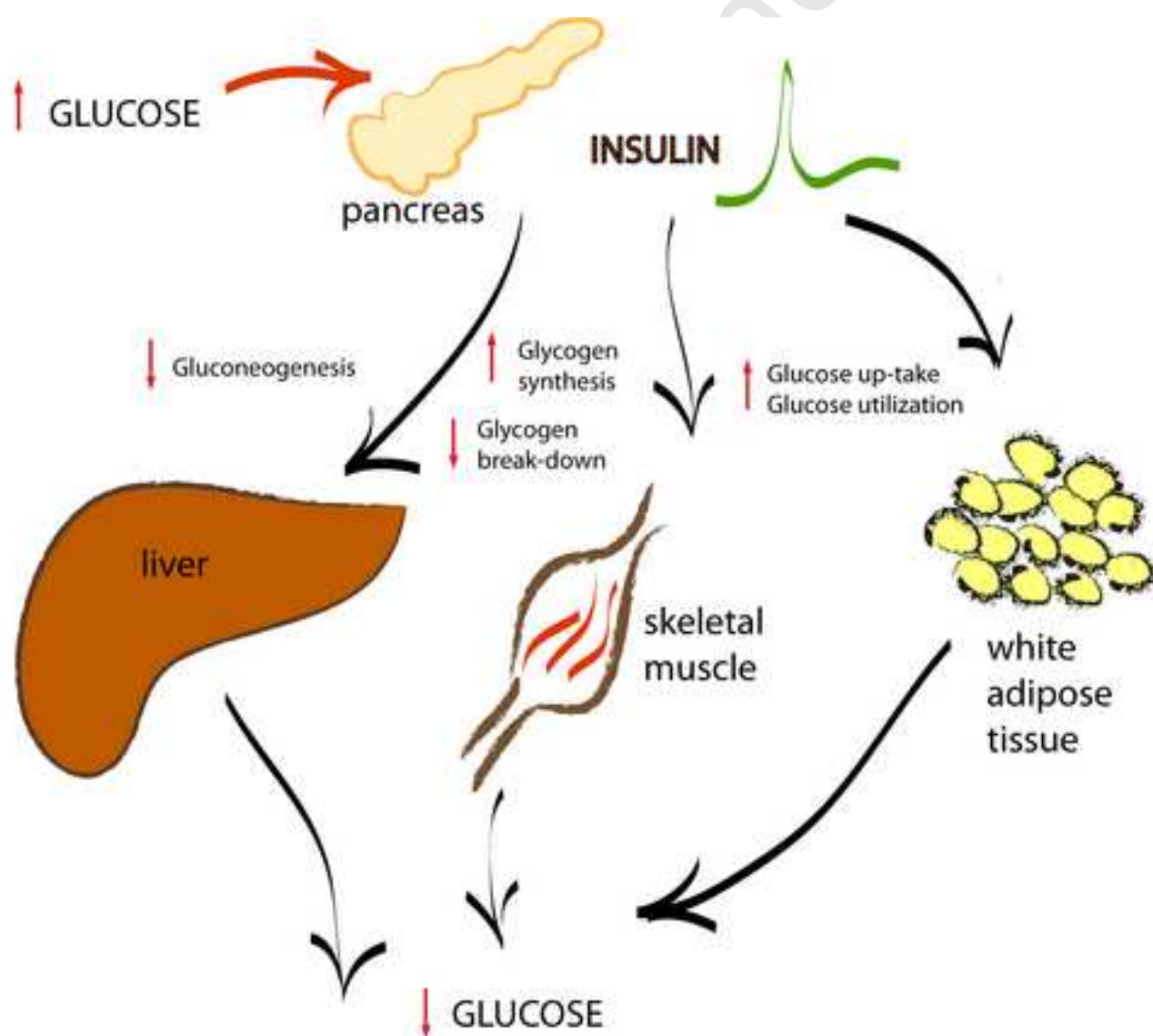
Schematic illustration of the glucose-lowering effects of insulin in different organs. Insulin lowers the blood glucose by targeting liver, skeletal muscle and adipose tissue. The glucose uptake and utilisation in muscle and adipose tissue is increased. In liver and muscle glycogen synthesis is enhanced and glycogen breakdown is suppressed, resulting in net storage of glycogen. Also liver gluconeogenesis is inhibited. All these effects lead to lowering of blood glucose concentration.

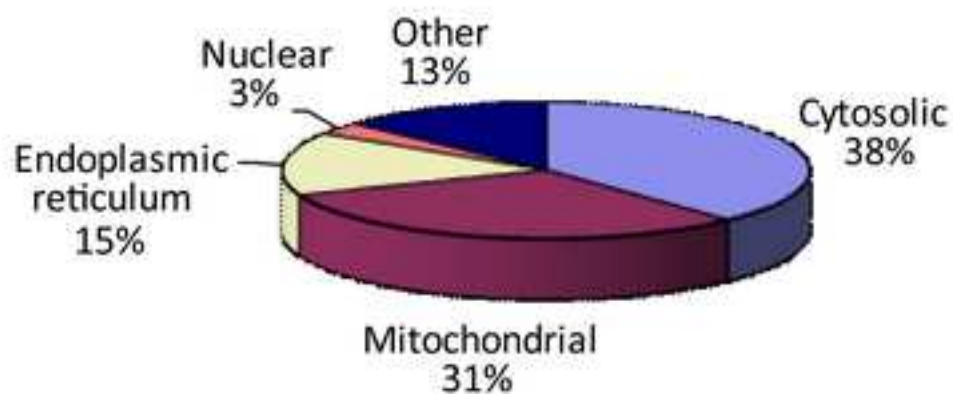
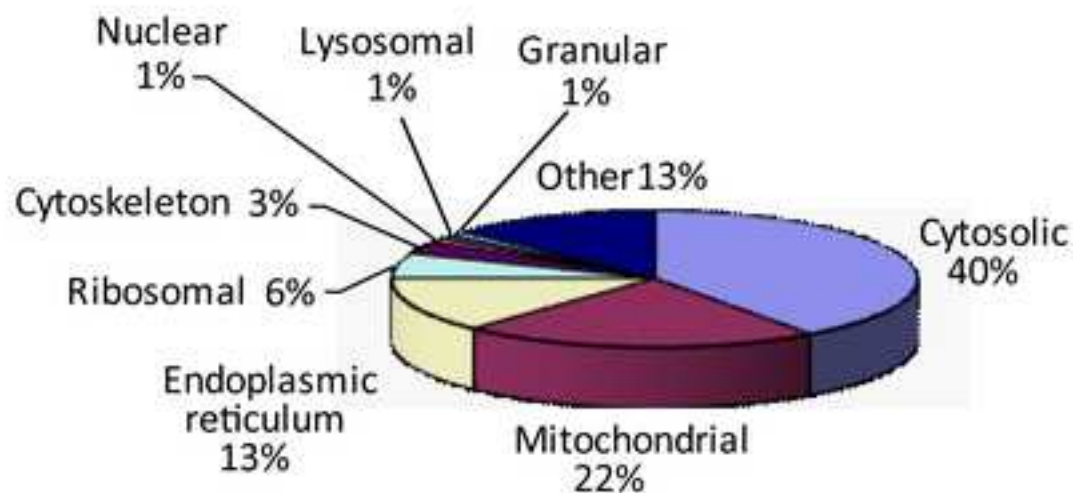
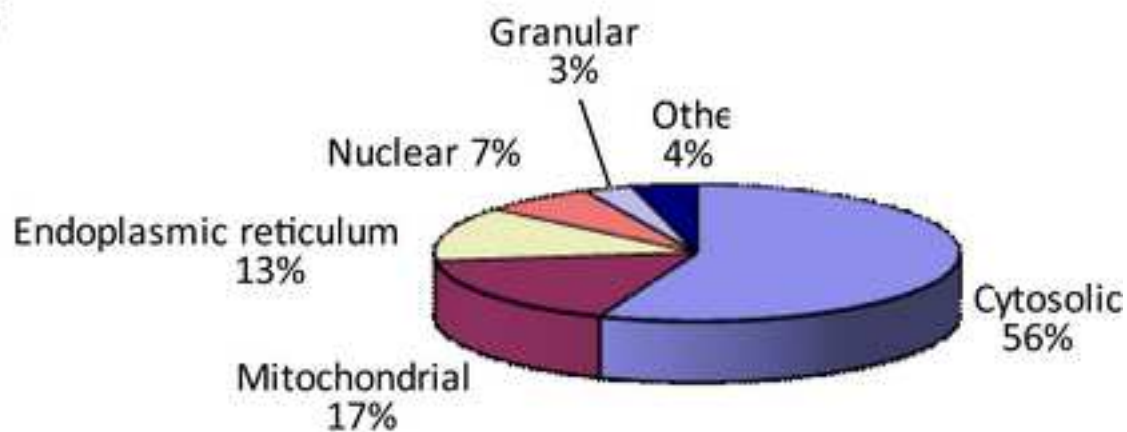
Figure 2

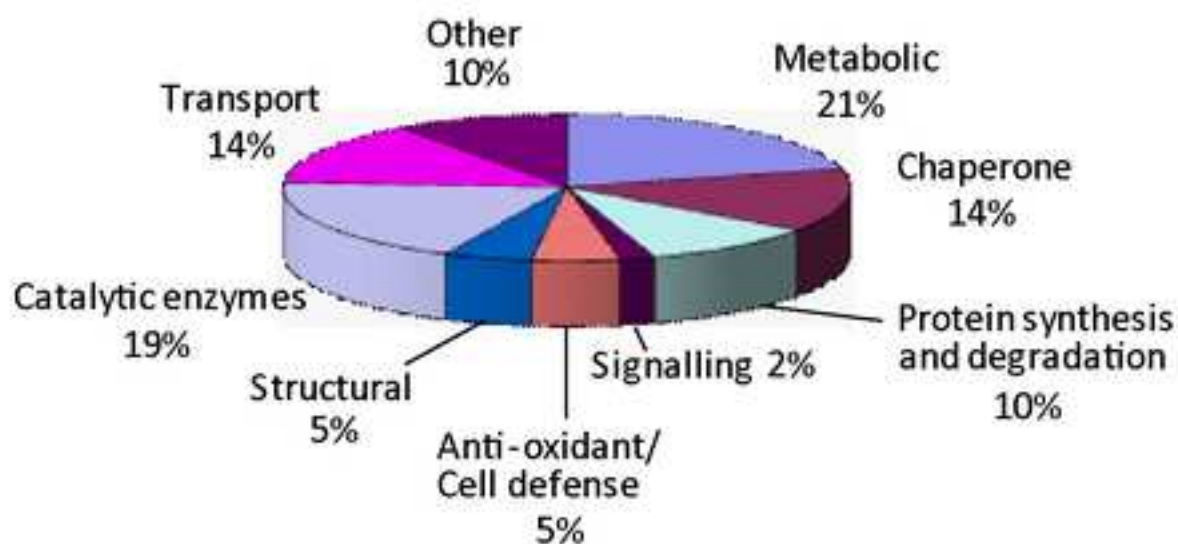
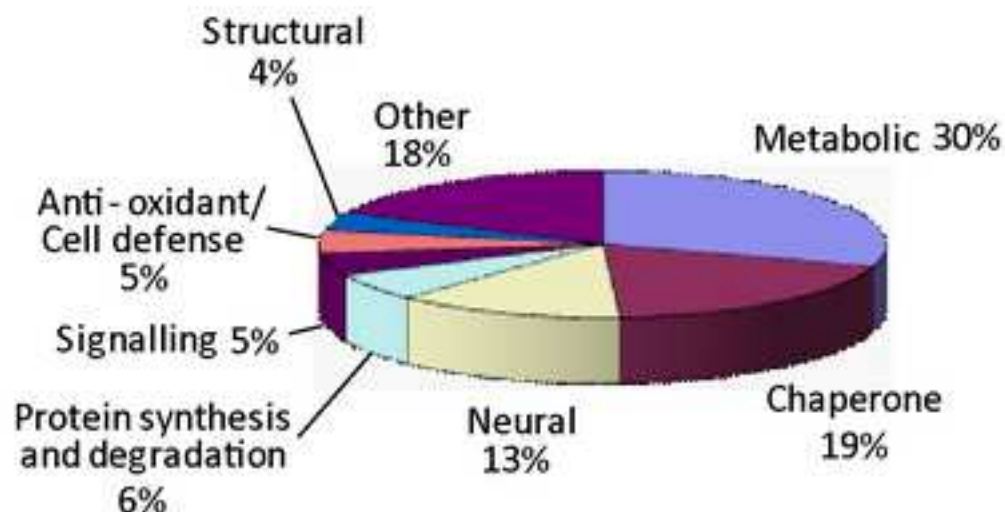
Classification of protein, based on their subcellular localisation, extracted and identified from (A) C57Bl/6J mouse islets (Sanchez et al. 2001), (B) BALB/c mouse islets (Nicolls et al. 2003) and (C) human islets (Ahmed et al. 2005).

Figure 3

Classification of protein, based on their putative function, extracted and identified from (A) C57Bl/6J mouse islets (Sanchez et al. 2001), (B) BALB/c mouse islets (Nicolls et al. 2003) and (C) human islets (Ahmed et al. 2005).



A**B****C**

A**B****C**