Diabetes Mellitus in the Era of Proteomics

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Diabetes mellitus is a disease that has been known to exist for thousands of years and that afflicts ~6% of the population. Because of its prevalence, chronicity, and propensity to cause end-organ damage, diabetes and its complications engenders about 100 billion dollars in health care costs each year in the United States.

Most individuals with diabetes have type 2 diabetes mellitus (T2DM).[1] These patients may have elevated, normal, or slightly decreased insulin levels. They have variable degrees of insulin resistance, in association with an inability of the beta cell to compensate for this resistance, and are not ketosis-prone under basal conditions. The age of onset of this disease is usually after age 40 but it often also occurs at age 20 or 30, or even at a younger age. Most, but not all, individuals with this form of diabetes are obese. Many also have hypertension, hyperlipidemia, heart disease, and peripheral vascular disease, exhibiting the so-called dysmetabolic syndrome. Furthermore, with the aging of our population and as a consequence of sedentary life styles, the incidence of T2DM will continue to increase.

Alarmingly, T2DM is also afflicting younger individuals more frequently than ever before, and this progression into the youthful segment of the population is independent of a specific type of diabetes termed maturity onset diabetes of the young or MODY. Thus, MODY is distinct from T2DM and represents, instead, an interesting group of monogenic forms of diabetes in which specific genetic alterations have been clearly delineated (1).

Another major form of diabetes mellitus is type 1 diabetes mellitus (T1DM). These patients frequently have very low or undetectable insulin levels, are dependent on exogenous insulin for survival, and are ketosis-prone. The onset of the disease usually occurs before age 20, but may occur at any age. This review will focus on the molecular alterations in T2DM and on several findings in cell lines and in animal models of diabetes that are based on proteomics-driven technology.

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[1] The abbreviations used are: T2DM, type 2 diabetes mellitus; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; GLUT4, glucose transporter 4; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; 2-D, two-dimensional.

TYPE 2 DIABETES MELLITUS

T2DM is characterized by abnormal glucose homeostasis leading to hyperglycemia, and its pathogenesis appears to involve complex interactions between genetic and environmental factors. It is believed by many that the primary defect in T2DM is represented by insulin resistance, which is already present at the very early stage of the prediabetic state. While initially the beta cell is able to compensate for this resistance, overt diabetes occurs when the beta cells become exhausted. It should be noted, however, that an alternative hypothesis proposes that the primary defect in T2DM is due to mild dysregulation in insulin secretory mechanisms that leads to overt diabetes following the secondary superimposition of insulin resistance (2). Some cases of gestational diabetes may indeed represent such a sequence of events.

Irrespective of whether the initial primary defect is due to insulin resistance of beta cell dysfunction, it is universally appreciated that T2DM is a complex and heterogeneous polygenic disease whose exact genetic causes continue to remain elusive. This complexity is underscored by the realization that in addition to peripheral tissue resistance to insulin action and a failure of the beta cell to overcome this resistance, T2DM is characterized by a variable inability of the liver to properly suppress hepatic glucose release and by adipose tissue-derived hormones and cytokines that antagonize insulin action (Fig. 1).

In theory, insulin resistance may arise through a variety of mechanisms (Fig. 2). Thus, it may result from defects in the insulin receptor gene, which encodes a transmembrane tyrosine kinase receptor, or as a consequence of post-receptor defects (3, 4). Insulin receptor mutations are rare, and homozygous deletions of the insulin receptor are exceedingly rare, resulting in intra-uterine growth retardation, hypoglycemia, failure to thrive, a peculiar phenotype that has been termed leprechaunism, and death during infancy (5). Insulin receptor activation can also be blocked by anti-insulin receptor antibodies that may arise in certain autoimmune disorders such as systemic lupus erythematosus (6, 7). In addition, certain hormones antagonize insulin action that may precipitate diabetes in susceptible individuals. Thus, patients with excess cortisol production (Cushing disease or syndrome) or patients who take glucocorticoids can develop insulin resistance and hyperglycemia. A similar phenomenon may occur in patients with acromegaly whose elevated growth hormone levels antagonize insulin action.

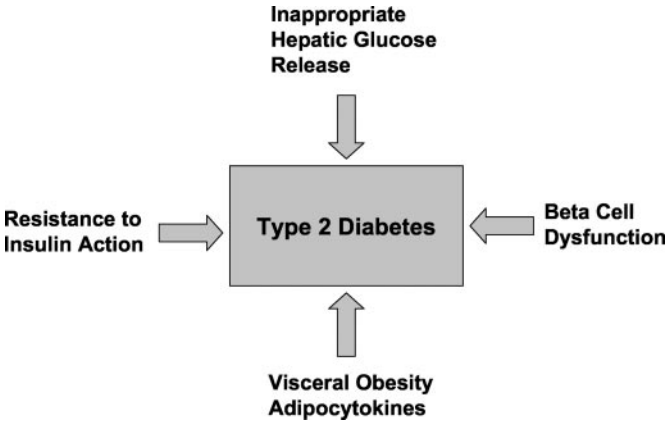

FIG. 1. **Pathophysiology of T2DM.** T2DM is a multi-gene disorder with four major areas of dysfunction. The hallmark of the disease that has been recognized for a long time is peripheral resistance to insulin action. This resistance occurs in a setting of variable levels of beta cell dysfunction, which leads to an inadequate capacity to release insulin in response to the degree of resistance, in conjunction with inappropriate hepatic glucose release in the setting of hyperglycemia. A major contributor to this resistance is visceral obesity and the production of adipocytokines that antagonize insulin action.

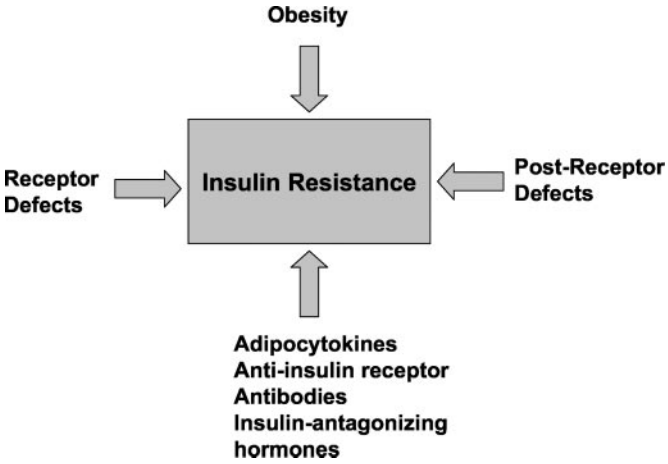

FIG. 2. **Mechanisms of insulin resistance.** There are numerous mechanisms that contribute to insulin resistance, some of which are listed in this figure. These include alterations that are associated with T2DM such as many types of post-receptor defects in the insulin signaling cascades, obesity, and the excessive production of fat cell-derived cytokines (adipocytokines). In addition, alterations that are not generally associated with T2DM include defects at the level of the insulin receptor, anti-insulin receptor antibodies that are distinct from anti-insulin antibodies, and insulin antagonizing hormones such as cortisol.

T2DM AND ALTERED INSULIN SIGNALING

The vast majority of causes of insulin resistance in T2DM are not related to the above alterations but, instead, are due to defects at the post-receptor level. It is important, therefore, to understand the mechanisms whereby the insulin receptor acts to modulate glucose homeostasis. This receptor is a complex composed of two identical alpha and beta half-receptors that are linked together by disulfide bonds (8). The first step in the initiation of insulin action is represented by its binding to the extracellular alpha subunits, leading to the activation of the tyrosine kinase activity of the receptor and to the autophosphorylation of specific tyrosine residues on the mostly intracellular beta subunits (9). This is followed by the phosphorylation of insulin receptor substrate (IRS) signaling proteins, and ultimately by the activation of phosphatidylinositol 3-kinase (PI 3-kinase). Numerous adapter and signaling proteins have been shown to associate with the IRS proteins and to mediate the multiple anabolic effects of insulin (10, 11), including the stimulation of glucose uptake in skeletal muscle and adipose tissues (Fig. 3). This stimulation occurs as a consequence of the cell-surface redistribution of a pool of intracellular glucose transporter 4 (GLUT4) proteins, and this effect is principally mediated by the activation of PI 3-kinase (12). PI 3-kinase activation leads to the generation of phosphatidylinositol 3,4,5-phosphate, which binds to the plekstrin homology domains of phosphoinositide-dependent kinase-1 (PDK1) and AKT (3). Activated PDK1 phosphorylates and activates the serine-threonine kinase AKT, which acts to induce the translocation of cytosolic GLUT4 to the cell membrane, thereby leading to increased glucose uptake (3). In addition, PI 3-kinase can activate atypical protein kinase C (PKC) isoforms (Fig. 3), such as PKC-λ, which also promote GLUT4 translocation to the membrane (13). The complexity of insulin action is further underscored by the observation that GLUT4 translocation can also occur through PI 3-kinase-independent mechanisms (14).

Defects in IRS-1 and/or PI 3-kinase functions may contribute to T2DM. For example, several silent polymorphisms and naturally occurring amino acid substitutions in IRS-1 have been identified (15). Although two IRS-1 alterations, a G922R variant and a T608R mutation, have been directly related to defective insulin action (16, 17), it is not clear that expression of these altered IRS-1 proteins is clinically relevant. Thus, some studies have reported that the G922R variant is more common in T2DM patients (18), others have reported that it is not (19), and still others have suggested that this variant may be more prevalent in patients with T2DM and severe insulin resistance (20). The potential role of other IRS proteins in T2DM and of PI 3-kinase polymorphisms is less clearly defined. Nonetheless, IRS-1 knockout mice exhibit insulin resistance and glucose intolerance (21), whereas IRS-2 knockout mice develop overt diabetes due to a marked decrease in insulin-stimulated glucose transport in conjunction with decreased beta cell mass (22). Moreover, the homology between the ataxia-telangiectasia (AT) gene product and PI 3-kinase, and the relatively high prevalence of T2DM in AT, suggest that perturbations in PI 3-kinase function may have a contributing role in some T2DM patients (23).

T2DM AND ALTERED NUCLEAR RECEPTOR SIGNALING

Nuclear receptors participate in the regulation of many cellular processes. One group of nuclear receptors that has an important role in modulating energy metabolism is repre-

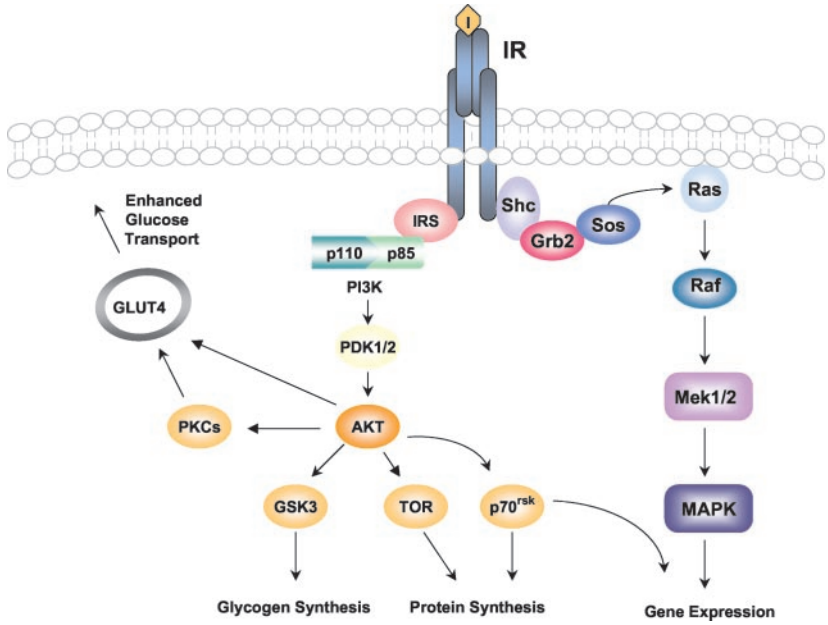

FIG. 3. **Schematic representation of insulin signaling.** Insulin (*I*) binding to the extracellular alpha subunits of the insulin receptor (*IR*) leads to the activation of the tyrosine kinase activity of the receptor and to the autophosphorylation of specific tyrosine residues on the beta subunits. This is followed by the phosphorylation of IRS signaling proteins, and other substrates such as Shc. The Shc-Grb2-SOS-Ras-Raf-mitogen activated protein kinase (MAPK) kinase (MEK1/2)-MAPK cascade has been well characterized in many signaling pathways. IRS recruits and activates PI 3-kinase (*PI3K*), which activates phosphoinositide-dependent kinase-1 (*PDK1*), PDK-2, and AKT. AKT, as well as atypical PKC isoforms (*PKCs*) induce the translocation of GLUT4 to the cell membrane, thereby leading to increased glucose uptake. AKT can also enhance protein synthesis by activating target of rapamycin (*TOR*) and $p70^{rsk}$, and glycogen synthesis by activating glycogen synthase kinase-3 (*GSK3*).

sented by peroxisome proliferator-activated receptors (PPARs). The existence of this group of receptors was surmised following the observation in the early 1960s that Clofibrate, an agent used to treat hypertriglyceridemia, induces the proliferation of peroxisomes in rodents (24). The receptor that mediates this effect was called PPARalpha or PPARα (25). Homologous receptors include PPARβ and PPARγ (25).

The PPARγ receptor, and specifically the PPARγ2 isoform, appears to have an especially important role in diabetes because it modulates adipocyte differentiation and energy storage by adipocytes (26). Rare mutations of this receptor (P115Q) are associated with obesity, as a consequence of constitutive gain of function of the mutated receptor (27). However, the consequences of loss of function mutations (P12A) in the PPARγ2 isoform have been controversial and have been associated with both decreased adiposity and enhanced insulin sensitivity (28), as well as with obesity and insulin resistance (29). Irrespective of these observations, the importance of PPARγ in the regulation of glucose homeostasis is underscored by the usefulness of thiazolidinediones, a group of drugs that bind with varying affinities to PPARγ, in the control of blood glucose levels in diabetic patients.

T2DM AND OBESITY

Approximately 80% of patients with T2DM are obese, and obesity is associated with insulin resistance. Adipose tissues induce insulin resistance through a variety of mechanisms. Thus, adipocytes are known to release free fatty acids that can directly interfere with efficient insulin signaling, partly as a result of their ability to stimulate PKC isoforms (30). Free fatty acids also have the capacity to enhance inappropriate hepatic glucose release (31). A major culprit with respect to readily available free fatty acid stores is represented by visceral obesity, because lipolysis at this site is very efficient and is more resistant to the suppressive effects of insulin than at other adipose sites (32).

Adipose tissues also synthesize and release several adipocytokines. The most important among these is tumor necrosis factor alpha, which acts to induce a state of insulin resistance by down-regulating GLUT4 and increasing free fatty acid release (33, 34). The potential roles of resistin and leptin in insulin resistance in humans remain to be firmly delineated. By contrast, adiponectin is a recently discovered adipocytokine that enhances insulin sensitivity (35), and its levels in the circulation are decreased in T2DM and in obesity, implying a causal relationship. In support of this hypothesis, adiponectin-deficient mice exhibit insulin resistance and diabetes (36), whereas the administration of adiponectin causes glucose-lowering effects and ameliorates insulin resistance in mice (37). Two adiponectin receptors (AdipoR1 and AdipoR2) were recently cloned, providing further evidence for an important role for adiponectin in the maintenance of glucose homeostasis (38). Similarly, the anti-inflammatory cytokine interleukin-10 may exert anti-diabetogenic effects, inasmuch as re-

duced levels of this interleukin have been correlated with an increased propensity for the development of the dysmetabolic syndrome in women (39).

Obesity *per se*, which is a polygene disorder that involves extensive gene-environment interactions, can also lead to T2DM. It has been known for a long time that a low resting metabolic rate is a risk factor for obesity (40). Because the β3-adrenergic receptor is localized in adipose tissues, including visceral fat, and because it has been implicated in the regulation of thermogenesis and lipolysis (41), its potential role in obesity and T2DM was investigated. Initial studies in Pima Indians indicated that the W64R mutation in the β3-adrenergic receptor was associated with and earlier onset of T2DM and a lower resting metabolic rate (42). Subsequent studies have demonstrated that this mutation is also associated with deficient insulin secretory capacity from the beta cell (43), underscoring the multifaceted aspects of the defects that lead to T2DM.

PROTEOMICS AND T2DM

Numerous studies of specific defects in insulin action, insulin secretion, hepatic glucose release, or adipocyte function have shed considerable light on the pathophysiology of T2DM. In recent years, in addition to focusing on specific candidate genes, the powerful tools of positional cloning and genomic analysis have been used to delineate novel pathways that contribute to T2DM (44). For example, a positional cloning strategy was used to identify a single nucleotide polymorphism in the *calpain 10* gene in Mexican-Americans with T2DM (45). This gene is located on chromosome 2q37 (45). A second locus, localized to chromosome 15, was subsequently shown to increase the susceptibility to T2DM of the calpain 10 single nucleotide polymorphism (46). *Calpain 10* gene alterations may also be associated with decreased β3-adrenergic receptor function (47), further underscoring the complexity of gene interactions in the pathogenesis of T2DM. In this context, it should be possible to take advantage of the tremendous power of proteomics to analyze protein expression profiles in tissues and serum from normal individuals and patients with T2DM in order to identify proteins that contribute to insulin resistance, adipocytokine production, beta cell dysfunction, inappropriate hepatic glucose release, and susceptibility to end organ damage.

To date, most studies of diabetes using a proteomics approach have been carried out in cells lines and in animal models of diabetes (48, 49), as discussed in the next section. It should be possible, however, to carry out protein profiling in a variety of tissues and in serum from patients with T2DM (50). For example, a very small percentage of patients with T2DM exhibit a maternal pattern of inheritance in conjunction with inherited deafness, as a consequence of mitochondrial gene defects (51). Because it is possible to perform high-throughput profiling of the mitochondrial proteome (52), it will be important to perform this type of analysis in patients with T2DM and mitochondrial alterations.

A recent analysis of the proteome of human skeletal muscle in normal controls and in patients with T2DM is illustrative of the power of proteomics in pointing toward new directions (53). In this study, eight potential markers of T2DM were identified in the skeletal muscle. Most notably, the levels of two proteins that have a crucial role in ATP synthesis were found to be decreased in the muscles derived from diabetic patients. However, as the authors indicate, it remains to be determined whether this down-regulation of creatine kinase B and ATP synthase β-subunit represents a primary defect in T2DM or is the consequence of diabetes-induced alterations.

LESSONS FROM CELL LINES AND ANIMAL MODELS OF DIABETES

A number of proteomics-based studies have been carried out in order to elucidate the mechanisms of action of insulin and the pathophysiology of insulin resistance. The mouse 3T3-L1 fibroblastic cell line has been widely investigated in this regard, inasmuch as it differentiates rapidly into an adipocyte phenotype when treated with insulin. For example, two-dimensional (2-D) gel electrophoresis of ^{32}P-labeled proteins from 3T3-L1 cells, before and after immunoprecipitation with an anti-calmodulin antibody, revealed that insulin does not stimulate calmodulin phosphorylation under conditions in which it stimulates the phosphorylation of other proteins (54). Moreover, giant 2-D gel electrophoresis delineated a series of cellular proteins involved in the differentiation process in these cells (55). However, none of these proteins was specifically identified. The same methodology was used to demonstrate that insulin stimulated the phosphorylation of multiple proteins in NIH 3T3 cells expressing high numbers of human insulin receptors, implicating these proteins as substrates for the kinase activity of the receptor (56). As in the previous studies, however, none of these proteins was identified.

Studies with animal models of diabetes have also been instructive. Thus, separation of adipocyte proteins from 7-wk-old lean and obese Zucker rats by 2-D gel electrophoresis followed by laser densitometry revealed an obesity-related decrease in the concentration of a 28-kDa cytosolic protein in two lipogenic tissues, liver and white fat, but not in soleus muscle (57). This protein was identified by microsequencing as carbonic anhydrase III (CA III), and its activity was also decreased in obesity (57). Inasmuch as the decrease in CA III activity was reversed following suppression of the hyperinsulinemia by the beta cell toxin streptozotocin, the authors suggested that CA III levels are decreased by hyperinsulinemia. The same laboratory also used protein microsequencing to detect increased adipocyte (but not hepatic) expression of pyruvate carboxylase during progression to obesity (58). They suggested that it was related to the lipogenic capacity of obese Zucker rat adipocytes and demonstrated that the increased levels of pyruvate carboxylase were attenuated in streptozotocin-treated rats, thus implicating the high insulin levels in the induction of the high levels of this enzyme.

Other studies have taken advantage of the availability of

PPAR agonists to assess protein expression profiles in specific tissues in various animal models of obesity or diabetes. For example, the PPARα agonist WY14,643, which tends to normalize both the elevated plasma triglycerides levels and the hyperglycemia in ob/ob mice, was shown by high-resolution 2-D electrophoresis to up-regulate 14 hepatic proteins that were identified by mass spectrometry to be components of the peroxisomal fatty acid metabolism pathway (59). Notably, acyl CoA oxidase, peroxisome bifunctional enzyme, and 3-ketoacyl thiolase were up-regulated by WY14,643 (57). Together, these findings indicate that WY14,643 induces peroxisomal fatty acid beta-oxidation in the liver of these mice. Rosiglitazone, a PPARγ selective agonist, induced similar changes in obese mice, but, in contrast to WY14,643, was without effect in lean mice (60).

The effects of Rosiglitazone were also studied in obese C57BL/6J lep/lep mice by comparison with lean littermates (61). As in other studies, this PPARγ agonist normalized the impaired glucose tolerance in lep/lep mice but had no significant effect on glucose tolerance in the lean mice. High-resolution 2-D electrophoresis was then used to analyze pancreatic islet-derived polypeptides, followed by mass spectrometric analysis of the differentially expressed proteins. Alterations in actin-binding proteins were noted in islet proteins from the lep/lep mice, which may contribute to defective islet cell function (61). Furthermore, Rosiglitazone increased carboxypeptidase B expression in both lep/lep and normal mice, raising the possibility that it may also ameliorate glucose homeostasis by improving insulin processing.

CONCLUSION

Irrespective of whether a patient has T2DM or T1DM, the principal goal of diabetes treatment is normalization of blood glucose levels and prevention of complications. The application of advanced proteomics to the field of diabetes should yield important new information on disease pathophysiology that could be useful for the prevention and treatment of diabetes, as well as provide new approaches for the prevention of the microvascular and macrovascular complications that are associated with this disease.

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