Metabolism and insulin signaling in common metabolic disorders and inherited insulin resistance

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THE THESIS IS BASED ON THE FOLLOWING ORIGINAL PAPERS:

I. Højlund K, Hansen T, Lajer M, Henriksen JE, Levin K, Lindholm J, Pedersen O, Beck-Nielsen H. A novel syndrome of autosomal-dominant hyperinsulinemic hypoglycemia linked to a mutation in the human insulin receptor gene. Diabetes 2004; 53: 1592-8.

II. Højlund K, Frystyk J, Levin K, Flyvbjerg A, Wojtaszewski JFP, Beck-Nielsen H. Reduced plasma adiponectin concentrations may contribute to impaired insulin activation of glycogen synthase in skeletal muscle of patients with type 2 diabetes. Diabetologia 2006; 49: 1283-91.

III. Højlund K, Wojtazsewski JFP, Birk J, Hansen BF, Vestergaard H, Beck-Nielsen H. Partial rescue of in vivo insulin signalling in skeletal muscle by impaired insulin clearance in heterozygote carriers of a mutation in the insulin receptor gene. Diabetologia 2006; 49: 1827-37.

IV. Højlund K, Glintborg D, Andersen NR, Birk JB, Treebak JT, Frøsig C, Beck-Nielsen H, Wojtaszewski JP. Impaired insulin-stimulated phosphorylation of Akt and AS160 in skeletal muscle of women with polycystic ovary syndrome is reversed by pioglitazone treatment. Diabetes 2008; 57: 357-66.

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INTRODUCTION

Understanding the physiological and cellular mechanisms responsible for common forms of insulin resistance in human individuals with type 2 diabetes and related metabolic disorders such as obesity and polycystic ovary syndrome (PCOS) is crucial to prevent the increased morbidity and mortality from cardiovascular disease associated with these disorders (1,2).

Insulin is a potent anabolic hormone, which exerts a variety of effects on many types of cells. The main metabolic actions of insulin are to stimulate glucose uptake in skeletal muscle and fat, promote glycogen synthesis in skeletal muscle, suppress hepatic glucose production, and inhibit lipolysis in adipocytes (3). A decreased sensitivity to these actions of insulin is often referred to as insulin resistance. More broadly, insulin resistance can be defined as an impaired biological response to either endogenous or exogenous insulin. In human individuals, insulin resistance is traditionally demonstrated by an impaired insulin action on whole-body glucose metabolism. Several methods for the in vivo assessment of insulin sensitivity have been developed (4). Of these, the euglycemic-hyperinsulinemic clamp test is generally accepted as the 'gold standard', whereas other less precise measures such as the insulin suppression test, the homeostasis model of glucose tolerance (HOMA), minimal model analysis of the frequently sampled intravenous glucose tolerance test (FSIVGTT), indices of insulin sensitivity derived from oral glucose tolerance test (OGTT) or simply fasting insulin levels are better suited for large cross-sectional or prospective studies (4).

Insulin resistance is not unique to metabolic disorders such as type 2 diabetes, obesity and PCOS. Thus, the application of in vivo methods for assessment of insulin sensitivity has demonstrated the presence of insulin resistance in a number of conditions. This ranges from mild to moderate insulin resistance in 1) normal and abnormal physiologic states such as prolonged fasting, pregnancy, sepsis, uremia and liver cirrhosis, 2) endocrinopathies due to states of growth hormone, glucocorticoid or catecholamine excess, 3) monogenic syndromes of obesity (5,6), and extreme

insulin resistance in genetic and acquired forms of lipodystrophies and insulin receptorpathies (5), including rare mutations in genes of post-receptor molecules, e.g. Akt2 (7). At the cellular level, human insulin resistance has been extensively studied in vivo in skeletal muscle biopsies (8), and to a lesser extent in biopsies from adipose tissue, liver and other tissues. Moreover, the possibility to establish cell lines, e.g. myotubes (8), from human tissue samples has enabled studies of the molecular mechanism of insulin resistance and potentially distinguish between genetic and environmental etiologies of insulin resistance.

Although skeletal muscle, liver and adipose tissue are believed to be the main target tissues of insulin action, there is increasing evidence that insulin has important physiological and biological functions in other tissues and cell types such as the brain, pancreatic β-cells, heart and endothelial cells (9,10). In addition, a number of studies support the concept of partial insulin resistance, in which only some of insulins pleiotropic effects are affected in common metabolic disorders. (11-15). Thus, insulin resistance in skeletal muscle and endothelial cells affects only the metabolic actions of insulin mediated through the phosphatidylinositol 3-kinase (PI3K)/Akt arm of the insulin signaling pathway, whereas Ras/mitogen-activated protein kinase dependent (MAPK) insulin signaling to mitogenesis, growth and differentiation remains intact (12,13). There is evidence that this partial insulin resistance may be more deleterious than generalized insulin resistance such as caused by mutations in the insulin receptor gene (INSR). In endothelial cells, the compensatory hyperinsulinemia may enhance pro-hypertensive and atherogenic actions of insulin (10). Similarly, partial insulin resistance in liver cells causes impaired suppression of gluconeogenesis while lipogenesis continues to be activated by hyperinsulinemia (14,15). In type 2 diabetes, this may be responsible for the deadly combination of hyperglycemia and hypertriglyceridemia.

There is growing evidence that an increased release of proinflammatory cytokines and reduced secretion of anti-inflammatory factors, in particular adiponectin, from adipose tissue are associated with the development of insulin resistance, and increases the risk of premature atherosclerosis, type 2 diabetes and cardiovascular disease (16). However, whether this state of chronic low-grade inflammation is a cause or consequence of insulin resistance in metabolic disorders like type 2 diabetes and PCOS, or solely related to the degree of obesity, e.g. visceral fat, remains to be determined. Although, the adipokine-mediated inflammatory cross-talk of adipose tissue with skeletal muscle and other peripheral tissues is interesting, a detailed description of this field is beyond the scope of this review; except for the role that circulating adiponectin may play for the metabolic and cellular actions of insulin in skeletal muscle.

The present review will focus on studies of insulin action in humans. In a series of studies, we have characterized the mechanisms of insulin resistance in individuals with type 2 diabetes, obesity and PCOS, and in individuals with inherited insulin resistance due to a mutation in the insulin receptor gene (*INSR*) (17-40). Here, differences and similarities in insulin action on glucose and lipid metabolism and molecular mechanisms underlying insulin resistance in skeletal muscle in these conditions will be pointed out. Emphasis is placed on describing defects in insulin signaling to glucose transport and glycogen synthesis (17-26,35, 40), and the potential role of adiponectin on AMP activated kinase (AMPK) and insulin action (25-30,37) in skeletal muscle of individuals with common metabolic disorders and an inherited form of insulin resistance.

COMMON METABOLIC DISORDERS AND INHERITED INSULIN RESISTANCE

Type 2 diabetes, obesity and PCOS are common complex disorders associated with insulin resistance and compensatory fasting hyperinsulinemia. Familial clustering, twin studies and increasing prevalences observed under the influence of life-style factors such as increased calorie intake and diminished physical activity, have shown that both inherited and environmental factors contribute to the pathogenesis of these disorders (41-45). Type 2 diabetes, obesity and PCOS are all considered to be a part of the metabolic syndrome, which is associated with an increased risk of e.g. hypertension, dyslipidemia and cardiovascular disease. Obesity and PCOS also show an increased risk of impaired glucose tolerance and type 2 diabetes, which further increases the risk of cardiovascular diseases and causes microvascular complications of diabetes. Together, these common metabolic disorders place a substantial economic burden on health-care systems worldwide. Below, a brief phenotypic and genotypic description of these common metabolic disorders is given together with a more detailed description of a novel syndrome characterized by postprandial hypoglycemia and severe insulin resistance. This syndrome, which is caused by a heterozygous mutation in the insulin receptor gene (*INSR*), provides an example of a monogenic cause of insulin resistance, although with different penetrance.

TYPE 2 DIABETES

Type 2 diabetes is the most common chronic metabolic disease in developed countries (46). The International Diabetes Federation (IDF) has estimated that in 2012 more than 371 million people worldwide have diabetes, with more than 90% suffering from type 2 diabetes. Previously, type 2 diabetes was thought of as a disorder of the elderly, but age at onset is rapidly decreasing under the influence of modern western lifestyle implicating a major role for environmental factors. A strong genetic component is evidenced by a higher concordance rate of type 2 diabetes in monozygotic twins compared with dizygotic twins (47-48). Moreover, first-degree relatives have a lifetime risk of developing type 2 diabetes of ~40% if one parent has type 2 diabetes (49), and significantly higher if both parents have type 2 diabetes (50-51). Type 2 diabetes is characterized by insulin resistance in major metabolic tissues such as skeletal muscle, liver and adipose tissue (52-54). The majority of patients with type 2 diabetes are obese, but the majority of obese individuals do not become diabetic even in the face of a high degree of insulin resistance. Thus, in addition to insulin resistance, failure of the pancreatic β-cells to compensate for this abnormality is required to cause hyperglycemia and overt type 2 diabetes (54). The degree of insulin resistance in type 2 diabetes is increased compared with weight-matched controls. It remains elusive whether this additional insulin resistance is inherited or acquired secondary to changes in the metabolic milieu associated with the progression to type 2 diabetes, e.g. (postprandial) hyperglycemia, hyperinsulinemia, increased circulating lipids, and proinflammatory molecules. Prospective studies of glucose tolerant, first-degree relatives of parents with type 2 diabetes (FDR) have demonstrated that insulin resistance is a significant predictor of type 2 diabetes (51,55, 56), but cross-sectional studies also support the hypothesis that both insulin resistance and β-cell dysfunction are early defects (57-63). Thus, at present it remains unclear whether one abnormality precedes the other(s) or whether a common causative mechanism for these abnormalities exists. Accelerated atherosclerosis and cardiovascular disease are major causes of morbidity

and mortality in type 2 diabetes. There is substantial experimental and epidemiological evidence that both hyperglycemia and insulin resistance/hyperinsulinemia either directly or indirectly promote atherosclerosis (2,64). However, recent clinical trials suggest that improvements of glycemia achieved without a concomitant improvement of insulin sensitivity may not be sufficient to cause a statistically significant delay in the progression of macrovascular complications in patients with type 2 diabetes (65-68). This emphasizes hat insulin resistance remains an important target to treat to avoid type 2 diabetes and prevent cardiovascular disease.

Dissecting the genetics of type 2 diabetes was until recently a slow and challenging task with limited success (41). Using traditional methods such as linkage analysis and the candidate gene approach, only a few variants have consistently been shown to confer an increased risk of type 2 diabetes (41). However, the introduction of Genome Wide Association (GWA) studies has within the past 5 years caused a breakthrough in the genetics of type 2 diabetes (42). To date, the results from several GWA studies and meta-analyses have shown ~65 common genetic variants, typically with a minor allele frequency (MAF) above 5%, to be robustly associated with type 2 diabetes (41,42,69,70). However, even in combination these risk variants explain no more than 5-10% of overall trait variance corresponding to 10-20% of overall heritability in type 2 diabetes (42). Most of these risk alleles are located in genes believed to influence β-cell function, while only a few variants seem to be associated with insulin resistance, e.g. a variant upstream of insulin receptor substrate-1 (IRS1) (71). This emphasizes the role of preserved β-cell function to compensate for insulin resistance in the pathogenesis of hyperglycemia and overt type 2 diabetes. Based on the fact that most of the genetic variance is unexplained, it has been proposed that much of the unexplained familial clustering could be attributable to several less common (MAF<5%) genetic variants or the accumulated effect of many hundreds of genetic variants weakly associated with type 2 diabetes (42). In both cases, this may involve variants in genes related to insulin resistance or associated phenotypic traits. However, there is also several lines of evidence suggesting a role for epigenetic mechanisms, and that the predisposition to develop type 2 diabetes reflects a complex interplay between genetics, epigenetics and environment (41,42).

OBESITY

According to the WHO more than 500 million people are obese. Obesity is a major risk factor for not only hypertension, dyslipidemia, type 2 diabetes, and cardiovascular disease (1), but also for several other disorders including sleep apnea, non-alcoholic fatty liver disease (NAFLD), and certain forms of cancer (72,73). This largely preventable condition is therefore considered one of the most serious healthcare problems (74). Obesity, particularly central obesity, is closely associated with peripheral insulin resistance. In contrast to type 2 diabetes, the hepatic glucose production and β-cell function are preserved to maintain normoglycemia in glucose tolerant, obese individuals (75,76). Insulin resistance is, however, not a simple function of overweight or obesity (77). There is a large variation of insulin resistance within obesity, and those with the highest degree of insulin resistance have the highest risk of cardiovascular and type 2 diabetes (78).

It is obvious that lifestyle factors such as excessive energy intake and a low level of physical activity play a significant role for the increasing prevalence of obesity. However, family and twin studies have shown that there is also a substantial heritability of obesity (44,45,79). Until recently, genetic variants influencing body mass index (BMI) were restricted to rare mutations in genes that cause monogenic or syndromic obesity (6,41,80). However, recent meta-analyses of GWA studies have led to the identification of more than 30 genetic variants for obesity and BMI explaining, however, only ~2% of trait variance for BMI (42,81-83). This includes several genetic variants known to influence hypothalamic function in the brain (81-83). This emphasizes the potential importance of genes that regulate food intake in the development of obesity. It does, however, not exclude that other genetic variants related insulin resistance in obese individuals are those that increases the risk of morbidity and mortality in these individuals. Moreover, as in type 2 diabetes, the familial clustering of obesity likely reflects a complex interplay between genetics, epigenetics and environment (41,42).

POLYCYSTIC OVARY SYNDROME

PCOS is a common endocrine disorder causing infertility in up to 10% of women of reproductive age (43). Although the diagnosis is based exclusively upon its reproductive manifestations such as hyperandrogenemia, oligo- or an-ovulation, and/or polycystic ovaries in the absence of related disorders, as defined by different diagnostic criteria (84), PCOS is also a metabolic disorder characterized by often profound peripheral insulin resistance (85,86).

The prevalence rates of impaired glucose tolerance (IGT) (20-35%) and type 2 diabetes (7.5-10%) among women with PCOS are markedly higher than in women of similar age (84,87,88). Moreover, obesity is a common feature of PCOS with a prevalence of ~80 % in the United States, and ~50% outside the United States (84,87,88). There is also convincing epidemiologic evidence that the prevalence of risk factors for atherosclerosis and cardiovascular disease is increased in PCOS (89). However, it remains to be established in prospective studies whether there is an increased incidence of cardiovascular disease and mortality in women with PCOS (89). In addition to peripheral insulin resistance, PCOS is associated with β-cell dysfunction, which explains the high rates of IGT and type 2 diabetes (90,91). Whether hepatic insulin resistance is a feature of PCOS is at present unclear. Thus, basal hepatic glucose production and the ED50 for insulin suppression of hepatic glucose production were increased only in obese women with PCOS (86,92). However, in a study using appropriate tracer technology, no defects in hepatic glucose production could be demonstrated in obese women with PCOS (85).

A significant component of insulin resistance in PCOS is independent of body weight (84,86), and there is strong evidence for a link between androgen excess and insulin resistance in the pathogenesis of PCOS (43,84). The compensatory fasting hyperinsulinemia associated with insulin resistance contributes not only to the metabolic abnormalities in PCOS but also to high androgen levels by stimulating the ovarian androgen production, and by inhibiting hepatic production of sex hormone binding globulin (SHBG) (84,93,94).

A role for genetic factors in PCOS is strongly implied by twin studies and familial clustering of the syndrome and related reproductive and metabolic abnormalities, the latter even in brothers of women with PCOS (43,84,95). Indeed, a large number of candidate genes involved in ovarian and adrenal steroidogenesis and insulin resistance and secretion have been positively linked to PCOS (84,95). However, lack of replication of these positive results together with lack of universally accepted diagnostic criteria, and the use of small study populations have made genetic studies of PCOS troublesome (84,95). While GWA studies have been

extensively used to identify genetic risk variants for type 2 diabetes and obesity, so far only two GWA studies localizing susceptibility genes for PCOS have been published (96,97). In these studies of women in large Han Chinese cohorts, genome-wide significance for association with PCOS was provided for a total of eleven novel risk loci, which contained susceptibility genes related to insulin signaling, sexual hormone function, and type 2 diabetes. While recent studies in European PCOS cohorts have replicated some of these associations, a larger GWA study in PCOS cohorts of European and of Korean ancestry are currently under way, and will likely further increase the number of genetic variants associated with PCOS (84).

INSULIN RECEPTOR MUTATIONS

Homozygous or compound heterozygous mutations in the human insulin receptor gene (*INSR*) are known to cause rare syndromic forms of extreme insulin resistance such as Leprechaunism and the Rabson-Mendenhall syndrome (98). These syndromes are characterized by growth retardation, several dysmorphic features, massive hyperinsulinemia, acanthosis nigricans, and, initially, paradoxical fasting hypoglycemia and postprandial hyperglycemia, followed by constant hyperglycemia, frank diabetes with poor glycemic control and often early death. A milder form, Type A insulin resistance, which can be caused by a loss-of-function mutation in either one or both alleles of *INSR*, shows essential normal growth and body composition (non-obese), but severe insulin resistance, acanthosis nigricans, and, in female patients, hyperandrogenism and polycystic ovaries (98). One of the lessons learned from these rare genetic syndromes is that extreme insulin resistance and hyperinsulinism per se is sufficient to cause full-blown PCOS in affected post-pubertal females. Mutations in *INSR* have almost exclusively been discovered by sequencing the *INSR* gene in patients with the above typical phenotypic characteristics of extreme insulin resistance (98-99). However, many patients diagnosed with Type A insulin resistance do not have *INSR* mutations (98-101) suggesting the possibility of selection bias with respect to the phenotypic characterization of individuals with heterozygote mutations in *INSR*. Moreover, biochemical analysis of mutant *INSR* does not reliably predict whether the phenotype will be Leprechaunism, the Rabson-Mendenhall syndrome or Type A insulin resistance (98,102). Mutations in the insulin receptor tyrosine kinase (IRTK) domain of *INSR* are characterized by decreased IRTK activity despite normal binding and affinity of insulin to the receptor in vitro (98). These mutations appear to cause insulin resistance in a dominant fashion, unlike mutations in other domains of the *INSR*. The dominant negative effect may result from the heterotetrameric ($\alpha_2\beta_2$) structure of the insulin receptor. Thus, in patients heterozygous for a single mutant allele, only the ~25% of insulin receptors assumed to be formed by the two wild-type (wt) alleles are expected to show normal IRTK activity (98,99). Normal IRTK activity is required for normal endocytosis of the insulin-insulin receptor complex (98). Therefore, mutations in the IRTK domain are usually associated with increased plasma levels of insulin due to impaired clearance of insulin.

It has been estimated that the prevalence of heterozygous carriers of *INSR* mutations is at least ~1:1000. It is likely that an increased frequency (up to 1%) is seen in patients with type 2 diabetes being heterozygous carriers (98). The long-term consequences of heterozygous mutations in *INSR* are unknown, but a single 30-year prospective follow-up of 11 patients with either Type A insulin resistance or the Rabson-Mendenhall syndrome, showed a very high morbidity and mortality (103). Nine patients had diabetes at presentation, and more than half suffered or died from severe microvascular diabetic complications before reaching 45 years of age. Interestingly, patients with *INSR* mutations have strikingly normal lipid profiles despite extreme insulin resistance (21,103), and there are no reports of macrovascular diseases.

A NOVEL SYNDROME OF HYPOGLYCEMIA WITH INSULIN RESISTANCE

In searching for the mechanisms responsible for hypoglycemic episodes in several family members of a large pedigree, we identified the Arg1174Gln mutation in the tyrosine kinase domain of the insulin receptor as the most likely cause of hypoglycemia (21). Several family members in three generations suffered from episodes with hypoglycemia ranging from moderate symptoms of hypoglycemia to episodes with loss of consciousness and convulsions. The latter was treated with anticonvulsant therapy in three individuals for years, however, without any relieving effect. Reported age of onset was between 3 and 30 years, and all affected family members were characterized by fasting hyperinsulinemia and an elevated insulin-to-C-peptide-ratio in the absence of increased fasting plasma glucose levels. These traits were distributed in an autosomally dominant pattern of inheritance. Affected family members reported symptoms of hypoglycemia in the presence of low plasma glucose (1.8-3.0 mmol/l) and hyperinsulinism (143-680 pmol/l) during the last part of a 5-h OGTT. Euglycemic-hyperinsulinemic clamp studies showed insulin resistance and markedly decreased clearance of serum insulin in affected family members (21). Although two had IGT, none of the 10 affected family members (age 7-80) had type 2 diabetes (21). This suggests a preserved β-cell function to compensate for insulin resistance in this family.

To identify the genetic cause of hypoglycemia and associated features of hyperinsulinism and insulin resistance, we first excluded hyperammonemia and disease-causing defects in the genes of insulin (*INS*) and the pancreatic β-cell KATP channel subunits, Kir6.2 (*KCNJ11*) and SUR1 (*ABCC8*) (104-108). Linkage analysis and subsequent mutation screening revealed a missense mutation (Arg1174Gln) in the IRTK domain of *INSR*, and showed that all ten family members affected by hypoglycemia were heterozygote carriers of this mutation. The complete co-segregation (LOD score 3.21) with the disease phenotype strongly suggested that the Arg1174Gln mutation was the cause of hypoglycemia and fasting hyperinsulinemia (21).

Although paradoxical fasting hypoglycemia has been described in the initial states of Leprechaunism and Rabson-Mendenhall´s syndrome (98), hypoglycemia (postprandial) in combination with insulin resistance in adults represent a novel phenotype linked to heterozygous mutations in *INSR*. Based on the fact that the majority of patients with features of the Type A syndrome have normal insulin receptors (100,101), and that no evidence of linkage between insulin receptor mutation and the Type A syndrome has been provided, we argued that other additional factors (genetic or environmental) may be responsible for the development of hyperglycemia and extreme insulin resistance (Type A syndrome) previously reported in three females with the Arg1174Gln mutation (100,109). In our study, none in three generations had diabetes mellitus, which suggests a selection bias of patients previously screened for *INSR* mutations. Our findings confirm the wide range of phenotypes observed in patients heterozygous for kinase-deficient *INSR* mutations, where some develop syndromes of severe insulin resistance and type 2 diabetes, whereas other even in the same family do not (98, 110). Moreover, our results

support animal studies showing that mice with a heterozygote *INSR* mutation develop diabetes with a frequency varying between 5-10% (111,112). This suggests that heterozygous *INSR* mutations exert only a predisposing role in the susceptibility to type 2 diabetes and that additional susceptibility genes and environmental factors are needed to give manifest diabetes. Whether heterozygote carriers of the Arg1174Gln mutation and other *INSR* mutations have an increased risk of cardiovascular disease remains to be established. However, two had IGT, and mean levels of HbA1c were in the upper range of normal. Epidemiological studies have indicated that an elevated glucose concentration per se increases the risk of type 2 diabetes and cardiovascular disease in apparently healthy men and women (113).

Despite lack of a clear cut evidence, the postprandial hypoglycemia observed in these heterozygous carriers of a *INSR* mutation seems to be explained by a temporary imbalance between insulin-mediated suppression of hepatic glucose output and glucose utilization in muscle and other tissues in the postprandial state, probably due to inappropriately high insulin levels even at low glucose levels.

METABOLISM IN COMMON METABOLIC DISORDERS AND INHERITED INSULIN RESISTANCE

Estimates of glucose and lipid metabolism in insulin resistant conditions can be obtained by different methodologies. In the present review, focus is placed on studies that have used the combination of the euglycemic-hyperinsulinemic clamp and indirect calorimetry to assess glucose disposal rates (GDR), glucose oxidation, lipid oxidation and non-oxidative glucose metabolism. These whole-body estimates are usually determined by systemic calorimetry, which is considered sufficient to provide measures of insulin action on glucose metabolism in skeletal muscle (114). In a few studies, local indirect calorimetry by the arteriovenous (A-V) leg-balance technique has been used to obtain more precise estimates of glucose and lipid metabolism in skeletal muscle in the resting, basal state, in which muscle glucose uptake accounts for less than 20% of whole body glucose disposal (115,116). Comparison of the results obtained in our versus other studies should be done with caution due to several factors such as regional differences and heterogeneity in the small sample sizes of study participants, application of different tracer-methodologies, insulin infusion concentrations and duration of clamp studies, and with respect to PCOS, also different diagnostic criteria (84). Therefore, a direct comparison between the different insulin resistant conditions discussed in this review will mainly rely on our own whole-body experiments. In all studies, we have combined a 3-4 hour euglycemic hyperinsulinemic clamp with systemic indirect calorimetry using an insulin infusion rate of 40 mU/min/m^2 (Table 1).

ABNORMALITIES IN INSULIN ACTION ON GLUCOSE METABOLISM

Skeletal muscle is the major site of glucose disposal in response to insulin accounting for up to 80% of whole-body glucose clearance in vivo (115,117). Furthermore, 80-90% of the glucose taken up in skeletal muscle during insulin stimulation is stored as glycogen (117). Correspondingly, skeletal muscle is the predominant site of peripheral insulin resistance, and quantitatively impaired muscle glycogen synthesis is considered the major defect of insulin-stimulated glucose metabolism in most insulin resistant conditions including common metabolic disorders such as obesity, type 2 diabetes (19,26,28,40,59,60,117-125), and PCOS (85,126,127). In patients with type 2 diabetes, reduced insulin-mediated GDR and non-oxidative glucose metabolism are accompanied by a smaller but significant reduction in insulin-stimulated glucose oxidation when compared with weight-matched, non-diabetic controls (19,26,28,35,40,59,116,121-125). In studies that include lean healthy individuals it has been demonstrated that obesity alone is associated with reduced insulin-mediated GDR (26,40,118,119) and non-oxidative glucose metabolism (26,28,40,121,123,125). In about half of these studies, insulin action on glucose oxidation was also lower than in lean controls (26,28,125). This suggests that obesity contributes to this defect in oxidative glucose metabolism. The consistent observation of reduced insulin action on glucose oxidation in type 2 diabetes versus obesity indicates that a part of this defect, similar to the defects in GDR and non-oxidative glucose metabolism, cannot be attributed to obesity alone, but rather should be explained either by genetic susceptibility or factors secondary to the development of hyperglycemia. The above-mentioned differences between lean, obese and type 2 diabetes individuals in insulin action on GDR, non-oxidative glucose metabolism and glucose oxidation are well-reflected in our studies (Table 1).

Insulin-mediated GDR is decreased by 35-50% in women with PCOS (85,86,126-131). It is generally agreed that obese women with PCOS are insulin resistant, and that this insulin resistance is independent of obesity alone (84). However, at least some studies have failed to demonstrate insulin resistance in lean women with PCOS using the euglycemic-hyperinsulinemic clamp technique (128,129). This could be explained by the use of different diagnostic criteria when including these women in the studies. Women with PCOS also show defects in both the oxidative and non-oxidative glucose metabolism (85,126,127). Consistent with a recent study (126), we observed significant reductions in both GDR, glucose oxidation and non-oxidative glucose metabolism during insulin infusion in obese women with PCOS (85). These findings suggest that not only reduced GDR in obese women with PCOS (86), but also insulin resistance in the non-oxidative and oxidative pathways is independent of obesity. In our study, these defects were demonstrated in the absence of fasting hyperglycemia. However, this does not exclude the presence of IGT in women with PCOS (91). Thus, IGT is reported in up to 20-35% of PCOS (87,88,132). As noted in a review recently (84), when we compare our studies of type 2 diabetes, obesity and PCOS directly, it is remarkably that the degree of insulin resistance observed in these relatively young obese women with PCOS is strikingly similar or even worse than in middle-aged, obese patients with type 2 diabetes (Table 1). These findings indicate that in women with PCOS other factors than those in type 2 diabetes and obesity contribute to insulin resistance, and support the hypothesis of a unique pathogenesis for insulin resistance in PCOS (86). In both the total study population of obese women with and without PCOS and in the subgroup of women with PCOS, we observed a significant inverse relationship between circulating free testosterone and insulin-stimulated values of GDR and non-oxidative glucose metabolism (25). This suggests that higher androgen levels at least in part contribute to the insulin resistance observed in women with PCOS.

Insulin resistance in heterozygote carriers of the Arg1174Gln mutation in the *INSR* was characterized by an isolated defect in insulin action on non-oxidative glucose metabolism (21). This strongly implies that the defect in glucose oxidation observed in type 2 diabetes, obesity and PCOS is not mediated by intrinsic defects in insulin signaling, but is secondary in nature to other abnormalities associated with these disorders. The isolated defect in non-oxidative glucose metabolism has also been reported in two other cases with mutation at the same site in the *INSR* (133,134). Although, at first sight, the insulin resistance in

Table 1. Metabolic characteristics of individuals with common metabolic disorders and inherited insulin resistance

	Control	Obese	T2D	Control	PCOS	Control	Arg1174Gln
n	10	21	20	14	24	6	6
Age (years)	50.8 ± 1.0	49.8 ± 1.2	50.2 ± 1.1	33.8 ± 2.1	31.6 ± 1.3	44.0 ± 2.3	41.7 ± 5.6
Body mass index (kg/m^2)	24.2 ± 0.5	31.8 ± 1.0^{A}	32.3 ± 0.8^{A}	33.7 ± 1.7	33.3 ± 0.9	24.5 ± 0.8	25.4 ± 1.0
Plasma triglycerides (mmol/l)	1.1 ± 0.2	1.4 ± 0.1	2.7 ± 0.5^{A,C}	0.9 ± 0.1	1.7 ± 0.2^{B}	1.2 ± 0.4	1.3 ± 0.2
Plasma glucose (mmol/l)	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.2^{A,B}	5.6 ± 0.1	5.9 ± 0.1	5.3 ± 0.1	5.6 ± 0.2
Serum insulin (pmol/l)	24 ± 6	51 ± 4^{A}	84 ± 7^{A,B}	51 ± 6	104 ± 12^{B}	18 ± 2	177 ± 30^{A}
HbA1c (%)	5.5 ± 0.1	5.2 ± 0.1	7.3 ± 0.4^{A,B}	0.54 ± 0.07	0.54 ± 0.07	4.8 ± 0.1	5.7 ± 0.2^{A}
Plasma FFA basal (mmol/l)	0.54 ± 0.07	0.52 ± 0.05	0.50 ± 0.03	0.47 ± 0.04	0.44 ± 0.03	0.50 ± 0.08	0.38 ± 0.07
Plasma FFA clamp (mmol/l)	0.03 ± 0.00	0.05 ± 0.01	0.09 ± 0.01^{A,B}	0.02 ± 0.00	0.06 ± 0.01^{B}	0.01 ± 0.00	0.12 ± 0.01^{A}
GDR basal	81 ± 5	77 ± 2	90 ± 3^{B}	72 ± 3	77 ± 2	77 ± 4	76 ± 2
GDR clamp	352 ± 18	266 ± 17^{A}	159 ± 16^{A,B}	297 ± 23	150 ± 9^{B}	346 ± 33	200 ± 34^{D}
Glucose oxidation basal	51 ± 7	49 ± 4	47 ± 5	52 ± 8	42 ± 3	45 ± 6	48 ± 6
Glucose oxidation clamp	137 ± 10	104 ± 10^{A}	72 ± 10^{A,B}	141 ± 17	86 ± 5^{B}	117 ± 5	100 ± 14
Lipid oxidation basal	44 ± 3	46 ± 2	52 ± 3	33 ± 3	39 ± 1	38 ± 4	37 ± 3
Lipid oxidation clamp	11 ± 3	23 ± 2^{A}	39 ± 2^{A,B}	1 ± 6	23 ± 2^{B}	13 ± 2	21 ± 6
NOX basal	30 ± 7	28 ± 4	40 ± 4	20 ± 7	35 ± 3	32 ± 5	27 ± 5
NOX clamp	215 ± 16	166 ± 14	91 ± 4^{A,B}	157 ± 22	65 ± 6^{B}	229 ± 28	100 ± 24^{D}
RER basal	0.80 ± 0.01	0.80 ± 0.01	0.79 ± 0.01	0.81 ± 0.01	0.79 ± 0.00	0.80 ± 0.01	0.80 ± 0.01
RER clamp	0.96 ± 0.02	0.90 ± 0.01	0.83 ± 0.01^{A,B}	0.94 ± 0.01	0.87 ± 0.01^{B}	0.91 ± 0.01	0.89 ± 0.03
Δ - RER	0.16 ± 0.02	0.10 ± 0.01^{A}	0.05 ± 0.01^{A,B}	0.13 ± 0.01	0.08 ± 0.01^{B}	0.12 ± 0.01	0.08 ± 0.02

Differences in clinical and metabolic characteristics between individuals with obesity, type 2 diabetes (T2D), women with PCOS or carriers of a INSR mutation (Arg1174Gln) and their respective controls. Results are as described previously (22,25,26). Metabolic rates are expressed as mg/min per m^2. Data represent means ± SEM. ^{A}P<0.01 and ^{D}P<0.05 vs. lean controls; ^{B}P<0.01 and ^{C}P<0.05 vs. obese controls. GDR; glucose disposal rates; NOX, non-oxidative glucose disposal; RER, respiratory exchange ratio.

Arg1174Gln carriers seems to be slightly less pronounced than in type 2 diabetes and PCOS (Table 1), a major difference between Arg1174Gln carriers and individuals with type 2 diabetes, PCOS or obesity was a 4-fold decrease in insulin clearance in Arg1174Gln carriers. This makes a direct comparison of insulin action on glucose metabolism difficulty. Thus, clamp insulin levels were 4-fold increased, and this probably rescued these individuals from extreme insulin resistance by counteracting the detrimental effects of decreased functional insulin receptors (21). A minor degree of impaired insulin clearance has also been reported in a few cohorts of PCOS (85,128,129), but not in others (86, 130). Nevertheless, in these studies clamp insulin levels were only slightly elevated, and in fact in none of the study cohorts compared directly here (Table 1), clamp insulin levels were higher in individuals with type 2 diabetes, PCOS or obesity than in the matched controls.
It remains to be established which of the abnormalities in insulin-stimulated glucose metabolism that represent primary defects, and which are secondary to changes in the metabolic milieu associated with obesity, type 2 diabetes and PCOS. Nevertheless, at least quantitatively, we have demonstrated a major defect in insulin action on non-oxidative glucose metabolism (glycogen synthesis) in all insulin resistant conditions ranging from 60-70% of the defect in GDR in obesity, type 2 diabetes and PCOS to 90% in carriers of the Arg1174Gln mutation in *INSR*. Moreover, studies of monozygotic and dizygotic twins have shown that the heritability of non-oxidative glucose metabolism is about 50% (135), and impaired insulin-stimulated glycogen synthesis has been reported in most studies of non-obese, glucose-tolerant FDR (58-60,136), and in skeletal muscle cell cultures (myotubes) established from patients with type 2 diabetes (18,137,138). These findings support the hypothesis of a primary defect in insulin-mediated glycogen synthesis in the pathogenesis of type 2 diabetes (114). Consistent with the isolated defect in insulin action on non-oxidative glucose metabolism in Arg1174Gln carriers, previous studies of glucose tolerant, FDR could not find a significant defect in insulin-stimulated oxidative glucose metabolism (55,58-60). However, similar to women with PCOS, obese FDR with IGT showed impaired insulin-stimulated glucose oxidation compared with glucose tolerant, obese control individuals (139,140). This defect may therefore represent changes associated with the prediabetic state itself such as postprandial hyperglycemia. As both groups are known to be at high risk for insulin resistance and future type 2 diabetes, this defect in insulin action on glucose oxidation could be viewed as an early marker of increased susceptibility to develop type 2 diabetes. The fact that insulin action on glucose oxidation is quantitatively smaller than the effect on glycogen synthesis, points out that it is more difficulty to detect a significant defect in glucose oxidation than in non-oxidative glucose metabolism. Therefore, it is impossible to rule out that abnormalities in insulin action on both non-oxidative glucose metabolism and glucose oxidation co-exist very early in the development of type 2 diabetes.

ABNORMALITIES IN INSULIN ACTION ON LIPID METABOLISM

Insulin plays a critical role in whole-body lipid oxidation by inhibiting the release of free fatty acids (FFA) from adipose tissue (lipolysis) in response to a meal. This inhibition of lipolysis and hence suppression of circulating FFA is a major cause of insulin-mediated suppression of lipid oxidation. The ability of insulin to suppress circulating FFA and lipid oxidation during a euglycemic-hyperinsulinemic clamp is compromised in type 2 diabetes, and to a lesser extent in obesity (19,26,28,40,118,121,123,125), and in women with PCOS (85,126,141). These findings indicate that impaired insulin action on lipolysis resulting in elevated FFA levels in the insulin-stimulated state is a major factor determining the ability of insulin to stimulate glucose oxidation. This is likely explained by the fact that excessive amounts of FFA, used as substrates for lipid oxidation, compete with glucose in muscle as a source of energy according to the hypothesis of the glucose-fatty acid cycle proposed by Sir Randle (142). In accordance with normal insulin action on glucose oxidation, no impairment in insulin-mediated suppression of FFA levels or lipid oxidation was reported in glucose tolerant, normal-weight FDR (58), or in isolated cases of carriers of Arg1174 mutations in *INSR* (133,134). How-

ever, in our small cohort of Arg1174Gln carriers, we did find an impaired insulin-mediated suppression of FFA, but without significant changes in insulin action on lipid oxidation (21). This suggest that in some of these studies, sample sizes are too small to draw any firm conclusions.

METABOLIC INFLEXIBILITY IN INSULIN RESISTANCE

As noted above, reliable estimates of substrate metabolism in human skeletal muscle in the resting, basal state cannot be obtained by whole-body systemic calorimetry. From indirect calorimetric studies using the leg balance technique it is, however, clear that abnormalities in muscle glucose oxidation and lipid oxidation in type 2 diabetes and obesity exist under basal conditions as well (116, 124). Thus, conversely, to impaired stimulation of glucose oxidation and suppression of lipid oxidation in response to insulin, muscle glucose oxidation is increased and reliance on lipid oxidation is decreased in type 2 diabetes and obesity during fasting conditions (116, 124, 125, 143). This impaired ability to switch between lipid oxidation and glucose oxidation in response to insulin and fasting has been described as "metabolic inflexibility" of skeletal muscle, and may be a major determinant of skeletal muscle insulin resistance (124,144). No studies of FDR using the leg-balance technique are available, and therefore, it remains to be established whether metabolic inflexibility in skeletal muscle is an early defect in the pathogenesis of type 2 diabetes. However, in a recent study, whole-body metabolic flexibility defined as the insulin-stimulated change in RQ during the clamp, was reported to be reduced in FDR (145). Moreover, whole-body metabolic flexibility was positively correlated with insulin sensitivity. Consistently, our whole-body calorimetric studies of obesity, PCOS and type 2 diabetes have shown a similar decrease in Δ-RQ in these common metabolic disorders (25,28,40), whereas only a tendency (p=0.12) was observed in Arg1174Gln carriers compared to controls (21) (Table 1). These results support a role for metabolic inflexibility in the pathogenesis of insulin resistance.

LIPID AVAILABILITY AND LIPID OXIDATION IN INSULIN RESISTANCE

Studies of healthy humans have shown that lipid-infusion for several hours impairs insulin action on not only glucose oxidation but also GDR and non-oxidative glucose metabolism (146). Moreover, there is evidence from at least one study in vivo, that infusion of lipids, which increased FFA levels 4-fold (1.8 mmol/l) inhibited insulin stimulation of IRS1 associated PI3K activity, the most proximal part of the insulin signaling (147). Indeed, we and others have reported elevated circulating triglyceride levels in both type 2 diabetes and PCOS (19,25,28,40). However, FFA levels even in type 2 diabetes rarely exceeds 1 mmol/l, and in several studies including those presented in table 1, we have been unable to detect increased levels of plasma FFA in either obesity, type 2 diabetes or PCOS (19,25,26,28,31,38,40). Thus, while lipid infusion studies in healthy humans have provided evidence for the existence of the glucose-fatty acid cycle as proposed by Randle et al (142), there is in fact no evidence that elevated circulating FFA alone can explain insulin resistance in skeletal muscle of individuals with type 2 diabetes, obesity or PCOS. This implies a larger role for the elevated circulating triglyceride levels in common forms of insulin resistance. Circulating triglyceride levels were not increased in patients with *INSR* mutations (21). This is consistent with another report (103,) and indicates that genetically determined general insulin resistance caused by e.g. mutations in the *INSR* gene may be less harmful than partial insulin resistance by protecting against enhanced insulin-stimulated hepatic triglyceride synthesis (14,15).

In obesity and type 2 diabetes, reduced lipid oxidation in the resting, basal state is observed despite higher circulating levels of lipids. This is in contrast with the hypothesis of the glucose-fatty acid cycle (142). In type 2 diabetes, this could be explained by increased glucose levels, which may increase muscle malonyl CoA concentrations leading to inhibition of carnitine palmitoyl transferase-1 (CPT1), and hence impaired uptake and oxidation of fatty acids in mitochondria (148-150). However, this mechanism is unlikely to be responsible for decreased lipid oxidation during fasting conditions in obese individuals. In a study of insulin resistant obese individuals, muscle CPT1 activity was reported to be reduced, and this was proportional to an overall reduction in oxidative enzyme activity (119). This implies a role for reduced mitochondrial content or function in insulin resistance. In human skeletal muscle, lipid is the predominate oxidative substrate during postabsorptive conditions, accounting for ~80% of oxygen consumption. It is therefore not surprising that defects in mitochondrial oxidative metabolism have been sought to explain impaired lipid oxidation in skeletal muscle of individuals with obesity and type 2 diabetes.

MUSCLE LIPIDS IN INSULIN RESISTANCE

A reduced reliance on lipid oxidation during fasting conditions is likely a key mechanism by which triglyceride and lipid metabolites accumulate within skeletal muscle, although a role for increased lipid availability cannot be excluded (144,151,152). In humans, an increased amount of muscle lipids is regarded as a key marker of insulin resistance (153), and has been reported not only in obesity and type 2 diabetes (32,154-158), but also in lean, glucose tolerant FDR (159). To our knowledge, reports of muscle triglyceride or lipid metabolites in women with PCOS or in patients with mutation in *INSR* are not available. However, at least for women with PCOS it is expected that muscle lipids are increased. Thus, a close relationship between intramyocellular lipid concentrations (IMCL) and insulin resistance has been reported in healthy individuals, FDR, and patients with type 2 diabetes (154,155). Intramyocellular triglyceride is regarded as a metabolically inert marker of other lipid intermediates known to suppress insulin sensitivity (151,152). Thus, increased levels of specific lipid metabolites such as long chain fatty Acyl CoAs (150,160), diacylglycerols (DAG) (161) and ceramides (118,162) have been demonstrated in obesity and type 2 diabetes, and linked to insulin resistance. Although, the mechanisms linking IMCL to insulin resistance in humans have not been fully established, it has been hypothesized that accumulation of these lipid metabolites impairs insulin signaling due to activation of certain serine/threonine (Ser/Thr) kinases (e.g. PKC) (161,163), which subsequently leads to inhibitory Ser phosphorylation of proximal components in the insulin signaling cascade (164), and glycogen synthase (165,166).

SUMMARY OF ABNORMALITIES IN METABOLISM

Metabolic studies are fundamental to understand the pathophysiology of skeletal muscle insulin resistance in humans with common metabolic disorders such as obesity, type 2 diabetes and PCOS. As outlined above, such studies have revealed a number of abnormalities in both the basal and insulin-stimulated state. Each of these may represent early markers for the development of insulin resistance and type 2 diabetes. The clear cut defects in insulin action on glucose metabolism have been a major driving force for many research groups including our own to search for defects in the insulin signaling cascade, and are a major focus of

this review. A common causative mechanism for the abnormalities observed in glucose and lipid metabolism in insulin resistant individuals could be perturbations in skeletal muscle mitochondrial oxidative metabolism (23,31,33,36,124,167). In particular, the abnormalities in the resting, basal state has recently prompted us and other researchers to look for other defects using global unbiased approaches such as genomics and transcriptomic and proteomic profiling of skeletal muscle (38,39,168-170) as well as more focused studies of isolated mitochondria from skeletal muscle (171,172). A discussion of these studies are, however, beyond the scope of this review.

INSULIN SIGNALING TO GLUCOSE TRANSPORT AND GLYCOGEN SYNTHESIS

At the cellular level, insulin resistance in skeletal muscle is characterized by impaired insulin stimulation of glucose uptake and glycogen synthesis (23,53,114,117). As will be outlined below, a number of abnormalities explaining these defects have been reported in skeletal muscle biopsies obtained from patients with type 2 diabetes and other insulin resistant conditions (8,23,84, 173). However, first a brief introduction to the current understanding of insulin signaling to glucose transport and glycogen synthesis in skeletal muscle will be given (Fig. 1). Despite extensive research, the continuous identification of novel players in insulin signaling shows that there is still much to learn. Insulin is a potent anabolic hormone that regulates a wide variety of biological processes in skeletal muscle including glycogen synthesis, glucose transport, protein synthesis, and gene expression (174,175). The intracellular actions of insulin are mediated by modification of the activity and/or the subcellular location of key regulatory proteins and enzymes (kinases and phosphatases) primarily by affecting their phosphorylation state (175,176). Promotion of glucose transport and glycogen synthesis are key biological actions of insulin in skeletal muscle, and as noted above, defects in these actions of insulin seem to be major determinants of skeletal muscle insulin resistance in type 2 diabetes, obesity, PCOS, and other high-risk individuals.

The insulin receptor is a heterotetrameric protein that consists of two extracellular α-subunits and two transmembrane β-subunits connected by disulfide bridges (174-177). Insulin signaling involves a cascade of events initiated by insulin binding to the extracellular α-subunits. This causes autophosphorylation of specific tyrosine (Tyr) residues in the IRTK domain of the intracellular part of the β-subunit, and subsequent recruitment, binding and Tyr phosphorylation of members of the insulin receptor substrate (IRS) family (174-177), of which the IRS1 isoform seems to be the most important in skeletal muscle (178). The Tyr phosphorylated residues of IRS1 mediate an association with the p85 regulatory subunit of PI3K leading to activation of the p110 catalytic subunit, which then catalyzes the formation of PI(3,4,5)-P3 from PI(4,5)-P2 in the inner part of the plasma membrane (174-177). These initial, proximal steps are necessary for initiating a

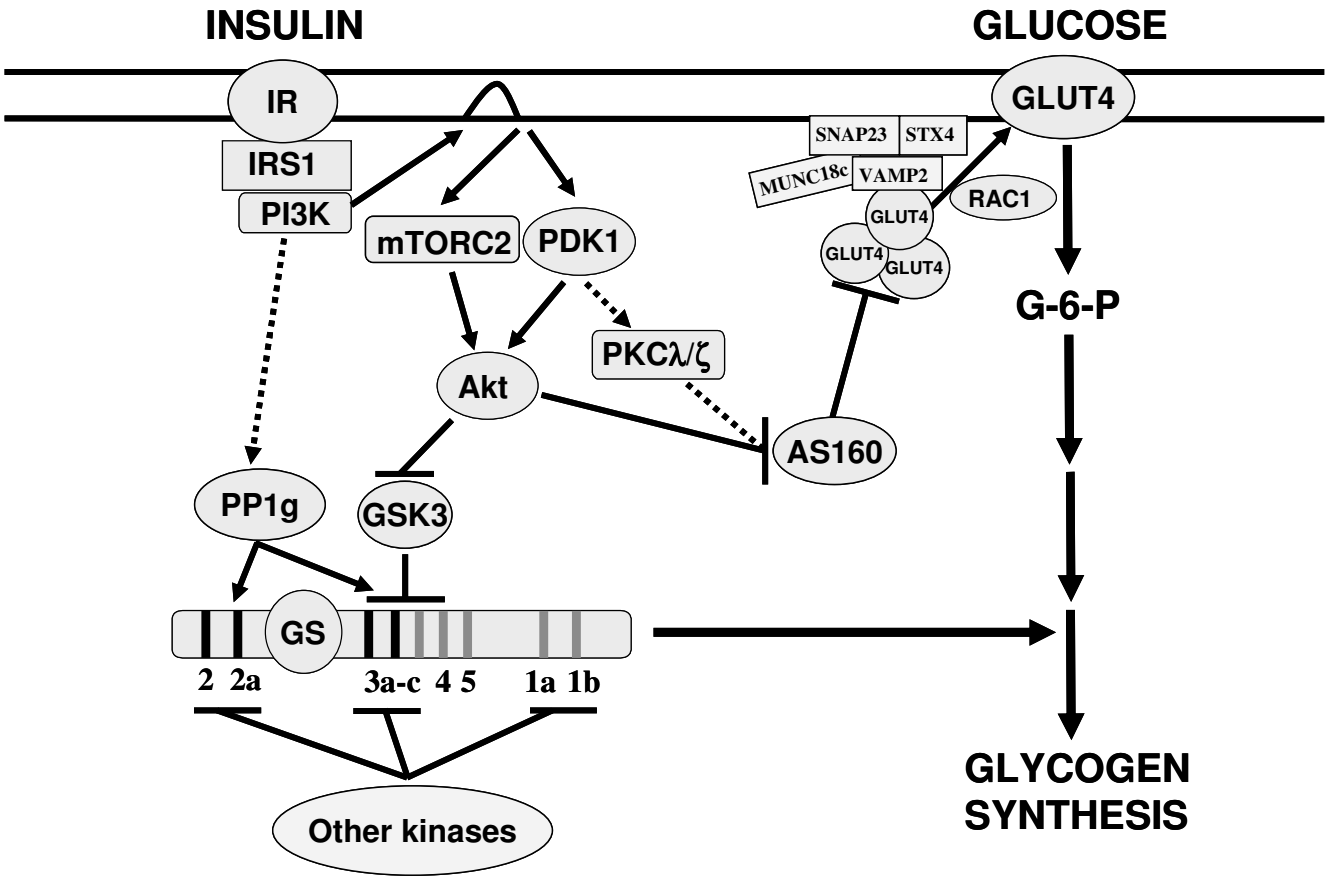

Figure 2. Insulin signaling to glucose transport and glycogen synthesis.

Insulin binds to the insulin receptor (IR), and causes recruitment and activation of IRS1 and PI3K. This leads to activation of PDK1 and mTORC2, which in turn stimulates Akt and probably PKCλ/ζ. Subsequent inactivation of AS160, as well as activation of RAC1, increases translocation of GLUT4 to and SNARE-mediated (VAMP2, STX4, SNAP23 and Munc18c) docking and fusion with the plasma membrane, and hence glucose transport. Activation of Akt causes inhibition of GSK3 leading to activation of GS and hence glycogen synthesis. Insulin may also activate GS by PI3K-dependent stimulation of a glycogen associated phosphatase, PP1G. See text for the abbreviations used.

number of divergent signaling cascades mediating nearly all the metabolic actions of insulin (175). IRS1 is regulated by phosphorylation at multiple sites of which Ser and Thr phosphorylation initially were thought to negatively modulate the association with PI3K (175,177), whereas Tyr phosphorylation promotes its association with PI3K, and hence insulin signaling. Within recent years up to 40 phosphorylation sites have been shown to be regulated in response to physiological insulin in human skeletal muscle, and this includes several Ser and Thr residues (179,180). However, the molecular consequences of most of these insulin-mediated changes in phosphorylation of IRS1 remain to be established (177).

The next important step in insulin signaling to both glucose transport and glycogen synthesis is activation of the Ser/Thr kinase, Akt (Fig. 1). It exists in three isoforms called Akt1, Akt2 and Akt3, of which Akt2 is highly expressed in skeletal muscle and is thought to mediate insulin action in this tissue (178,181). Akt is considered a critical node in insulin signaling (175). Thus, it serves as a highly regulated point of divergence for downstream signaling to 1) glucose transport through Akt substrate of 160 kDa (AS160), also known as TBC1D4, 2) glycogen synthesis through glycogen synthase kinase 3 (GSK3) and glycogen synthase (GS), and 3) protein synthesis through the mammalian target of rapamycin (mTOR) pathway. Full activation of Akt is obtained by phosphorylation of both Thr308 and Ser473 (182). Thr308 is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) in response to increases in PI(3,4,5)P3 from PI(4,5)P2, and Ser473 is believed to be phosphorylated by the mTOR-Rictor complex (or mTORC2) (175,176,183,184). Another mechanism that may play a significant role for insulin activation of Akt2 is its sorting to the plasma membrane in response to insulin. Thus, the generation of PI(3,4,5)P3 by PI3K recruits Akt to the membrane leading to its apposition to its stimulatory kinases, PDK1 and mTORC2 (185). This has been suggested to contribute to the isoform-specific signaling of Akt2 to the insulin responsive glucose transporter type 4 (GLUT4), and hence glucose transport (186).

INSULIN-MEDIATED TRANSLOCATION OF GLUT4

In the further downstream signaling from Akt to glucose transport (Fig. 1), there is evidence that insulin-mediated translocation of GLUT4 from its intracellular localization to the plasma membrane is dependent on phosphorylation of AS160/TBC1D4 (181,187-189). AS160/TBC1D4 contains two phosphotyrosine binding domains, and a Rab GTPase-activating protein (GAP) domain, the activity of which under basal conditions is sufficient to inhibit a Rab protein required for GLUT4 translocation. AS160/TBC1D4 is regulated by phosphorylation of several Ser and Thr residues in response to insulin and contraction (181,190). Currently, at least ten phosphorylation sites have been identified (Ser318, Ser341, Thr568, Ser570, Ser588, Thr642, Ser666, Ser704, Ser711, Ser751), some of which are also regulated by binding of 14-3-3 proteins (181,188,191-193). Of all these sites, Thr642 and Ser588 seem to be the key residues regulating Akt-mediated GLUT4 translocation in response to insulin (188). Upon insulin stimulation, phosphorylation of AS160/TBC1D4 at several specific sites by Akt, also in human skeletal muscle (194), suppresses its GAP-activity to a degree that permits exocytosis of GLUT4 vesicles to the plasma membrane. AMPK has been identified as another upstream kinase for AS160/TBC1D4 in response to contraction in skeletal muscle suggesting that AS160/TBC1D4 may be a convergent point for stimuli regulating GLUT4 translocation and glucose transport (181,190,194-196). In muscle cells, Rab8A, Rab14 and most recently Rab13 have been identified as downstream mediators of GLUT4 translocation in response to inhibition of AS160/TBC1D4 (197,198).

In the basal state, the majority of GLUT4 protein is localized intracellularly. An increase in the plasma membrane content of GLUT4 can result from either increasing exocytosis or decreasing endocytosis (197). Insulin stimulates glucose transport by promoting GLUT4 exocytosis. Some of the best established mechanisms will in brief be outlined below. Downstream of PI3K the insulin signal diverges into the Akt2 $\rightarrow$ AS160/TBC1D4 $\rightarrow$ Rab axis as outlined above, and a Rac1 $\rightarrow$ actin $\rightarrow$ α-actinin-4 (actin remodeling) axis (197) (Fig. 1). The Rho family GTPase, Rac1, has been shown to regulate insulin-stimulated GLUT4 translocation and glucose transport in cultured muscle cells (197,199). Insulin activation of Rac1 leads to activation of its downstream target, p21-activated kinase (PAK) by facilitating its autophosphorylation of Thr423. This pathway induces actin-remodeling of the cortical actin-cytoskeleton (200), which is necessary for insulin to induce GLUT4 translocation in these cells. Very recently, it was confirmed that Rac1 and its downstream target, PAK, are indeed regulators of insulin-stimulated glucose uptake in mature mouse and human skeletal muscle (201). Furthermore, there is data to suggest that full PI3K-dependent stimulation of glucose transport requires the activation of atypical forms of the protein kinase C family (PKC ζ/λ) (183,197). Insulin has also been suggested to promote tethering, docking and hence fusion of GLUT4 vesicles with the membrane (197). Docking and fusion of GLUT4 vesicles to the cell membrane is mediated by the SNAP-associated receptor (SNARE) proteins VAMP2, syntaxin-4 and SNAP23, and their regulatory partners Munc18c and Synip (197,202). Syntaxin-4 along with SNAP23 forms a functional SNARE complex with the v-SNARE VAMP2, carried by GLUT4-containing vesicles (Fig. 1). Most studies, but not all, indicate that Munc18c negatively regulates GLUT4 exocytosis (203). Thus, in the basal state, interaction between the SNAREs is thought to be prevented by Munc18c and Synip (202). As reviewed recently (204), muscle contraction, membrane depolarization and energy deprivation can also increase the density of surface GLUT4 mainly by slowing GLUT4 endocytosis. This seems to involve AMPK and Ca2+-dependent mechanisms. Recently, a role for Rac1 in contraction-mediated GLUT4 translocation was also demonstrated (205). In addition, a number of other molecules and mechanisms are possibly involved in the insulin-mediated translocation of GLUT4 to the plasma membrane (204). However, some of these mechanisms are not fully established, or have only been demonstrated in adipocytes, and, therefore, are outside the scope of this review.

REGULATION OF GLYCOGEN SYNTHASE

GS is a key enzyme in muscle glycogen synthesis catalyzing the final step in the synthesis of glycogen by the formation of α-1-4-glucosidic linkages with UDP-glucose as the glucosyl donor (176,208). The regulation of GS is highly complex. Thus, skeletal muscle GS activity is controlled extensively by both covalent modifications (multisite phosphorylation) and allosteric effectors, of which glucose-6-phosphate (G6P) seems to be the most important (176,206,208). Phosphorylation leads to inactivation of GS (Fig. 2), but full activity can be restored in the presence of G6P. These properties of GS regulation are used when assaying the phospho-dependent activity of GS (206,208). Thus, in studies of human skeletal muscle biopsies, GS-activity is measured ex vivo as the amount of the substrate UDP-glucose incorporated into glycogen in the presence of no, low or high concentrations of G6P (209,210). The activity at no or low G6P concentrations divided by

activity at saturated G6P concentrations are given as G6P-independent GS-activity (I-form) and as fractional velocity of GS (GS-FV), respectively. Already in 1963 it was demonstrated that insulin activated GS by promoting its dephosphorylation (176). However, here 50 years after this discovery, the relative importance of allosteric and covalent modification in the regulation of GS activity and glycogen synthesis remains to be established (se below). Mammalian GS is phosphorylated at at least nine Ser residues in vivo (211,212) (Fig. 2). Site-directed mutagenesis studies (Ser→Ala) have shown that sites 2 and 2a in the NH_2-terminus and sites 3a and 3b in the COOH-terminus are the most important sites for GS activity (176,206,208,212,213). The effects seem to be additive so that almost complete inhibition of GS occurs if all four sites are phosphorylated (206). In contrast, phosphorylation of GS at sites 1a, 1b, 4 and 5 is thought to have little effect on the enzyme activity, although is has to be mentioned that Ser→Ala mutagenesis of site 1a and 1b was not performed (176,206,208). However, there is evidence that site 1b as well as sites 2 and 2a could play a role for the subcellular localization of GS, and play a role for GS activity in response to e.g. contraction (214). The identification of the kinases and phosphatases regulating phosphorylation of GS at these multiple sites has been a major challenge. Thus, many of the kinases and phosphatases identified in vitro may have no physiological relevance in vivo (176,206,208). Currently, about eleven kinases and one phosphatase are believed to be potential regulators of GS activity (208) (Fig. 2). Of these, GSK3 is the most active kinase phosphorylating sites 4, 3c, 3b and 3a in vitro, and it seems to do so in a sequential manner (176,215,216). Moreover, these four sites are only phosphorylated by GSK3 if site 5 has already been phosphorylated by casein kinase-2 (CK2), a phenomenon termed hierarchal phosphorylation (176,206,215-217). Recent work in cell models has indicated that sites 3a and 3b can be directly phosphorylated by other kinases than GSK3. Thus, the dual-specificity tyrosine phosphorylated and regulated protein kinase (DYRK) and the Per/Arnt/Sim domain-containing protein kinase (PASK) may phosphorylate site 3a, and p38β MAPK may phosphorylate site 3b on GS (208,218,219).

Protein kinase A (PKA), which is known to be activated by increases in the second messenger cAMP, e.g. in response to adrenalin, preferentially phosphorylates sites 2, 1a and 1b in vitro (176). Site 2 can be phosphorylated by several other protein kinases in vitro, of which PKC, AMPK, calmodulin-dependent protein kinase-2 (CaM-KII), and phosphorylase kinase (PhK) may have physiological relevance under different conditions (165,176,208,220). Casein kinase-1 (CK1), which is constitutively active, is the only detectable site 2a kinase in skeletal muscle extracts (221). Prior phosphorylation of site 2 has been shown to increase several fold the phosphorylation of site 2a by CK1, demonstrating another example of hierarchal phosphorylation in the regulation of GS activity (221). Dephosphorylation of GS is believed to be mediated by members of a family of glycogen-associated PP1Gs. In muscle, protein phosphatase 1 (PP1) has been reported to be the dominating protein phosphatase dephosphorylating and activating GS (222,223). Moreover, PP1 is the only phosphatase, which specifically associates with glycogen particles, and which is thus localized in the compartment of muscle where it is expected to work (223). The glycogen associated form from muscle, termed PP1G.GM, is thought to be responsible for activation of GS by direct dephosphorylation (176,208,224). The inhibition of PP1G.GM by PKA is thought to be a major mechanism by which adrenalin increases phosphorylation of other sites (2a, 3a, 3b and 3c) than site 2, 1a, and 1b (221). At least in vitro, PP1G.GM can dephosphorylate all of the sites in GS (221).

EFFECT OF INSULIN ON GLYCOGEN SYNTHASE PHOSPHORYLATION

Despite decades of extensive research the molecular mechanisms by which insulin activates GS in vivo remains controversial (208,225). Earlier studies of rabbit skeletal muscle have shown that most of the phosphate released from GS in response to insulin in vivo is removed from the tryptic peptide containing sites 3a and 3b (211). Accordingly, several earlier studies have demonstrated that insulin decreases the activity of glycogen synthase

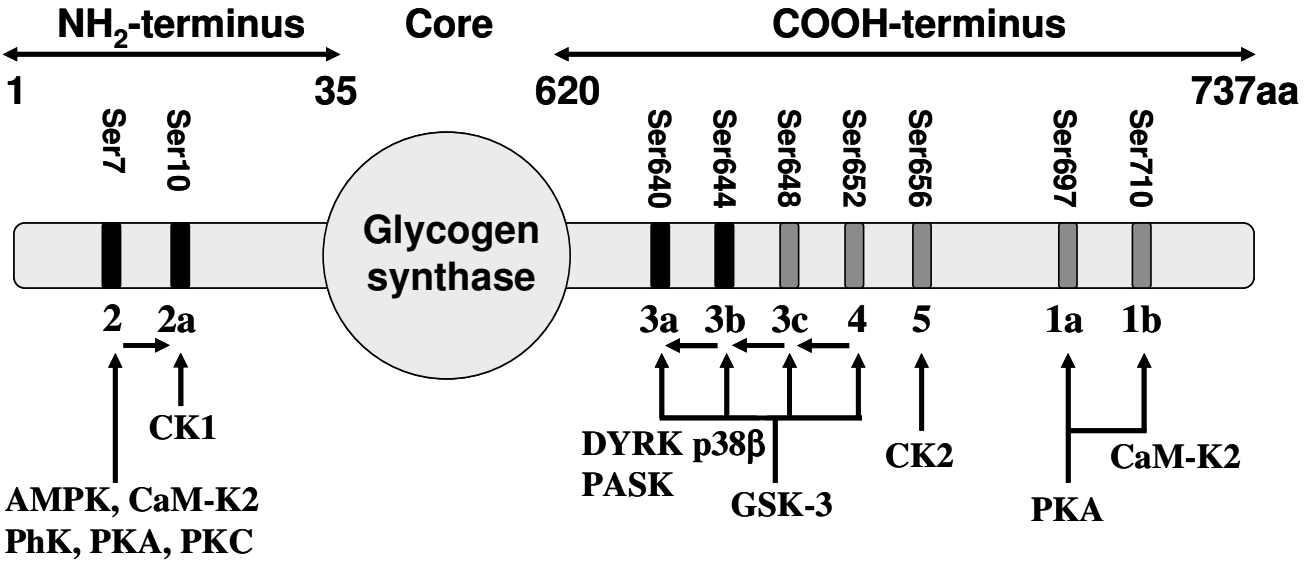

Figure 3. Phosphorylation of glycogen synthase.

Phosphorylation of glycogen synthase (GS) at nine serine residues in the NH_2- and COOH- terminal ends of GS, and the potential serine kinases believed to play a role in the regulation of GS activity. The glycogen-associated phosphatases PP1Gs can dephosphorylate all nine serine residues on GS. The most important sites for GS activity are marked with black, and horizontal arrows denote hierarchal phosphorylation. See text for the abbreviations used.

kinase-3 (GSK3), and that this inhibition is almost sufficient to account for the observed GS activation (176,208,226,227). The inhibition of GSK3 is mediated through phosphorylation of Ser21 in GSK3α and Ser9 in GSK3β (176). Insulin-mediated phosphorylation and inhibition of GSK3 is catalyzed by Akt, whose activation is dependent on PI3K activity (176,183,206,226). Thus, it is currently believed that insulin signaling from the insulin receptor into GS involves activation of IRS1, PI3K, PDK1 and mTORC2, and Akt, which in turn leads to inhibition of GSK3, and hence dephosphorylation and activation of GS ((176,183,184,206) (Fig. 1-2). Also, in human skeletal muscle, insulin in physiological concentrations causes dephosphorylation and inhibition of GSK3 activity, which is accompanied by dephosphorylation and activation of GS, and a robust increase in glycogen synthesis (19,24,26,40,228,229). Studies in cell models expressing rabbit skeletal muscle GS and in rat skeletal muscle in vitro have demonstrated that insulin in high concentrations also promotes dephosphorylation of sites 2 and 2a as well (176,213,230,231). Therefore, insulin-mediated stimulation of PP1G.GM was originally thought to be responsible for GS activation by promoting dephosphorylation of both NH_2- and COOH-terminal sites on GS (176,206,211,224). Although, some reports have questioned the ability of insulin to activate GS by PP1G.GM (232,233, as well as the mechanism by which PP1G.GM is activated by insulin (234), a recent study indicates that insulin does in fact activate PP1G.GM (235). Moreover, dephosphorylation of site 2+2a has been observed in human skeletal muscle even in response to physiological concentrations of insulin (24,26,40). However, the molecular events linking insulin activation of PI3K with stimulation of PP1G.GM remain to be established.

On the other hand, recent studies taking advantage of knockin mice in which the Akt-responsive phosphorylation sites on GSK3 were changed to Ala, have shown that inhibition of GSK3 is sufficient to account for the insulin activation of GS (236,237), and the authors concluded that if a phosphatase contributes to activation of GS it has to be controlled by GSK3 (236). However, while insulin activation of GS was fully accounted for by genetically mediated inhibition of GSK3, results from the same study indicated that insulin stimulation of glucose uptake does not require Ser9/21 phosphorylation of GSK3 (236). Moreover, in a follow-up study, it was shown that insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation (237). This dissociation between the effects of insulin on the phosphorylation and activation of GS, and insulin stimulation of glycogen synthesis and glucose uptake raised the possibility that allosteric activation of GS by G6P could play a major role in glycogen synthesis (237). Finally, using the generation of knockin mice in which a residue that was shown to play an important role for the allosteric activation of GS by G6P, Bouskila et al provided genetic evidence that the allosteric activation of GS by G6P is the primary mechanism by which insulin promotes glycogen synthesis in skeletal muscle at least in mice (238). One of the problems interpreting these data in the light of previous results is that measurement of GS activity almost always reflect the phosphorylation state, but that within the muscle cell, the true activity of GS is also dependent of the concentrations of UDP-glucose, G6P and glycogen in the cell (208,225). Moreover, much of the evidence put forward in the study of Bouskila et al is based on the result that blocking G6P-binding to GS abolished 80% of the insulin activation of glycogen synthesis in vitro using stimulation with 100.000 pmol/l insulin (238). However, glucose uptake was not affected due to a simultaneous increase in insulin-stimulated glycolysis. Intriguingly, insulin-stimulated glycolysis in the wild-type mice accounted for more than 75% of glucose utilization with less than 25% left for glycogen storage (238). This is far more than normally seen in e.g. human individuals using physiological concentrations of insulin, where glycogen synthesis accounts for up to 90% of the insulin-stimulated glucose uptake (117). As noted by Bouskila et al, glycogen content in mouse muscle is only 10% of that in human muscle (239), and therefore insulin-stimulated glycogen synthesis in skeletal muscle may not play a similar large role for glucose homeostasis as in humans. Finally, as seen in numerous other studies using whole-body genetic manipulations of crucial enzymes, these manipulations often create compensatory and artifactual responses, and the results from such studies therefore need to be interpreted with caution. In the future, it will be more than interesting to see if insulin can stimulate glycogen synthesis in knockin studies of mice, in which Site 2 +2a and Site 3a +3 b are replaced by Ala. Although, there is definitely still some lack of clarity in this field, GSK3 is currently believed to be the best candidate for a kinase responsible for the insulin-mediated dephosphorylation and activation of GS, and hence glycogen synthesis. However, with respect to skeletal muscle insulin resistance, increases in any kinase capable of keeping the phosphorylation of GS high may be of pathophysiological relevance. Thus, increased GS phosphorylation at sites not regulated by insulin in vivo may counteract the effect on sites known to be regulated by insulin and thus impair glycogen synthesis and glucose disposal.

RATE-DETERMINING STEPS OF GLYCOGEN SYNTHESIS

Despite progress in the unraveling of the signal transduction pathways mediating insulin activation of GS and glucose transport, it has remained a major issue, whether the rate of glycogen synthesis in muscle in response to insulin is determined by the rate of glucose entry into the cell or the activity of GS and its upstream enzymes. In other words, is insulin stimulation of glycogen synthesis primarily determined by pushing glucose into cell by stimulating glucose transport, or by pulling glucose into the cell by stimulating GS - or both? A wide variety of approaches have been used to solve this issue, and as reviewed (114,206,208, 240-243), there are several arguments for and against each of these hypothesis. In summary, convincing support for both hypotheses has been provided, and it is now well accepted, by most, that both glucose transport and glycogen synthase contribute to the control of glycogen synthesis in skeletal muscle (114,206,208, 241-243). The promotion of the hypothesis that glucose transport is the rate-determining step may, however, explain why the Akt-GSK3-GS axis in many studies and reviews of insulin action in muscle in vivo are almost forgotten or neglected (240), despite the convincing amount of data showing that impaired insulin stimulation of glycogen synthesis quantitatively is the major defect in insulin resistant conditions.

MODULATORS OF INSULIN SIGNALING

Except from a genetic variant near the IRS1 gene (244), there is currently no evidence supporting a role for common genetic variants in the coding regions of enzymes involved in classical insulin signaling to glucose transport or glycogen synthesis (42). Therefore, impaired insulin activation of components in the insulin signaling cascade is likely to be explained by other factors modulating insulin signaling in skeletal muscle (245). This could include epigenetic, transcriptional, posttranscriptional, and post-translational mechanisms related to obesity, excessive intake of nutrients, decreased physical activity, or factors associated with e.g. type 2 diabetes and PCOS such as increased circulating glucose and lipids, hyperinsulinemia, chronic-low grade inflamma-

tion, and altered levels of sex hormones. The role of epigenetics and control of protein expression by microRNA on insulin signaling enzymes is an emerging field of research, but remains to be determined (42,246,247). Moreover, most studies of gene expression and, in particular, protein abundance have reported normal levels of IRS1, p85α, and p110α of PI3K, Akt1/2, AS160/TBC1D4, GLUT4, GSK3, and GS in human skeletal muscle obtained from individuals with common and inherited forms of insulin resistance (12,19,22,24-26,35,40,131,139,228,248-265). At the posttranslational level, which is the major focus of this review, increased activity in a number of modulating signaling pathways has been linked to insulin resistance due to an effect on the phosphorylation and activity of different components of the classical insulin signaling pathway. Thus, chronic low-grade inflammation caused by changes in adipose tissue, in part mediated by macrophage infiltration, and, perhaps, dysbiotic microbiota, causes increased secretion of proinflammatory adipocytokines (e.g., TNFα, IL-1β, IL-6) to the circulation, as well as reduced levels of adiponectin (266). This can activate proinflammatory pathways in skeletal muscle with increased signaling through c-Jun NH_2-terminal kinase (JNK), IKKβ, and NFκB, which has been shown to cause inhibition of insulin signaling by Ser/Thr phosphorylation of IRS1 (266). Moreover, an increased supply of energy substrates, in particular high levels of glucose and lipids, but also amino acids, may contribute to activation of several pathways. This include activation of the hexosamine biosynthetic pathway, which via increased levels of UDP-GlcNAc may cause O-GlcNAc site-specific regulation of several components in the insulin signaling cascade including GS (267-270). High glucose and lipids levels may also increase oxidative stress due to excessive production of mitochondrial ROS with subsequent activation of stress-activated signaling pathways including JNK, p38 MAPK, and NFκB, which all are thought to suppress insulin signaling at the level of IRS1 (271,272). Elevated circulating amino acids may cause hyperactivation of the mTOR signaling pathway, which via S6K1 and mTORC1 causes a negative feedback on insulin signaling with Ser phosphorylation of IRS1 and a subsequent decline in the IRS1–associated PI3K activity (273). Furthermore, an increased availability of lipids and decreased lipid oxidation, due to an impaired mitochondrial oxidative metabolism (23,33,167), leads to accumulation of lipid metabolites such as ceramides and DAG (151,152,274). Increased ceramides may impair insulin signaling by the activation of PP2A, which causes decreased phosphorylation of Akt and perhaps GSK3 (275). Increased DAG is thought to be responsible for activation of certain members of the PKC family, which may then impair insulin signaling via inhibitory Ser/Thr phosphorylation of IRS1 and perhaps the insulin receptor (151,152,274), as well as Ser phosphorylation of GS at site 2 (165,166). Abnormalities in proteins involved in lipid droplet formation may also play a role for the increase in these lipid metabolites may involve (276). Thus, modulation of different components of the insulin signaling cascade may take place at several levels involving a wide array of molecular mechanisms. However, much of the evidence comes from experimental studies in cells or animals, whereas data from studies in humans are either lacking, or showing conflicting results. The effect of interventions such as e.g. changes in diet and exercise training or the use of different insulin sensitizers may exert their effect on insulin resistance and insulin signaling in skeletal muscle primarily by affecting one or more of the above-mentioned mechanisms rather than directly affecting the enzymes involved in insulin signaling. However, except from a more detailed description of adiponectin, this review will primarily focus on abnormalities identified in insulin action on insulin signaling enzymes in skeletal muscle obtained without any intervention in the resting state from individuals with obesity, type 2 diabetes, PCOS or mutation in the insulin receptor.

CHALLENGES IN STUDIES OF INSULIN SIGNALING IN HUMAN SKELETAL MUSCLE

Genetic manipulation of animal models have significantly provided insight into the molecular mechanisms involved in insulin signaling and underlying the development of insulin resistance in skeletal muscle under different conditions (277). However, even in animal models, the nature of skeletal muscle insulin resistance and the relative contribution of other organs may be quite different from that seen in humans. This is exemplified by the observation that the glycogen content of mouse muscle is only about 10% of that of human muscle, when expressed as a percentage of total body glycogen (239). Moreover, mouse strains can differ substantially in their metabolic response under different conditions depending on their genetic background (278,279). Studies of the activity and phosphorylation of enzymes in the insulin signaling pathway in human skeletal muscle in vivo are therefore of crucial importance to translate findings in animal studies, and most importantly to increase our understanding of the pathogenesis of insulin resistance in humans.

A major challenge in studying insulin signaling in human skeletal muscle is a larger biological variation than observed in specific strains of mice or rats. Furthermore, the interpretation of the results is clouded by the use of different approaches to determine the response to insulin. Thus, in some studies stimulation with physiological concentrations of insulin (400-1000 pmol/l) was used, whereas in others insulin levels were raised to supraphysiological levels (3000-6000 pmol/l), possibly to obtain a more pronounced activation of the insulin signaling components (8). In some studies, biopsies from the m. vastus lateralis were obtained in the steady-state period of a clamp (typically after 2-4 hours), whereas in others biopsies were taken 30-40 min after initiation of insulin infusion (8). It has been argued that early peaks in the response of enzymes to insulin may be transient in nature and, therefore, not present in the steady-state period of a clamp. However, studies of human skeletal muscle in vivo have shown that the effects of insulin on signaling components are sustained for several hours (120,229,280,281). A number of other factors may contribute to different results. This includes differences in the handling of the muscle biopsies, removal of contaminating tissue components e.g. by freeze-dissection, homogenization, the use of different buffers, gels, and primary and secondary antibodies. However, most importantly, the sample sizes used in most studies of insulin signaling in human skeletal muscle are small, especially when considering the large heterogeneity within obese and type 2 diabetic individuals. All these factors increase the risk of type 2 errors, and the abnormalities and lack of differences reported in studies of insulin signaling in human skeletal muscle in vivo should be interpreted in this context. In the following sections, such studies are reviewed together with a comparison of insulin signaling in common metabolic disorders versus an inherited form of insulin resistance (Table 2).

Table 2. Results of studies of insulin signaling in human skeletal muscle

Cases	Type 2 diabetes			Obesity		Prediabetes*		PCOS		Arg1174Gln	
versus	matched	lean	any control	lean		control		matched		control	
Insulin action	↓	↓	→	↓	→	↓	→	↓	→	↓	→
IRTK	282; 284	282; 283; 284	256; 285	283	284	286	256				22
pIR Tyr		12; 288	249; 289	12; 287	289		255				
pIRS1 Tyr	249; 254; 288; 289	289; 291	12	287; 291	12; 289	139; 255					
IRS1-ass.-PI3K	120; 249; 254; 288; 289; 290	12; 120; 289; 291	19; 26; 256; 287	287;291; 292;293	12; 26; 120; 131; 289	139; 255; 294	256; 290	295	25; 131	22	
PDK1			289		289						
Akt	26; 300	26	120; 290; 300#	293	120; 131	88			131	22	
pAkt Thr308	35; 40; 264; 303	26; 40; 303	19; 256; 291; 302	26	40; 291		256; 265	25		22	
pAkt Ser473	40; 303	32; 40; 302; 303	19; 26; 35; 264; 290; 291	118	26; 32; 40; 290	290;301	139; 255; 265	25	307; 308		22
PKC λ/ζ	290	289; 290; 291; 309		131; 289; 291		290			131		
pAS160 PAS	35; 264		26; 310		26		265	25			
pAS160 Ser/Thr	35										
GLUT4	312; 314	311; 313		311		265					
GSK3 α/β			19; 228	293	228						22
pGSK3 pSer9/21			26; 40	303	26; 40				24		
GS-FV/I-form	19; 28; 35; 40; 120	26; 121; 228	249	26; 28	40; 120; 121; 228	58; 60; 294	255; 256	24		22	
pGS Site 2+2a	19; 35; 40	26; 40		26	40			24			22
pGS Site 3a+3b			19; 26; 35; 40		26				24		22

*An overview of results from studies of the effect of insulin on the activity of proximal and distal enzymes involved in insulin signaling to glucose transport and glycogen synthesis in skeletal muscle biopsies obtained from humans with common metabolic disorders and inherited insulin resistance versus controls. The numbers refer to references in the reference list. The prefix "p" refers to phosphorylation of the enzymes listed. *either FDR and/or IGT; # at 2 nM insulin. For abbreviations see text.*

DEFECTS IN INSULIN ACTION ON IRTK, IRS1 AND PI3K

Impaired insulin activation of IRTK in skeletal muscle of patients with type 2 diabetes has been reported in some (282-284), but not all studies (256,285). In one of these studies (283), IRTK activity was similarly reduced in obese non-diabetic controls. In young, non-obese glucose tolerant FDR both basal and insulin-stimulated IRTK activity was reduced (286), but in a later study of young, obese FDR there was no reduction in insulin activation of IRTK (256). Likewise, Tyr phosphorylation of the insulin receptor in response to insulin stimulation has been found to be impaired in obese type 2 diabetic patients and obese non-diabetic controls (12,287,288), but normal in other studies of obese type 2 diabetic patients and in lean FDR (249,255,289). More consistent is the finding that Tyr phosphorylation of IRS1 and the association of p85/PI3K activity with IRS1 in response to insulin are attenuated in obese patients with type 2 diabetes (12,120,249,254,288-292), in obese non-diabetic controls (287,291-293), and in FDR with or without IGT (139,255,294). In some studies, including some of our own, we found, however, no significant differences between obese patients with type 2 diabetes and obese controls (19,26, 256,287), between obese non-diabetic controls and lean controls (12,26,120,131,289), or between obese FDR and matched controls (256,290). Some of the most marked (clear) differences, were observed when using muscle strips stimulated with 10-7 M insulin (287,293), or 60 nM (249), or using supraphysiological levels of insulin during clamp (~6000 pmol/l) (288-291). We and others have reported that increased basal Tyr phosphorylation of IRS1 and/or IRS1-associated PI3K activity may contribute to the attenuated incremental effect of insulin in patients with type 2 diabetes and FDR (12,19,139,256,288,294). Thus, although the response to insulin was impaired, no difference in the insulin-stimulated state was detectable. This could reflect fasting hyperinsulinemia in type 2 diabetes, and to a lesser extent in obesity. The molecular mechanisms underlying skeletal muscle insulin resistance in women with PCOS in vivo are less well characterized than in obesity or type 2 diabetes. In an earlier study of skeletal muscle in vivo, insulin-stimulated IRS1 associated PI3K activity was shown to be impaired after 30 min, but with no difference after 90 min of physiological hyperinsulinemia in morbidly obese women with PCOS (295). However, in another study using supraphysiological insulin levels, the insulin-induced increase in IRS1-associated PI3K activity was not significantly diminished in obese women with or without PCOS compared with lean controls (131). Consistently, we observed an intact insulin action on IRS1-associated PI3K activity in obese PCOS patients compared with BMI-matched women (25). Although, we cannot rule out the possibility that we missed a transient lower IRS1-associated PI3K activity, there is convincing evidence that insulin action on proximal insulin signaling components are sustained for several hours in human skeletal muscle (120,229,280,281). Thus, impaired insulin signal-

ing through IRS1 and PI3K in skeletal muscle may not explain the marked reduction in insulin-stimulated glucose metabolism in obese PCOS patients.

Previous studies of the naturally occurring Arg1174Gln mutation in *INSR* in vitro have shown normal insulin binding and affinity to the insulin receptor on the plasma membrane but diminished rates of internalization and degradation of the insulin-insulin receptor complex probably due to a 70% reduction in insulin-stimulated IRTK activity (109,296-299). In cell lines transfected with the Arg1174Gln mutation, thus expressing the mutant *INSR* in a homozygous state, insulin in physiological concentrations (1000 pmol/l) showed impaired ability to increase Tyr phosphorylation of IRS1, PI3K activity, GLUT4 translocation and glycogen synthesis (298,299). However, in the first case-control study of the effect of a heterozygous mutation in the IRTK domain of the *INSR* on downstream insulin signaling in skeletal muscle in humans in vivo, we observed that insulin infusion caused a comparable increase in IRTK activity in the control (42%) and the Arg1174Gln (33%) groups, but in neither of the groups this achieved statistical significance (22). In controls, insulin infusion increased IRS1-associated PI3K activity, whereas in the Arg1174Gln group no significant response to insulin was observed. However, there was no significant difference in IRS1-associated PI3K activity in either the basal or the insulin-stimulated state between the groups (22).

In summary, these studies have provided evidence that insulin stimulation of glucose uptake and glycogen synthesis is mediated by activation of IRTK, Tyr phosphorylation of the insulin receptor and IRS1 as well as IRS1 association with PI3K of human skeletal muscle in vivo. Several studies of Tyr phosphorylation of the insulin receptor and IRS1 and IRS1-associated PI3K activity have shown impaired response to insulin in skeletal muscle of patients with type 2 diabetes, and to a lesser extent in obese non-diabetic and prediabetic individuals, women with PCOS and inherited insulin resistance. Except from studies using supraphysiological or extreme levels of insulin, the observed abnormalities are rather inconsistent, and sometimes with increased basal activity as the major cause of impaired response. At least, based on currently available data, it is impossible to point out clear differences in insulin action at the level of IRS1 or PI3K between common forms and an inherited form of insulin resistance, although insulin action in Arg1174Gln clearly were partially rescued by the 4-fold decrease in insulin clearance, and hence 4-fold higher insulin levels during the clamp.

DEFECTS IN INSULIN ACTION ON AKT, ATYPICAL PKCs AND PDK1

In contrast to the many initial reports that showed impaired insulin activation of IRS1 association with PI3K in patients with type 2 diabetes and high-risk individuals (12,120,287-291), the majority, but not all (293,300) of the first studies of insulin signaling to the level of Akt were characterized by the absence of abnormalities. Thus, insulin induced a robust increase in the phosphorylation of Akt at Ser473 and Thr308, and in activation of Akt 1, 2 and 3 in human skeletal muscle in vivo, and none of these responses were diminished in patients with type 2 diabetes or obese non-diabetic individuals (19,120,131,256,290,291). Furthermore, studies of muscle biopsies from both normoglycemic, non-obese FDR and obese FDR with IGT failed to demonstrate a significant impairment in insulin activation of Akt (139,255,256, 265). These reports were disappointing given that Akt is thought to be a critical node in the downstream signaling to both glucose transport and glycogen synthesis. It was proposed that only a small amount of PI3K activity is necessary to activate Akt (120). However, it was subsequently demonstrated that insulin action on Akt Ser473 phosphorylation was impaired in relation to obesity, and that this was related to the muscle content of ceramides (118). It was also shown that in a small group of FDR with severe insulin resistance, insulin induced phosphorylation of Akt at Ser473 was 60% reduced (301). Moreover, a study of non-obese patients with type 2 diabetes, showed that insulin action on Thr308 phosphorylation was reduced, and that this was associated with an impaired insulin action on the down-stream substrate, AS160/TBC1D4 (264). Consistent with our first study (19), we found no difference in insulin induced phosphorylation of Akt at Ser473 and Thr308 between patients with type 2 diabetes and BMI-matched non-diabetic controls in our second study (26). However, compared with lean controls, insulin-mediated phosphorylation of Akt at Thr308 was impaired in both obese patients with type 2 diabetes and obese non-diabetic controls (26) (Fig. 3). Because of slightly higher basal levels of Ser473 phosphorylation in obesity and type 2 diabetes, the incremental increase in Akt Ser473 phosphorylation was also found to be significantly diminished in patients with type 2 diabetes compared to lean controls (26). More recently, we have also revealed a decrease in insulin-mediated phosphorylation of Akt Thr308 in obese patients with type 2 diabetes versus weight-matched controls (35). In that study, we used an insulin infusion rate (80 mU/min/m^2) during the clamp that was 2-fold higher than in our previous studies (19,22,25,26), which could be the reason for detecting a significant difference. This is supported by previous ex vivo studies of human muscle strips, in which a defect in insulin activation of Akt was detected in morbidly obese individuals (293) and patients with type 2 diabetes (300) when using 60 nM insulin, but not when using 2 nM insulin (300). Moreover, in more recent studies, also using an insulin infusion rate of 80 mU/min/m^2, a reduced insulin-induced phosphorylation of Akt Ser473 (32,302,303), and an even more pronounced defect phosphorylation of Akt Thr308 (303) were demonstrated in obese individuals with and without type 2 diabetes compared with lean controls. Nevertheless, in our most recent studies of obese patients with type 2 diabetes, we actually found that insulin action on both Akt Thr308 and Ser473 phosphorylation, as well as Akt2 activity was impaired when compared to both obese non-diabetic and lean controls, even using an insulin infusion rate of 40 mU/min/m^2 during clamp (40). It must, however, be said that the insulin sensitivity in the obese non-diabetic controls in that study were higher than previously observed. Thus, even in our own hands, the discrepancies demonstrate that the results depend on both the conditions used as well as the sample sizes and the heterogeneity within different cohorts of patients with type 2 diabetes and lean and obese controls.

In skeletal muscle of obese women with PCOS, we observed a pronounced defect in insulin-mediated phosphorylation of Akt at both Thr308 and Ser473 (25) (Fig. 3). Of interest, these defects were completely reversed after 16 weeks treatment with the insulin sensitizer, pioglitazone, despite only a modest improvement in insulin-stimulated GDR in these women with PCOS. This effect of thiazolidinediones (TZDs) on insulin action on Akt has also been reported in muscle from patients with type 2 diabetes and FDR (304,305), although not in newly diagnosed patients with type 2 diabetes (306). However, in another study, 10 weeks training did not normalize the defect in Akt phosphorylation at Thr308 in patients with type 2 diabetes, despite a modest increase in insulin stimulated GDR (35). These results provide evidence that not all interventions leading to improved insulin sensitivity is accompanied by improved signaling through Akt, and that nor-

malization of insulin action on Akt does not necessarily fully restore insulin sensitivity. In contrast with our results, another study failed to demonstrate impaired insulin induced Akt activity in women with PCOS (131) using supraphysiological insulin levels (~4000 pmol/l). Moreover, no significant impairment at the level of Ser473 was found in overweight/obese women with PCOS compared with obese controls in two more recent studies (307,308) using insulin stimulation with ~6000 pmol/l. In our study of women with PCOS, we observed a strong positive relationship between GDR and non-oxidative glucose metabolism and Akt phosphorylation at Thr308 and Ser473 during insulin stimulation (25). This association was recently reproduced in a study of muscle from lean, obese/overweight and type 2 diabetes individuals, in which insulin-stimulated glucose infusion rates (GIR) was found to be best predicted by insulin-stimulated phosphorylation of Akt at Thr309 (303). These findings provide correlative evidence that Akt is an important mediator of insulin-stimulated glucose metabolism in skeletal muscle in vivo. In PCOS patients, reduced insulin action on Akt in skeletal muscle seems to be independent of obesity and hyperglycemia. These findings may support the hypothesis of a unique sub-phenotype of skeletal muscle insulin resistance in PCOS (86).

In carriers of the heterozygous dominant-negative mutation, Arg1174Gln, in the IRTK domain of *INSR*, insulin caused a significant increase in Akt Thr308 and Ser473 phosphorylation (22). However, compared to matched controls insulin-stimulated Akt Thr308 phosphorylation and Akt2 activity were reduced in these Arg1174Gln carriers (Fig. 3). The relatively intact insulin signaling through Akt in skeletal muscle of patients harboring the Arg1174Gln mutation suggests that impaired insulin clearance in part rescues in vivo insulin signaling in muscle in these carriers of a mutant *INSR* probably by increasing insulin action on the fraction of non-mutated insulin receptors. The blunted Akt Thr308 phosphorylation and Akt2 activation in response to insulin may explain lower glucose transport in Arg1174Gln carriers, but did not seem to be responsible for the impaired insulin activation of GS, because insulin-mediated inhibition of GSK3 and subsequent dephosphorylation of GS at sites 3a+3b was normal (22).

So far, the effect of insulin on atypical PKC isoforms in insulin resistant human skeletal muscle in vivo have exclusively been studied using supraphysiologal levels of insulin (~4000-6000 pmol/l). In these studies, it has been demonstrated that insulin-stimulated aPKC activity is diminished in obese women with and without PCOS compared with lean women (131), and in obese individuals with and without IGT or type 2 diabetes compared with lean controls (289-291,309). These results suggest that impaired insulin activation of aPKC may play a special role for the impact of obesity alone on insulin signaling to glucose transport. In one of these studies, insulin action on PDK1 activity was also examined (289), but no difference between lean, obese and type 2 diabetes individuals could be demonstrated.

In summary, these data have shown that insulin activation of Akt by phosphorylation of Thr308 and Ser473, and perhaps PDK1 and atypical PKCs in human skeletal muscle in vivo are accompanied by and likely critical for insulin-mediated glucose transport and/or storage. Despite initial disappointing results, there is accumulating evidence that impaired insulin action on Akt2 by phosphorylation of Thr308 and perhaps Ser473 in skeletal muscle could play a critical role for impaired insulin action on both glucose transport and glycogen synthesis in obesity, type 2 diabetes, PCOS as well as carriers of a *INSR* mutation. Taking the more recent results into consideration, no really difference between common metabolic disorders or the studied inherited form of insulin resistance can be pointed out. This suggests the possibility that insulin signaling in carriers of a dominant-negative mutation in *INSR* may be modulated by some of the same mechanisms as in obesity, PCOS and type 2 diabetes.

DEFECTS IN INSULIN ACTION ON AS160/TBC1D4

It was recently shown that Akt-dependent insulin stimulation of glucose transport is accompanied by phosphorylation of AS160/TBC1D4 at multiple specific sites in human skeletal muscle in vivo (35,194). To what extent diminished insulin-mediated phosphorylation of AS160/TBC1D4 could play a role for impaired insulin stimulation of glucose transport in common forms of insulin resistance has been examined in a number of recent studies. In a study of non-obese patients with type 2 diabetes and matched controls, it was shown that impaired insulin-induced (600 pmol/l) phosphorylation of Akt at Thr308 was accompanied by a reduction in the phosphorylation of AS160/TBC1D4 in diabetic muscle (264). Phosphorylation of AS160/TBC1D4 was measured by the phospho (Ser/Thr) Akt substrate (PAS) antibody, which is believed to reflect primarily Thr642 phosphorylation (191). In a subsequent study (265), the same research group reported a comparable insulin-mediated increase in the phosphorylation of both Akt and AS160/TBC1D4 in skeletal muscle from glucose tolerant FDR and matched controls. In that study, muscle strips were stimulated ex vivo with 0.6, 1.2 and 120 nM insulin, respectively. At maximal insulin doses 3-O-methylglucose transport was impaired in muscle from FDR despite normal phosphorylation of Akt at Thr308 and Ser473 as well as AS160/TBC1D4 phosphorylation. They concluded that the dissociation between these events was an early defect in the pathogenesis to type 2 diabetes (265). However, in another study of obese patients with type 2 diabetes and matched obese non-diabetic controls, the effect of insulin on AS160/TBC1D4 phosphorylation in muscle was comparable using physiological hyperinsulinemia (310). This lack of difference could be explained by the fact that there was no difference in insulin sensitivity (GIR) between the two groups.

We have measured insulin action on AS160/TBC1D4 phosphorylation in three studies (25,26,35). In our first study of patients with type 2 diabetes, and obese and lean controls (26), we could not demonstrate any significant differences between the groups in insulin induced AS160/TBC1D4 phosphorylation measured by PAS-antibody in skeletal muscle. This lack of difference was observed despite significantly reduced insulin-stimulated GDR in patients with type 2 diabetes versus lean and obese controls, and in obese versus lean controls, and despite significantly reduced insulin action on Akt Thr308 and to a lesser extent Ser473 phosphorylation in muscle from obese individuals with and without type 2 diabetes (26). In a subsequent study, we applied both the PAS antibody and newly developed phospho-specific antibodies against AS160/TBC1D4 phosphorylated at Ser318, ser341, Ser588, Thr642 and Ser751 (35). In both patients with type 2 diabetes and matched obese controls, insulin significantly increased phosphorylation of AS160/TBC1D4 at all 5 sites in skeletal muscle, also when measured with the PAS-antibody. Consistent with a lower insulin-mediated phosphorylation of Akt Thr308, we observed a reduced insulin action on the phosphorylation of AS160/TBC1D4 at Ser318, Ser588 and Ser751 in skeletal muscle of patients with type 2 diabetes (35). In that study, insulin-induced PAS phosphorylation of AS160/TBC1D4 was also reduced in patients with type 2 diabetes. However, phosphorylation of Thr642 was not. Of interest, 10 weeks training normalized phosphorylation of AS160/TBC1D4 in patients with type 2 diabetes at all sites measured without a similar improvement in obese controls (35). This

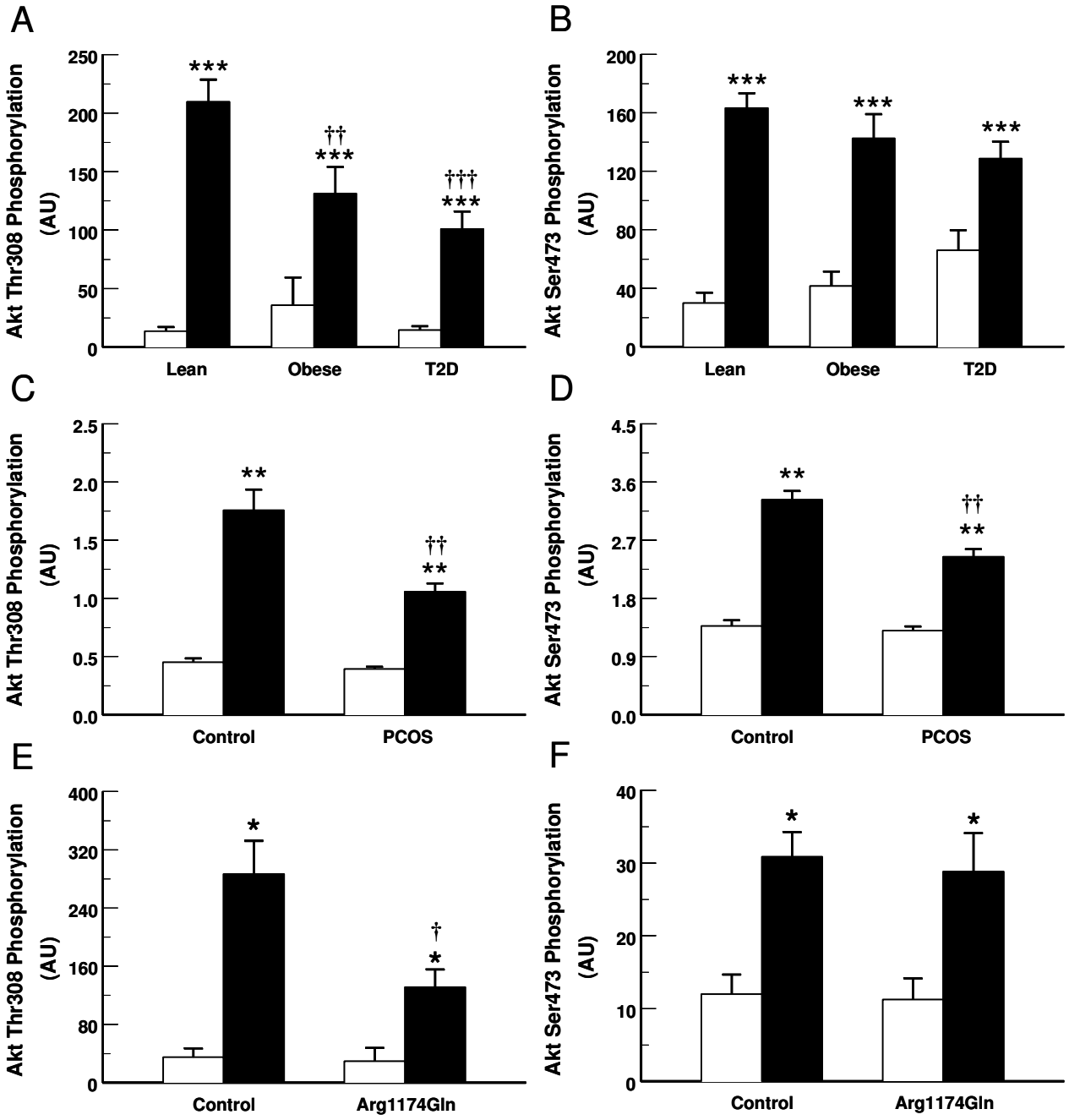

Figure 3. Akt phosphorylation.

*Phosphorylation of Akt at Thr 308 and Ser473 (A-B) in lean (n=10), obese (n=10), and type 2 diabetic (T2D) (n=10) individuals, (C-D) in control subjects (n=14) and women with PCOS (n=24), and (E-F) in control subjects and individuals with the Arg11174Gln mutation in the insulin receptor gene (INSR). Measurements were performed in skeletal muscle biopsies obtained during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 3-4 hour euglycemic-hyperinsulinemic clamp using an insulin infusion rate of 40 mU/min per m^2. Data are means ± SEM. ***P < 0.001, **P < 0.01 and * P < 0.05 vs. corresponding basal values; †††P < 0.001, ††P < 0.01, and †P < 0.05 vs. insulin-stimulated values in controls/lean individuals. Results are adapted from references (22,24,26).*

was seen in the absence of an rescuing effect on insulin-induced Akt Thr308 phosphorylation, and insulin sensitivity in patients with type 2 diabetes was still significantly lower than in obese controls after training. This suggest some dissociation both between signaling through Akt and AS160/TBC1D4, and between insulin signaling along this cascade and insulin sensitivity measured as GDR. In the most recent study, insulin-induced PAS phosphorylation of AS160/TBC1D4 in skeletal muscle was reported to be reduced in insulin resistant obese individuals, but not significantly in patients with type 2 diabetes when compared to lean controls, despite impaired insulin-mediated phosphorylation of Akt at Thr308 and Ser473 in both groups (303). Moreover, it was reported that only phosphorylation of Akt, but not phosphorylation of AS160/TBC1D4 was correlated with insulin sensitivity. We have also examined AS160/TBC1D4 phosphorylation in obese women with PCOS (25), and found a significant reduction in insulin-mediated PAS phosphorylation of AS160/TBC1D4 when adjusted for protein content. This was accompanied by impaired

phosphorylation of Akt at Thr308 and Ser473, and suggest that reduced insulin action on Akt and AS160/TBC1D4 in skeletal muscle of women with PCOS are early defects responsible for insulin resistance, at least in this condition, and which are independent of obesity and hyperglycemia. In the total cohort of obese women with and without PCOS, phosphorylation of Akt at Thr308 and Ser473 sites correlated not only with GDR and non-oxidative glucose metabolism, but also with PAS phosphorylation of AS160/TBC1D4 during insulin stimulation. However, somewhat similar to the report of Tonks et al (303), AS160/TBC1D4 phosphorylation only tended to correlate with insulin-stimulated GDR indicating that phosphorylation of AS160/TBC1D4 not only reflects insulin stimulation, but also other stimuli affecting its phosphorylation. A subgroup of these PCOS patients were treated 12 weeks with pioglitazone (25), and this treatment completely normalized insulin action on AS160/TBC1D4 phosphorylation, as was seen with Akt phosphorylation (see above).

Taken together, current available studies have demonstrated that stimulation with insulin in physiological concentrations causes a marked increase in Akt-dependent phosphorylation of AS160/TBC1D4 in human skeletal muscle in vivo, and this inhibition of AS160/TBC1D4 is likely to play an important role in insulin-mediated glucose uptake. While conclusive data are lacking, at least some studies have indicated that common forms of insulin resistance are also accompanied by impaired insulin action on AS160/TBC1D4. Further studies are, however, warranted, in particular, studies using phosphospecific antibodies.

DEFECTS IN GLUT4 TRANSLOCATION, SNAREs AND RAC1

As noted above, most studies, including our own, have shown that basal protein levels of GLUT4 in crude membrane fractions (plasma membrane + microsome) of skeletal muscle biopsies are normal in obesity, women with PCOS, Arg1174Gln carriers, patients with type 2 diabetes and FDR (22,25,35,257-263) and that GLUT4 content in these crude-membrane fractions do not change in response to insulin (22,25,35). However, this does not exclude impaired insulin-mediated GLUT4 translocation in these common and inherited forms of insulin resistance. Thus, in a previous study, ex vivo studies of muscle strips have shown an impaired 3-O-methylglucose transport in response to 10-7 M insulin in muscle of morbidly obese patients with and without type 2 diabetes (311). More recently, an impaired 3-O-methylglucose transport in response to 120 nM insulin has also been demonstrated in muscle strips obtained from non-obese patients with type 2 diabetes (312), and FDR compared with matched controls (265). In accordance, two studies have reported that insulin in physiological concentrations stimulates GLUT4 content in purified plasma membrane fractions of muscle from healthy controls, but that this response is impaired in insulin resistant and type 2 diabetic individuals (313,314). In another study, this effect of insulin on GLUT4 content in purified plasma membrane was not observed (261). However, using a new bis-mannose photolabelling technique, Ryder et al recently confirmed an impaired increase in cell surface content of GLUT4 in muscle strips from patients with type 2 diabetes in response to ex vivo stimulation with 120 nM insulin (312). Although further studies using this technology are warranted, this defect in insulin induced translocation of GLUT4 is consistent with the impaired activation of the upstream insulin signaling components observed in most studies, as discussed above.

Insulin-mediated docking and fusion of GLUT4 vesicles to the plasma membrane is mediated by a complex interplay between several SNARE proteins including VAMP2, syntaxin-4, SNAP23 and Munc18c (197,202,203). Recently it was demonstrated that SNAP23 is also critically involved in the fusion process by which lipid droplets are formed in cardiomyocytes (315). Of interest, it was shown that treatment of these cardiomyocytes with fatty acids resulted in a redistribution of SNAP23 from the plasma membrane to the cell interior, which was accompanied by accumulation of lipid droplets and impaired insulin action on cellular glucose transport (315). Thus, it appears that an increase in lipid droplets induced by fatty acid treatment impairs insulin-stimulated glucose uptake by "highjacking" SNAP23 from the plasma membrane. We, therefore, studied whether SNAP23 redistribution could play a role for insulin resistance and lipid accumulation in skeletal muscle of patients with type 2 diabetes compared with lean and obese controls (32). We found increased levels of lipid droplets in diabetic muscle, and this was associated with impaired insulin action on Akt Ser473 phosphorylation. There was no difference in mRNA levels of SNAP23, but somewhat surprisingly total SNAP23 protein was significantly increased in muscle of patients with type 2 diabetes. However, in a subset of lean controls and patients with type 2 diabetes, we fractionated muscle homogenates into a microsomal/cytosolic fraction containing all lipid droplets, and a membrane fraction enriched in plasma membrane markers. This subcellular fractionation showed that SNAP23 protein was increased in the microsomal fraction, but reduced in the membrane fraction in patients with type 2 diabetes (32). These results confirmed the findings in cardiomyocytes, by showing that higher levels of lipid droplets and insulin resistance are associated with a redistribution of SNAP23 from the membrane to the lipid droplets. We also found increased mRNA levels and protein abundance of the SNARE-interacting protein Munc18c in muscle of patients with type 2 diabetes, with no difference between obese and lean controls, as reported previously (316). In a previous study, we were, however, unable to detect a significant difference in Munc18c protein in skeletal muscle form patients with type 2 diabetes when compared to obese and lean controls (26). This could be explained by a higher degree of insulin resistance in the type 2 diabetic patients in the last studied cohort (32,40). Thus, only in that cohort we could demonstrate an impaired insulin action on Akt Thr308 and Ser473 phosphorylation in patients with type 2 diabetes compared to both lean and obese controls (40). We found no difference in protein content of SNAP23 or Munc18c between monozygotic twins discordant for type 2 diabetes, and treatment of human myotubes with high concentrations of insulin, glucose and/or fatty acids did not influence the protein content of SNAP23 (35). Therefore, we cannot exclude a genetic background for the observed changes in SNAP23 protein content and redistribution. As with measurement of GLUT4 translocation in human skeletal muscle, measurement of SNAP23 redistribution requires a rather large amount of muscle tissue, and therefore a sufficient amount of tissue was available only from 3 patients with type 2 diabetes and 3 lean controls. If these results can be confirmed in future studies, this redistribution of SNAP23 will represent an important area of research in the discovery of novel mechanisms and targets for treatment of insulin resistance.

Insulin activation of Rac1 and its downstream target, PAK, has been shown to induce GLUT4 translocation in cultured muscle cells (197-200), and more recently also insulin-stimulated glucose uptake in mature mouse and human skeletal muscle (201). The potential role of RAC1 and PAK Thr423 phosphorylation in insulin resistance was further investigated by Sylow et al, and included studies in human skeletal muscle (201). It was demonstrated that insulin resistance induced by intralipid infusion in healthy indi-

viduals (317) significantly impaired insulin-mediated phosphorylation of PAK Thr423. In accordance, insulin-stimulated phosphorylation of PAK Thr423 was impaired in skeletal muscle from obese non-diabetic individuals and patients with type 2 diabetes (201). No differences in Rac1 protein abundance were seen between the groups, but protein content of PAK1 was higher in type 2 diabetic compared to lean and obese individuals. These findings provide evidence that GLUT4 translocation mediated by Rac1-dependent signaling towards PAK is impaired in insulin resistant human skeletal muscle.

Taken together, there is evidence that insulin stimulates glucose uptake in human skeletal muscle by increasing GLUT4 translocation. Also in humans, this seems to involve activation of RAC1 with subsequent phosphorylation of PAK Thr423. In skeletal muscle from insulin resistant individuals, an impaired insulin action on glucose utilization seems to involve impaired stimulation of GLUT4 translocation, and diminished Rac1-dependent phosphorylation of PAK, as well as redistribution of SNAP23 from the plasma membrane to lipid droplets.

DEFECTS IN INSULIN ACTION ON GSK3

Downstream of Akt, studies in vitro, and more recently use of genetically manipulated mice, have emphasized the role of GSK3 in the regulation of GS phosphorylation and activity (226,227,236-238). Impaired insulin inhibition of GSK3 has therefore been suggested to play a potential key role in diabetes and insulin resistance (176,206). In murine skeletal muscle, insulin inhibits the activity of both the GSK3α and the GSK3β isoforms, but the inhibition of the GSK3β isoform is thought to play the most important role (318,236). However, in human skeletal muscle only the activity of the GSK3α isoform was significantly inhibited by physiological concentrations of insulin in vivo (22,228,319). In the first study of GSK3 activity in human skeletal muscle in vivo, the protein content of both GSK3 isoforms was increased in muscle of patients with type 2 diabetes compared with lean and obese controls, and this explained why total GSK3α activity was increased both in the basal and the insulin-stimulated state in muscle from patients with type 2 diabetes (228). However, consistent with the normal activation of Akt in the similar groups observed by the same research group (120), the insulin-mediated change in total GSK3α activity, the basal and insulin-stimulated specific GSK3α activity, and insulin-mediated Ser phosphorylation of GSK3 were not different between non-diabetic and type 2 diabetic individuals (228). In contrast, we found no differences in the total GSK3α activity in the basal state or the insulin-mediated inhibition of GSK3α activity in skeletal muscle of patients with type 2 diabetes versus obese non-diabetic controls (19). Thus, in our first study, absent insulin activation of GS was associated with normal activation of Akt and normal inhibition of GSK3α (19). Consistent with this report, we have not been able to demonstrate any differences in insulin action on the phosphorylation of GSK3α or GSK3β isoforms in our subsequent studies of insulin signaling in skeletal muscle of patients with type 2 diabetes, and matched lean and obese controls, despite impaired insulin-induced phosphorylation of Akt at Thr308 and Ser473 (26,40). Moreover, in a recent study of lean, obese and type 2 diabetic individuals, insulin-induced phosphorylation of GSK3 was not diminished in patients with type 2 diabetes or insulin-sensitive obese individuals, but only slightly reduced in insulin resistant obese individuals (303). Consistently, insulin action on GSK3 phosphorylation studied in muscle strips ex vivo was reported to be diminished in morbidly obese individuals(293). In our studies of muscle from women with PCOS and carriers of a mutation in *INSR* (22,24), in which insulin-mediated activation andphosphorylation of Akt was reduced, we could not demonstrate any impairment in insulin action on GSK3 activity or phosphorylation compared with matched controls. Thus, the majority of data shows that insulin causes a robust inhibition of GSK3 activity in human skeletal muscle in vivo, but that there is no major intrinsic defect in this part of the insulin signaling cascade to GS downstream of Akt in common metabolic disorders or inherited insulin resistance due to a mutation in *INSR*. Consistently, a recent study of insulin signaling components in muscle samples from almost 200 individuals, revealed that insulin-mediated Akt Thr308 phosphorylation and Akt2 activity correlated with GS-FV activity, but not with GSK3α activity (320).

DEFECTS IN INSULIN ACTION ON GLYCOGEN SYNTHASE ACTIVITY

As discussed above, impaired glycogen synthesis (non-oxidative glucose metabolism) is quantitatively the major abnormality in insulin-stimulated glucose metabolism in patients with type 2 diabetes and to a lesser extent in obesity (12,19,26,28,40,59,60, 117-125). It is therefore not surprising that impaired insulin activation of GS seems to be one of the most well-established signaling defects in skeletal muscle from patients with type 2 diabetes (19,26,28,35,40,60,120,121, 228,321). Only in a single study, in which muscle strips from non-obese patients with type 2 diabetes and matched controls were stimulated with 1.2, 2.4 or 60 nM insulin ex vivo, no differences in insulin activation of GS FV activity was reported (249). In some of these studies (19,28,35,40,120), but not in all (26,121,228), a significant difference in insulin action on GS-FV activity between obese non-diabetic individuals and patients with type 2 diabetes was demonstrated (Fig. 4). Although, we have shown reduced insulin-induced GS-FV activity in obese vs lean non-diabetic individual (26,28), most studies (40,120,121,228), however, indicate that obesity alone does not consistently impair insulin-stimulated GS FV activity. This abnormality in GS activation by insulin has also been demonstrated in most (58,60,294), but not all (255,256) studies of skeletal muscle from non-obese, glucose-tolerant FDR indicating that this may be an early defect in the pathogenesis of insulin resistance and type 2 diabetes.

In our study of women with PCOS, insulin-stimulated GS I-form and FV activities in muscle were markedly reduced compared with matched obese women (24) (Fig. 4). This finding further supports the hypothesis that impaired insulin activation of GS is an early defect in the pathogenesis of insulin resistance independent of fasting hyperglycemia and obesity (58,60,243). Importantly, we showed that treatment of these PCOS patients with pioglitazone for 16 weeks, caused a significant improvement in insulin-stimulated GS I-form and FV activities in muscle. While this positive effect of TZDs on GS FV activity has been reported in a study of insulin-resistant rhesus monkeys (322), the only other study of the effect of TZD showed no improvement on GS activity in response to troglitazone in skeletal muscle from obese, FDR (256). This ability of pioglitazone to normalize the defect insulin action on GS contrasts with studies showing an irreversible defect in insulin-induced GS FV activity in muscle of patients with type 2 diabetes after 10 weeks endurance training (40), or after 3 months treatment with metformin (323), despite significant improvements in whole-body insulin sensitivity in these studies. In carriers of the Arg1174Gln mutation in *INSR*, insulin did stimulate GS I-form activity in skeletal muscle, but compared to controls this response was significantly reduced (22) (Fig. 4). This provides

evidence that disruption of the upstream signaling components do result in impaired insulin action on GS.

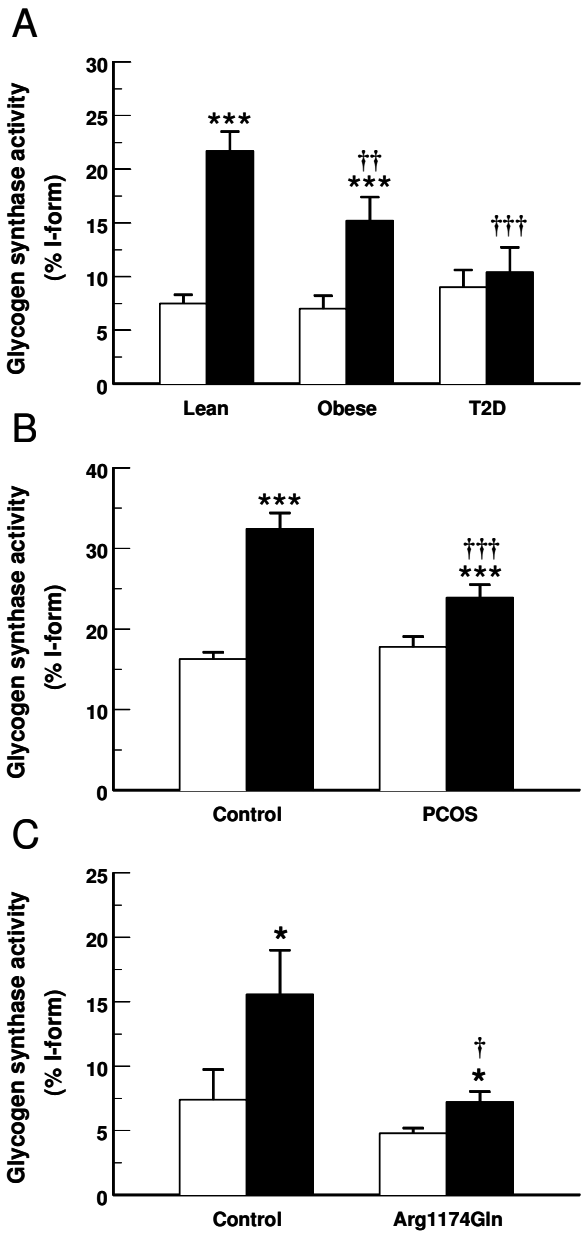

Figure 4. Glycogen synthase activity.

*Glycogen synthase (GS) activity given as % I-form activity (A) in lean (n=10), obese (n=10), and type 2 diabetic (T2D) (n=10) individuals, (B) in control subjects (n=14) and women with PCOS (n=24), and (B) in control subjects and individuals with the Arg11174Gln mutation in the insulin receptor gene (INSR). Measurements were performed in skeletal muscle biopsies obtained during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 3-4 hour euglycemic-hyperinsulinemic clamp using an insulin infusion rate of 40 mU/min per m^2. Data are means ± SEM. ***P < 0.001, and *P < 0.05 vs. corresponding basal values; †††P < 0.001, ††P < 0.01, and †P < 0.05 vs. insulin-stimulated values in controls/lean individuals. Results are adapted from references (22,24,26).*

In several of the above-mentioned studies (24,26,58,60), insulin-stimulated GS FV and I-form activities were shown to correlate strongly (r = 0.52-0.76) with insulin action on both GDR and non-oxidative glucose metabolism. This provides correlative evidence that in human skeletal muscle, insulin-mediated dephosphorylation of GS can explain a substantial fraction of insulin-stimulated glycogen synthesis. While activation of GS by insulin is an important signal in human skeletal muscle to store glucose as glycogen, the studies reviewed above also demonstrate that most, if not all, forms of insulin resistance are accompanied by impaired insulin activation of GS.

DEFECTS IN INSULIN ACTION ON GLYCOGEN SYNTHASE PHOSPHORYLATION

Of at least nine Ser residues phosphorylated in mammalian GS in vivo (176), insulin activation of GS is to a major extent mediated by dephosphorylation of the NH_2-terminal sites 2 and 2a, and the COOH-terminal sites 3a and 3b (23,176,206,208). Until recently, the effect of insulin on the phosphorylation of GS at these sites in human skeletal muscle, and their potential role for impaired insulin activation of GS in insulin resistant conditions had not been addressed. The finding of a normal insulin-mediated inhibition of muscle GSK3 in most studies of insulin resistant cohorts (19,22,24,26,40), did not a priori exclude impaired dephosphorylation of GS at sites 3a and 3b as the cause of impaired GS activity. Thus, although GSK3 is thought to be the major kinase of sites 3a and 3b (176), these sites are also phosphorylated by other kinases (23,206,208). To further investigate this, we have generated a panel of phosphoepitope-specific antibodies that specifically recognize GS when phosphorylated at both sites 3a and 3b (3a+3b), both sites 2 and 2a (2+2a), or only at site 2, 3a, 3b, 1a or 1b. Application of these antibodies in a series of studies have demonstrated that GS is dephosphorylated at site 3a+3b as well as site 2+2a in response to physiological doses of insulin in human skeletal muscle in vivo (24,26,40,324).

In the first study using these antibodies, we showed that infusion of insulin decreased phosphorylation of GS at sites 3a+3b equally and significantly in skeletal muscle from type 2 diabetic and obese non-diabetic individuals (19). This response was accompanied by inhibition of GSK3α. In patients with type 2 diabetes, insulin infusion, however, caused a marked increase in the phosphorylation of muscle GS at sites 2+2a, whereas no change in the phosphorylation of GS at sites 2+2a was observed in obese non-diabetic controls (19). These data indicated that the intact effect of insulin on GS site 3a+3b was counteracted by a simultaneous increase in the phosphorylation of the NH_2-terminal sites (sites 2+2a) in patients with type 2 diabetes. In a subsequent study of patients with type 2 diabetes, and lean and obese controls, we also found a robust and similar insulin-induced dephosphorylation of GS at sites 3a alone, 3b alone and 3a+3b in all groups (26) (Fig. 5). In lean controls, insulin induced GS dephosphorylation on site 2+2a. This is consistent with a recent study of a large cohort of non-diabetic twins, in which insulin was shown to dephosphorylate GS at sites 2+2a by ~20% in skeletal muscle (324). However, again there was no effect of insulin on the phosphorylation of GS site 2+2a in patients with type 2 diabetes or obese controls (26) (Fig. 6). GS site 2+2a phosphorylation tended to be increased in patients with type 2 diabetes compared with both lean and obese controls (26). These results suggested that both in obese individuals with and without type 2 diabetes, the lack of dephosphorylation at GS site 2+2a counteracts the activating effect of the site 3a+3b dephosphorylation during insulin stimulation, and thus contributes to the impaired insulin action on GS. Surprisingly, we observed that phosphorylation of GS at site 1b was markedly elevated (~2 fold) by insulin in muscle of patients with type 2 diabetes, and this was accompanied by hyperphosphorylation of the CaMKII target, phospholamban at Thr17 (26). As CaMKII phosphorylates both sites 1b and site 2 in vitro (23,206,208), we pro-

posed that an increase in the endogenous activity of CaMKII could play a role for abnormal phosphorylation of GS at these sites in muscle of patients with type 2 diabetes. Alternatively, hyperphosphorylation of GS at site 1b could regulate the cellular localization of GS, and prohibit its activation by insulin signaling (214).

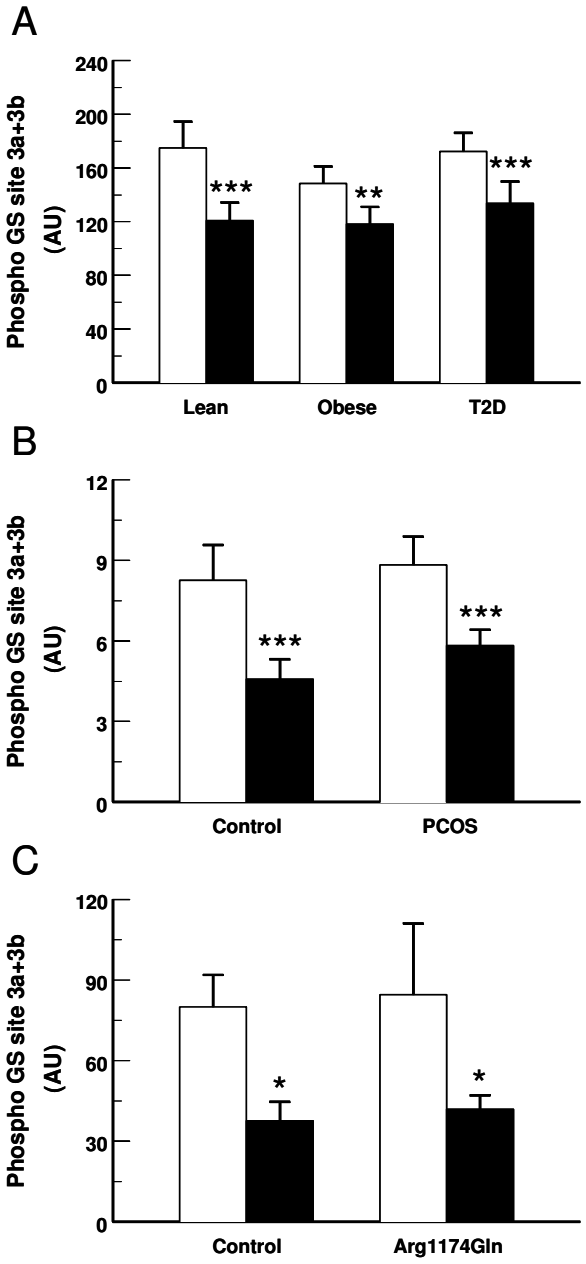

Figure 5. Glycogen synthase phosphorylation at site 3a+3b.
*Phosphorylation of glycogen synthase (GS) at site 3a+3b (A) in lean (n=10), obese (n=10), and type 2 diabetic (T2D) (n=10) individuals, (B) in control subjects (n=14) and women with PCOS (n=24), and (C) in control subjects and individuals with the Arg11174Gln mutation in the insulin receptor gene (INSR). Measurements were performed in skeletal muscle biopsies obtained during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 3-4 hour euglycemic-hyperinsulinemic clamp using an insulin infusion rate of 40 mU/min per m^2. Data are means ± SEM. ***P < 0.001, **P < 0.01 and *P < 0.05 vs. corresponding basal values. Results are adapted from references (22,24,26).*

In muscle extracts from all three groups, we also found more than 90% recovery of GS activity after phosphatase treatment in vitro (26). This indicates a major role of phosphorylation abnormalities in the mechanism for the impaired activation of GS by insulin in obesity and type 2 diabetes.

In a recent study of obese patients with type 2 diabetes and obese controls, we again observed a significant and similar decrease of GS site 3a phosphorylation in both groups (35). In patients with type 2 diabetes, phosphorylation of GS at site 2+2a tended to increase in response to insulin, and was higher after insulin than in obese controls (35). Thus, these results confirmed our initial observations (19). In our most recent study, however, physiological hyperinsulinemia significantly reduced phosphorylation of muscle GS at sites 2+2a and 3a+3b in both lean and obese controls (40). As mentioned before, the obese individuals in this study were relatively insulin sensitive, and also had normal insulin activation of GS FV activity (49). Consistent with our previous studies (19,26), there was no effect of insulin on GS phosphorylation at sites 2+2a in patients with type 2 diabetes during a euglycemic clamp (40). Of interest, we could not reproduce the finding of increased phosphorylation of muscle GS at site 1b in these patients with type 2 diabetes (26). This likely reflects the large heterogeneity in both type 2 diabetic and obese non-diabetic individuals combined with the relatively small sample sizes often used in such studies.

In a study of skeletal muscle from obese women with and without PCOS, we observed, that insulin caused a significant decrease in the phosphorylation of GS at sites 2+2a, site 3a alone and 3a+3b in obese controls, but only at sites 3a+3b in women with PCOS (24) (Fig. 5-6). Thus, also in muscle of women with PCOS an absent insulin-mediated dephosphorylation of GS at sites 2 and 2a seems to explain impaired insulin activation of GS, whereas dephosphorylation at sites 3a and 3b was normal consistent with adequate inhibition of the major upstream kinase, GSK3 (24). In our study of Arg1174Gln carriers, insulin infusion reduced phosphorylation of GS at sites 3a+3b by ~50% in both groups, whereas the phosphorylation of GS at sites 2+2a was unaffected by insulin infusion in both groups (22) (Fig. 5-6). While a diminished insulin activation of Akt provides an explanation for a reduced glucose transport in these Arg1174Gln carriers, we were unable to demonstrate the mechanism causing impaired insulin activation of GS, and hence glycogen synthesis (22). Thus, both insulin-mediated inhibition of GSK3 and subsequent dephosphorylation of GS at sites 3a+3b was normal. Perhaps the sample sizes were too small to identify abnormalities in GS phosphorylation. Another possibility is that increased phosphorylation at site 3a or 3b alone, or at sites believed to be less important for GS activity, such as sites 1a,1b, 3c, 4 and 5 are involved.

Several studies using these antibodies have provided evidence for a strong relationship between insulin action on GS activity and dephosphorylation of GS at site 2+2a and 3a+3b. Thus, we found that insulin-stimulated values of GS activity measured both as %I-form and %FV correlated inversely with GS phosphorylation at site 2+2a and site 3a (r=0.56-0.65) in a mixed cohort of patients with type 2 diabetes, and lean and obese controls (26). In our study of obese women with and without PCOS, multiple linear regression analysis showed that, insulin-mediated dephosphorylation of both site 2+2a and sites 3a+3b plays a major role for activation of GS in response to physiological hyperinsulinemia (24). However, in healthy obese women, dephosphorylation at sites 2+2a seems to be the best predictor of insulin activation of GS, whereas in women with PCOS, insulin activation of GS seems to be reliant upon dephosphorylation at sites 3a+3b and perhaps site 1a (24). In a recent evaluation of results from a large cohort of twins (324), it was demonstrated that measures of insulin-

induced Akt Thr308 phosphorylation, Akt2 activity and GS-FV all correlated inversely with phosphorylation of GS at site 2+2a, but not with phosphorylation of GS at sites 3a+3b (320). The authors concluded, that the association between proximal insulin signaling and GS activity in healthy individuals may be mediated primarily through Akt-dependent regulation of NH_2-terminal GS phosphorylation (320).

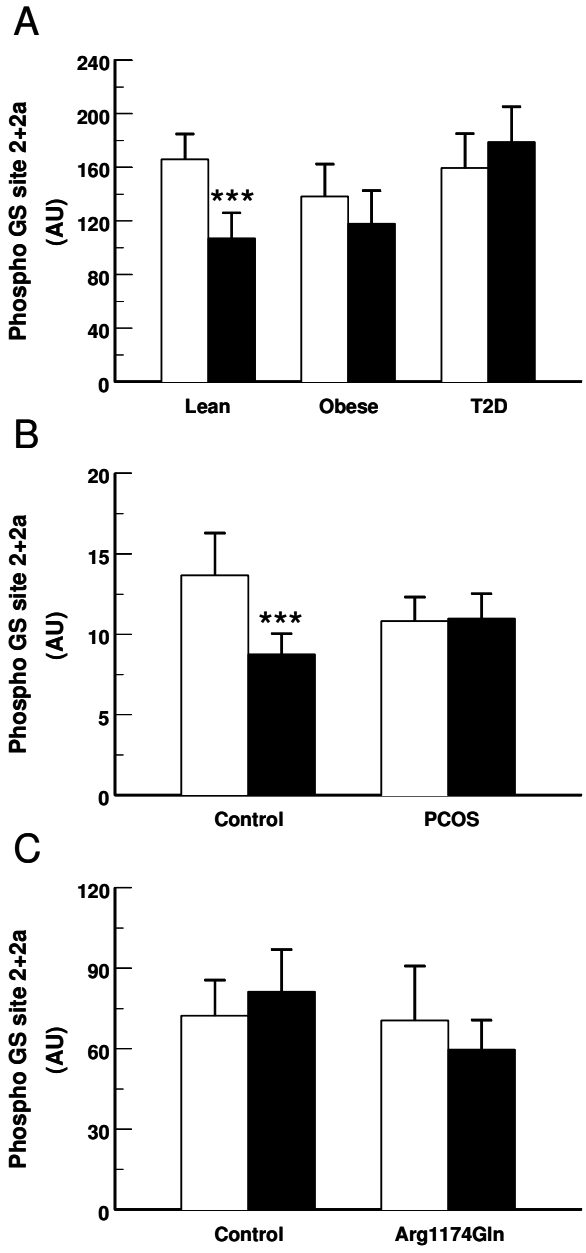

Figure 6. Glycogen synthase phosphorylation at site 2+2a.
*Phosphorylation of glycogen synthase (GS) at site 2+2a (A) in lean (n=10), obese (n=10), and type 2 diabetic (T2D) (n=10) individuals, (B) in control subjects (n=14) and women with PCOS (n=24), and (C) in control subjects and individuals with the Arg11174Gln mutation in the insulin receptor gene (INSR). Measurements were performed in skeletal muscle biopsies obtained during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 3-4 hour euglycemic-hyperinsulinemic clamp using an insulin infusion rate of 40 mU/min per m^2. Data are means ± SEM. ***P < 0.001 vs. corresponding basal values. Results are adapted from references (22,24,26).*

Taken together, current available studies show that insulin-mediated activation of Akt and inhibition of GSK3, and subsequent dephosphorylation of GS at both sites 2+2a and sites 3a+3b, are likely to be important mechanisms by which insulin stimulates GS activity and hence glycogen synthesis in healthy human skeletal muscle in vivo. However, the relative amount of GS phosphorylated at sites 2+2a compared with sites 3a+3b in human skeletal muscle, and hence the relative contribution of these phosphorylation sites to GS activity in vivo is at present unknown. The impaired insulin activation of muscle GS and the hyperphosphorylation or lack of dephosphorylation of GS at sites 2+2a seems to be a rather consistent finding in common forms of insulin resistance such as obesity, type 2 diabetes and PCOS (19,24,26,35,40). Moreover, in both common forms of insulin resistance and insulin resistance caused by a mutation in *INSR*, we were unable to demonstrate a significant reduction in the insulin-mediated dephosphorylation of GS at sites 3a+3b. These sites are to a major extent regulated by GSK3 during insulin stimulation, and in none of our studies, we have found any evidence that the ability of insulin to inhibit GSK3 is compromised in skeletal muscle of type 2 diabetic patients.

ADIPONECTIN AND ITS INTRACELLULAR EFFECTS IN MUSCLE

Adiponectin is an adipokine with anti-inflammatory, antiatherogenic and insulin sensitizing properties, which is synthesized and secreted mainly by adipocytes (325). It is abundantly present in the circulation of humans (5-10 µg/ml), and in contrast to other adipokines, plasma levels of adiponectin decrease with increasing adiposity, and correlate inversely with multiple indices of insulin resistance in humans (326,327). Moreover, low levels of adiponectin predict an increased risk of type 2 diabetes and cardiovascular disease (328-330). Since its discovery in the mid-1990s (331-333), these findings have prompted researchers to identify the actions of adiponectin and its use as a biomarker. Adiponectin circulates predominantly as full-length adiponectin in different oligomeric forms such as low-molecular weight (LMW) trimers, medium-molecular weight (MMW) hexamers and high-molecular weight (HMW) multimers (325,327), whereas the proteolytic cleavage product of the full-length form, globular adiponectin, represents less than 1% of total adiponectin (334). It has been suggested that globular adiponectin is the biological active form, in particular, in muscle (335). However, HMW adiponectin seems to be the most active and predominant form, and may be the most physiological relevant form since it is the only form (325,327,336,337), which shows the same strong inverse relationship with insulin resistance and type 2 diabetes as total adiponectin (338).

Initial studies of murine models and C2C12 myocytes treated with globular or full-length adiponectin have provided evidence that at a major part of the insulin-sensitizing effect of adiponectin could be explained by its ability to stimulate glucose uptake and lipid oxidation in muscle cells, and decrease hepatic glucose production (339-343). Of these actions, the insulin-sensitizing properties of adiponectin in skeletal muscle, will be the focus of this review. The intracellular actions of adiponectin are thought to be mediated by binding of adiponectin to one of its two transmembrane receptors, AdipoR1 or AdipoR2, of which AdipoR1 are abundantly expressed in skeletal muscle and liver of mice, whereas AdipoR2 is predominantly expressed in liver (325,340). In human skeletal muscle, AdipoR2 also seems to be highly expressed (344). The signaling mechanisms by which adiponectin exerts its metabolic effects in skeletal muscle were initially shown to be mediated by activation of AMPK and inhibition of its down-stream effector,

acetyl-CoA-carboxylase (ACC), as well as stimulation of p38 MAPK and PPARα in muscle from murine models or cultured muscle cells (340-343).

The evidence that adiponectin activates AMPK was interesting, because this enzyme is known to regulate a number of metabolic and cellular pathways, which have been implicated in the pathogenesis of insulin resistance in skeletal muscle. As reviewed elsewhere (345,346), AMPK is an αβγ heterotrimer that is activated by metabolic stresses such as increases in cellular AMP, ADP or Ca2+. In humans, AMPK subunits are encoded by seven genes (α1, α2, β1, β2, γ1, γ2, and γ3) that can form at least 12 heterotrimers, of which only three (α1β2γ1,α2β2γ1, and α2β2γ3) are present to a significant extent in skeletal muscle in vivo (347). AMPK is activated by phosphorylation of its catalytic α-subunit at Thr172 by its upstream kinases, the LKB1-STRAD-MO25 complex and CamK kinase II (CaMKKII) (345). AMPK stimulates fatty acid oxidation by inhibition of acetyl-CoA carboxylase-β (ACCβ). This causes a decrease in malonyl-CoA, which relieves the inhibition of carnitine palmitoyltransferase-1 (CPT1), the enzyme that controls the transfer of fatty acids into mitochondria (345). Acute activation of AMPK in skeletal muscle is also involved in contraction-mediated glucose transport and GLUT4 translocation via inhibition of AS160/TBC1D4 and TBC1D1, and activation of RABs (348). Moreover, chronic activation of AMPK induces an increased mitochondrial biogenesis and GLUT4 content (349-352). This seems to be mediated by an increased abundance, as well as activation of PGC-1α by phosphorylation and deacetylation by SIRT1 (345). Thus, the AMPK system provides a possible link between low adiponectin, insulin resistance and mitochondrial dysfunction (327,353).

Within the past five years, several studies have provided support for a crosstalk between adiponectin and insulin signaling pathways mediating the insulin sensitizing effects of adiponectin. First, the adaptor protein, APPL1, was shown to interact with AdipoR1 in response to adiponectin in C2C12 myocytes in vitro, and this caused activation of AMPK, p38 MAPK as well as increased GLUT4 translocation (354). Moreover, adiponectin and insulin had a synergistic effect on Akt activation, which was abolished by APPL1 downregulation (354). The insulin-sensitizing effects was recently supported by a study of APPL1 overexpression in single muscles of mice in vivo. Thus, APLL1 overexpression caused increased insulin-stimulated glycogen synthesis, a modest increase in glucose uptake, and an increase in both basal and insulin-stimulated phosphorylation of IRS1 Tyr608, Akt Ser473, AS160/TBC1D4 Thr642 and GSK3β Ser9 (355). However, in contrast to the results in vitro, APPL1 overexpression in vivo reduced AMPK and ACC phosphorylation, as well as PGC-1α abundance (355). These results, together with the finding of increased APPL1 mRNA and protein content in human skeletal muscle from obese individuals with and without type 2 diabetes (356), questions the role of APPL1 in mediating the insulin-sensitizing effects of adiponectin in vivo, although further studies are needed. However, a recent study reported that overexpression of AdipoR1 in single muscles of rats in vivo caused increased glucose disposal and glycogen synthesis, and that this was explained by an increased phosphorylation of IRS1 Tyr608, pAkt Ser473 and GSK3β Ser9 (357). Moreover, an additive effect of insulin on these measures was demonstrated. In contrast to APPL1 overexpression, the AdipoR1 overexpression increased AMPK and ACC phosphorylation and PGC-1α levels. Consistently, a study of mice with muscle-specific knock-out of AdipoR1 also reported decreased insulin-mediated glucose disposal, which was associated with impaired Tyr phosphorylation of IRS1, and Akt Ser473 phosphorylation (358). They also demonstrated increased Ser phosphorylation of IRS1, which seemed to be mediated by increased phosphorylation of JNK and S6K1, the latter indicating a role for inhibition of mTORC1 by AMPK in insulin signaling (358). This insulin-sensitizing effect of adiponectin mediated via activation of AMPK and inhibition S6K1 was also suggested by another recent study in myocytes (359), but phosphorylation of S6K1 was in fact increased in muscle of mice with overexpression of AdipoR1 suggesting a role for AMPK-independent mechanisms in the cross-talk of adiponectin with insulin signaling (357). Importantly, disruption of AdipoR1 in mice together with several silencing experiments in C2C12 myocytes demonstrated a role for increased in intracellular Ca2+ and activation of CaMKII as major mediators of AMPK activation and subsequent activation of PGC-1α and mitochondrial biogenesis (358).

In summary, these recent studies in murine models and myocytes have provided insight into the molecular mechanisms by which adiponectin exerts its beneficial effects on fatty acid oxidation, mitochondrial oxidative capacity, and glucose disposal in skeletal muscle. The following chapters will focus on studies in humans, which support a role for low adiponectin in the pathogenesis of common forms of insulin resistance, and also provide correlative evidence for a positive effect of adiponectin on several aspects of glucose and lipid metabolism, as well as components of insulin signaling and AMPK in skeletal muscle.

ADIPONECTIN AND GLUCOSE AND LIPID METABOLISM IN INSULIN RESISTANCE

Twins and family studies have shown that circulating adiponectin are highly heritable (30–70%) (360-362), and recent GWA studies have demonstrated that variants in the adiponectin gene (*ADIPOQ*), as well as variants in other genes/loci contribute to the variability of circulating adiponectin (363-365). However, none of these genetic variants are among the ~65 common genetic variants presently known to be robustly associated with type 2 diabetes, or the more than ~30 common genetic variants found to be associated with obesity and BMI (41,42). Therefore, it is less likely that common genetic variants that contribute to the variability in circulating adiponectin play a major role in insulin resistance in common metabolic disorders such as obesity, PCOS and type 2 diabetes. It is well-established that obesity, PCOS and type 2 diabetes are associated with lower levels of circulating adiponectin, and due to its strong association with multiple indices of insulin sensitivity, plasma adiponectin is now routinely reported as a biomarker of insulin resistance in most studies of common forms of insulin resistance. We will therefore focus our discussion on studies, which have evaluated the relationship between plasma adiponectin and measures of insulin action on glucose and lipid metabolism using euglycemic-hyperinsulinemic clamp studies and indirect calorimetry.

In our first study of plasma adiponectin, we pooled results from clamp studies of two smaller cohorts of patients with type 2 diabetes, and matched obese and lean controls (28). In agreement with other studies (366,367), we observed reduced plasma adiponectin in patients with type 2 diabetes, whereas no significant difference between obese and lean controls could be demonstrated (28). In a subsequent study of plasma adiponectin in patients with type 2 diabetes and BMI matched, younger and glucose tolerant FDR, we found comparable low levels in both groups. Lower adiponectin levels have been reported in most (344,368-371), but not all (372,373), studies of both lean and obese FDR compared with weight-matched controls. Our results

suggested that low adiponectin is an early abnormality in the pathogenesis of type 2 diabetes, which in part is independent of obesity. Consistent with several other studies (374-378), we also reported lower levels of total adiponectin in obese women with PCOS compared with weight-matched controls. This again suggested a relation between low adiponectin and insulin resistance, which is in part independent of obesity. Some previous studies have claimed that the HMW form of adiponectin is a better predictor of insulin sensitivity and metabolic disorders than total adiponectin. (336,337,379). However, as noted above, most of these studies, as well as a more recent study, do not support the superiority of HMW over total adiponectin (338). In our study of PCOS patients, we were in fact unable to demonstrate a significant reduction in HMW adiponectin, despite lower total adiponectin.

Most recently, we have examined plasma adiponectin and its multimers together with other known markers of insulin resistance such as leptin, IGFBP1 and IGFBP2 (380-385), and the IGF axis in carriers of a dominant-negative mutation (Arg1174Gln) in *INSR* (37). We did that because recent studies have reported a paradoxical increase in plasma adiponectin and IGFBP1 in patients with homozygous or compound heterozygous (biallelic) *INSR* mutations (386-388). Based on previous studies showing that long-term infusion of insulin suppresses plasma adiponectin and its expression and/or secretion from adipocytes (28,386,389-391), we hypothesized that this action of insulin is relatively intact in common forms of insulin resistance, and that fasting hyperinsulinemia in these conditions may contribute to their lower levels of plasma adiponectin, whereas in individuals with kinase-deficient or biallelic mutations in *INSR* this effect of insulin is reduced or abolished (386-388). In the face of highly elevated fasting insulin levels and marked insulin resistance, total plasma levels of adiponectin, IGFBP1 and IGFBP2 were surprisingly normal in carriers of a dominant-negative *INSR* mutation compared with lean, healthy controls (37). Moreover, insulin infusion, which raised serum insulin to ~1300 pmol/l in Arg1174Gln carriers, failed to suppress plasma adiponectin. This suggested an impaired ability of insulin to suppress plasma adiponectin, as well as known markers of common insulin resistance in these individuals. However, the HMW fraction of adiponectin was increased, while the total levels and fraction of LMW adiponectin were reduced, and we also found elevated levels of total IGF1, IGF2 and free IGF1. We proposed that these changes in the multimeric distribution of adiponectin and the IGF1-axis could contribute to rescue insulin action in these individuals.

To explore the mechanisms underlying the insulin-sensitizing effect of circulating adiponectin in humans in vivo, we have examined the relationship between plasma adiponectin and insulin action on whole-body parameters of lipid and glucose metabolism during euglycemic-hyperinsulinemic clamp studies in a mixed population of lean, obese and type 2 diabetic individuals (28), women with and without PCOS (29), and a cohort of young FDR and patients with type 2 diabetes (30). In all studies, we demonstrated a strong relationship between plasma adiponectin and insulin action on GDR and non-oxidative glucose metabolism (28-30), which was also present in the subgroups of women with PCOS (29), young FDR and patients with type 2 diabetes (30). Similar relationships have been shown in several other mixed populations of healthy, obese and type 2 diabetic individuals (366,367,392-396), in women with PCOS (397,398) and in young individuals with and without a family history of type 2 diabetes (368). These studies provide correlative evidence that adiponectin improves insulin sensitivity by increasing insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle. In the mixed population of lean, obese and type 2 diabetic individuals and the pooled cohort of young FDR and patients with type 2 diabetes, we also observed that plasma adiponectin correlated positively with glucose oxidation and RQ, and negatively with lipid oxidation and FFA during insulin stimulation (28,30). These findings, which were also reported in young individuals with and without a family history of type 2 diabetes (368), and more recently a large cohort of lean and obese healthy individuals (396), suggest that the insulin-sensitizing effect of plasma adiponectin may involve an improved metabolic flexibility. This is supported by a study showing a strong relationship between plasma adiponectin and markers of mitochondrial content in a cohort of individuals with and without a family history of type 2 diabetes (399). However, the results are based on measures of whole-body substrate utilization, and the strong inverse relationship between basal plasma adiponectin and the ability of insulin to suppress FFA could also indicate a role for adiponectin in sensitizing adipocytes to the antilipolytic effect of insulin. In the small group of carriers of a dominant-negative mutation in *INSR*, we observed an even stronger relationship between plasma adiponectin and insulin action on GDR, glucose oxidation, lipid oxidation and FFA suggesting a role for the altered multimeric distribution of adiponectin in the preservation of insulin sensitivity in these individuals (37)

The insulin-sensitizing effects of treatment with TZDs may, in part, involve the ability to induce a robust increase in plasma adiponectin. Thus, treatment with TZDs causes a substantial increase in plasma adiponectin in both lean healthy, obese non-diabetic and type 2 diabetic individuals, as well as in women with PCOS (337,376,397,400-406). Consistently, we have reported that treatment with TZDs caused a ~2-fold increase in plasma adiponectin in both patients with type 2 diabetes, FDR and women with PCOS (29,30). This increase was accompanied by an increased insulin action on GDR and non-oxidative glucose metabolism in all cohorts, whereas an improved insulin action on glucose oxidation and lipid oxidation was seen only in women with PCOS. Moreover, we found a tight correlation between the increase in plasma adiponectin induced by troglitazone and improvements in Si and insulin-stimulated GDR in FDR, but not in type 2 diabetic patients (30). The lack of a correlation between the TZD-induced increases in plasma adiponectin and insulin-stimulated GDR has also been observed in a mixed population of lean, obese and type 2 diabetic patients, and in obese women with PCOS (367,397). However, in another study of patients with type 2 diabetes, improved insulin sensitivity in response to TZDs correlated with increased HMW/total adiponectin-ratio, but not with changes in total adiponectin implying a special role for HMW adiponectin (337). In our study of women with PCOS, we showed that the pioglitazone induced increase in total adiponectin correlated significantly with improvements in insulin action GDR, glucose oxidation, and FFA, whereas the increase in HMW only correlated with the incremental increase in insulin-stimulated GDR (29). While these studies show somewhat conflicting results, they suggest that a potential adiponectin-dependent insulin-sensitizing effect of thiazolidinediones is exerted mainly on insulin-mediated glucose metabolism in skeletal muscle.

ADIPONECTIN CROSS-TALK WITH INSULIN SIGNALING AND AMPK IN HUMAN MUSCLE

As reviewed above, studies in humans have provided correlative evidence that lower plasma adiponectin in common forms of insulin resistance such as obesity, PCOS, FDR and type 2 diabetes

contribute to impaired insulin action on glucose disposal and glycogen synthesis in skeletal muscle, and to a lesser extent reduced insulin action on lipid metabolism. In insulin resistance caused by partial disruption of insulin signaling downstream of the insulin receptor, plasma adiponectin was surprisingly high, but nonetheless showed strong relationship with insulin action on glucose and lipid metabolism. These findings have prompted us and other researchers to look for abnormalities in the intracellular actions of adiponectin in human skeletal muscle.

Based on earlier reports showing that adiponectin enhances insulin-stimulated Tyr phosphorylation of the insulin receptor and IRS1 in rat muscle (339), and that plasma adiponectin correlates with insulin-stimulated Tyr phosphorylation of the insulin receptor in a cross-sectional study of lean, obese and type 2 diabetic individuals (392), we hypothesized that low plasma adiponectin levels could contribute to impaired insulin activation of muscle GS, one of the most consistent findings in common forms of insulin resistance (23,243). In a mixed population of lean, obese and type 2 diabetic individuals (28), we demonstrated that plasma adiponectin correlated positively with insulin-stimulated GS FV activity as well as the incremental increase in GS FV activity. Consistently, we found a significant relationship between plasma adiponectin and insulin action on GS activity in young FDR matched to a group of obese patients with type 2 diabetes (30). However, in the subgroup of patients with type 2 diabetes (30), and also in obese women with and without PCOS (29), we were unable to demonstrate a correlation between plasma adiponectin and insulin activation of GS (unpublished results). The lack of correlation in the rather large group (n=54) of women could be explained by a general less tight association of plasma adiponectin with many of its known effects in women as shown recently (407). Thus, in our first study (28), and also among FDR (30), more than two-thirds of the participants were men. Nevertheless, we proposed that the correlation between plasma adiponectin and insulin action on GS provided a potential explanation at the molecular level for the insulin-sensitizing effect of adiponectin (28,30). This is now strongly supported by studies of murine models and cultured muscle cells, in which genetic manipulation of AdipoR1 and APPL have demonstrated that adiponectin certainly cross-talks with the insulin signaling cascade (354,355,357,358).

Although AMPK is not the focus of this review, it is believed to be an important mediator of the response to adiponectin in skeletal muscle (345,346). We have, therefore, also measured activity, phosphorylation and protein content of AMPK subunits in several of our studies of obesity, type 2 diabetes and PCOS (25,27,28,35). Besides from a correlation between plasma adiponectin and $\alpha 2$ AMPK activity in a small subgroup of obese, non-diabetic individuals (28), we have not found any evidence for a relationship between plasma adiponectin and measures of muscle AMPK activity or content in any of these mixed populations or subgroups, despite the strong correlation of adiponectin with insulin action on glucose and lipid metabolism, and evidence for down-regulation of markers of mitochondrial biogenesis in similar study cohorts (31,36,38,39). Moreover, α-AMPK activities and Thr172 phosphorylation of α-AMPK, as well as protein content of most AMPK subunits in skeletal muscle showed no difference between obese men with and without type 2 diabetes and matched obese non-diabetic individuals, or between obese women with and without PCOS (25,28). Finally, we found no effect of treatment with pioglitazone on these measures of AMPK activity and content in a subgroup of women with PCOS, despite a robust increase in plasma adiponectin, and also evidence of an improved PGC-1α mediated mitochondrial biogenesis (38,39). In agreement with our data, other studies of human skeletal muscle in vivo have been unable to detect abnormalities in measures of AMPK activity in obese women and patients with type 2 diabetes compared to matched controls (347,408-410). However, a recent study reported lower total AMPK activity and ACC phosphorylation associated with lower fatty acid oxidation rates in muscle of obese male individuals with and without type 2 diabetes compared with lean controls (406). This suggests that at least in males obesity may impair AMPK activity. Moreover, this and another study have demonstrated that treatment of patients with type 2 diabetes with either rosiglitazone or pioglitazone for 3-6 months causes increased activation of AMPK, phosphorylation of ACC, and increased expression of markers of mitochondrial biogenesis and fat metabolism in skeletal muscle (405,406).

Thus, although most data do not support a major relationship between AMPK and the insulin-sensitizing effects of adiponectin in human skeletal muscle, at least some reports demonstrate that the AMPK-system is an interesting target of treatment in common metabolic disorders. Of interest, we have unpublished results (Kristensen et al) showing that AMPK activity and content are normal in carriers of a mutation in *INSR* despite reduced PGC-1α levels and markers of mitochondrial biogenesis. This further indicates that a normal AMPK activity is not dispensable for a normal mitochondrial biogenesis.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Insulin resistance plays an important role in the risk of type 2 diabetes and cardiovascular disease. Quantitatively, skeletal muscle is the major site of impaired insulin-stimulated glucose metabolism and, hence, insulin resistance in common metabolic disorders such as obesity, type 2 diabetes and PCOS. As reviewed above, several research groups have investigated the molecular mechanisms underlying skeletal muscle insulin resistance within the past 15 years. In general, impaired insulin activation of glucose transport and glycogen synthesis, and an association of low plasma adiponectin with impaired insulin action on glucose metabolism appear as consistent and early markers of insulin resistance. Although results from studies of human skeletal muscle are observational, and at best can provide correlative evidence for known and potential novel mechanisms, they are critical to confirm or reject findings in murine muscle and cultured muscle cells. Thus, the relative importance of different components in the insulin signaling cascade for the effect of insulin on glucose transport and glycogen synthesis may differ significantly between muscle in such models and human skeletal muscle. Indeed, the insights gained from studies of human skeletal muscle have substantially increased our understanding of impaired insulin signal transduction and the role of low adiponectin in insulin resistance. Insulin in physiological concentrations causes a robust activation of IRS1, PI3K, Akt2, AS160/TBC1D4 and RAC1 as well as inhibition of GSK3 and activation of GS by dephosphorylation of both site 2+2a and site 3a+3b in healthy human skeletal muscle in vivo. These effects of insulin are accompanied by, often correlate with and likely explain the majority of insulin-stimulated glucose uptake and glycogen synthesis. Correspondingly, insulin resistance in common metabolic disorders and inherited insulin resistance is reported to be accompanied by abnormalities in the insulin signaling cascade. This includes observations of impaired insulin action on IRS1, PI3K, Akt and AS160/TBC1D4, and, more consistently, a defect in insulin activation of GS, which is not caused by

reduced inhibition of GSK3 and its target sites (site 3a+3b) on GS. At least in obesity, PCOS and type 2 diabetes, impaired insulin action on GS seems to involve a lack of dephosphorylation of GS at site 2+2a (24,26,35,40). These results are supported by data indicating that Akt2 may mediate its effect on GS primarily by enhancing dephosphorylation at site 2+2a rather than inhibition of GSK3 in human skeletal muscle (320). Further characterization of the phosphorylation of GS at multiple sites, the potential kinases and/or phosphatases involved, as well as the mechanism by which impaired insulin activation of Akt2 causes defect insulin action on GS in insulin resistant conditions are warranted in future studies of human skeletal muscle.

Although several studies have been unable to demonstrate impaired insulin activation of IRS1, PI3K, Akt and/or AS160/TBC1D4 in skeletal muscle in obesity, type 2 diabetes and PCOS, the vast majority of studies have shown either impaired insulin action or no difference in effect, and only rarely an enhanced insulin action on these insulin signaling enzymes has been reported. This indicates that defects in these components of the insulin signaling cascade do play a role in insulin resistance in human skeletal muscle, but that the huge overlap in insulin sensitivity between healthy and insulin resistant individuals, differences in study designs, and the use of small sample sizes often hamper the ability to detect a significant difference. Sometimes the magnitude of reported differences in measures of insulin signaling in obesity, type 2 diabetes and PCOS appear much lower than the accompanying differences in insulin sensitivity, and sometimes even fully reversal of the insulin signaling defect, e.g. by TZDs, does not fully restore insulin sensitivity. This suggests the possibility that currently applied methods are unable to fully detect impairment in the investigated enzymes, or that larger defects are localized more distal in signaling to glucose transport and glycogen synthesis.

In favor of the former, the involvement of critical nodes such as IRS1, PI3K and Akt in down-stream signaling to a variety of biological processes suggests that simple measurement of activity and phosphorylation of these enzymes may not perfectly reflect their involvement in the stimulation of glucose transport and glycogen synthesis (175). Moreover, there is emerging evidence that intracellular compartmentalization plays a key role in skeletal muscle glycogen metabolism and insulin signaling, and, therefore, that measurement of the abundance and activity of enzymes in muscle homogenates and lysates may not necessarily reflect the regulation investigated (411). In the future, increased application of e.g. confocal immunoflourescence microscopy and transmission electron microscopy in studies of human skeletal muscle biopsies may help to resolve such issues. On the other hand, recent reports of abnormalities in the intracellular distribution of the SNARE protein SNAP23 as well as impaired insulin activation of RAC1 in skeletal muscle of patients with type 2 diabetes strongly imply that there is still much to learn about distal components in the regulation of GLUT4 translocation (32,201). In the future, studies of the site-specific phosphorylation of AS160/TBC1D4 and TBC1D1, the abundance and activation of different Rabs and RAC1, and redistribution of SNAP23 to lipid droplets in human skeletal muscle from insulin resistant individuals are needed to further address the role of these molecules in the pathogenesis of insulin resistance.

In inherited insulin resistance caused by mutation in the IRTK domain of *INSR*, we could not demonstrate a defect in insulin activation of IRTK, but observed impaired insulin signaling along IRS1, PI3K, Akt2 and GS to a magnitude surprisingly similar to that observed in common metabolic disorders (22). Thus, whereas an impaired insulin clearance appeared to fully preserve insulin action on IRTK, it only partially preserved insulin action on down-stream signaling molecules. One could therefore speculate that the observed defects in skeletal muscle are not directly caused by mutant *INSR* within muscle, but may be a consequence of mutant *INSR* in other tissues. Despite normal circulating levels of adiponectin in carriers of a mutation in *INSR* (37), we can not exclude that an increased secretion of factors from other tissues could negatively modulate insulin signaling in skeletal muscle. We are currently recruiting individuals with the same mutation in *INSR* to examine the expression of proinflammatory molecules in adipose tissue, circulating levels of a panel of adipokines/cytokines in the circulation, as well as microbiota, and plasma and urine metabolites.

Another important finding in studies of insulin resistant conditions is that although plasma adiponectin is decreased in obesity, type 2 diabetes and PCOS, this is not associated with decreased AMPK activity (25,27,409). Instead, a strong association of plasma adiponectin with insulin-stimulated glucose disposal and glycogen synthesis in several studies, and with insulin activation of the insulin receptor and GS, at least in some studies (26,30,392), support recent studies of murine models and muscle cell lines, which have shown that adiponectin may modulate insulin sensitivity in skeletal muscle by cross-talking with the insulin signaling cascade (354,355,357,358). However, a more complete understanding of the molecular mechanism involved are needed, and will require further mechanistic studies in animal models and muscle cells before they can be tested in human skeletal muscle in vivo.

To gain a more comprehensive understanding of the molecular mechanisms underlying insulin resistance in human skeletal muscle in vivo, a number of issues remain to be investigated. This includes better studies of biological processes and signaling pathways thought to modulate insulin signaling such as accumulation of lipid metabolites, impaired mitochondrial oxidative capacity, oxidative stress, inflammation, as well as novel mechanisms such as autophagy and unfolded protein response (274,412-414). Studies of metabolic and signaling enzymes in several pathways in a muscle biopsy specimen are usually restricted to a limited number when using classical protein technologies. However, recent advances in mass spectrometry-based proteomics have enabled targeted quantification of a large number of proteins and the phosphorylation degree at specific sites simultaneously using a methodology called multiple or selected reaction monitoring (MRM/SRM) (415,416). Novel quantitative proteomic approaches can be used to screen for abnormalities in multiple pathways in small skeletal muscle biopsies obtained from insulin resistant individuals, and will together with classical protein technologies help to identify novel mechanisms and possible targets for treatment of insulin resistance in the near future.

SUMMARY

Type 2 diabetes, obesity and polycystic ovary syndrome (PCOS) are common metabolic disorders which are observed with increasing prevalences, and which are caused by a complex interplay between genetic and environmental factors, including increased calorie intake and physical inactivity. These metabolic disorders are all characterized by reduced plasma adiponectin and insulin resistance in peripheral tissues. Quantitatively skeletal muscle is the major site of insulin resistance. Both low plasma adiponectin and insulin resistance contribute to an increased risk of type 2 diabetes and cardiovascular disease.

In several studies, we have investigated insulin action on glucose and lipid metabolism, and at the molecular level, insulin signaling to glucose transport and glycogen synthesis in skeletal muscle from healthy individuals and in obesity, PCOS and type 2 diabetes. Moreover, we have described a novel syndrome characterized by postprandial hyperinsulinemic hypoglycemia and insulin resistance. This syndrome is caused by a mutation in the tyrosine kinase domain of the insulin receptor gene (*INSR*). We have studied individuals with this mutation as a model of inherited insulin resistance.

Type 2 diabetes, obesity and PCOS are characterized by pronounced defects in the insulin-stimulated glucose uptake, in particular glycogen synthesis and to a lesser extent glucose oxidation, and the ability of insulin to suppress lipid oxidation. In inherited insulin resistance, however, only insulin action on glucose uptake and glycogen synthesis is impaired. This suggests that the defects in glucose and lipid oxidation in the common metabolic disorders are secondary to other factors. In young women with PCOS, the degree of insulin resistance was similar to that seen in middle-aged patients with type 2 diabetes. This supports the hypothesis of an unique pathogenesis of insulin resistance in PCOS.

Insulin in physiological concentrations stimulates glucose uptake in human skeletal muscle in vivo by activation of the insulin signaling cascade to glucose transport through the enzymes IRS1, PI3K, Akt2, AS160/TBC1D4 and RAC1, and to glycogen synthesis through Akt2, inhibition of GSK3 and activation of glycogen synthase (GS) via dephosphorylation of serine residues in both the NH_2-terminal (site 2+2a) and the COOH-terminal end (site 3a+3b). In type 2 diabetes, obesity and PCOS, there is, although with some variation from study to study, defects in insulin signaling through IRS1, PI3K, Akt2 and AS160/TBC1D4, which can explain reduced insulin action on glucose transport. In type 2 diabetes an altered intracellular distribution of SNAP23 and impaired activation of RAC1 also seem to play a role for reduced insulin action on glucose transport. In all common metabolic disorders, we observed an impaired insulin activation of GS, which seems to be caused by attenuated dephosphorylation of GS at site 2+2a, whereas as the inhibition of GSK3 and the dephosphorylation of GS at its target sites, site 3a+3a, appeared to be completely normal. In individuals with inherited insulin resistance, we observed largely the same defects in insulin action on IRS1, PI3K, Akt2 and GS, as well as a normal inhibition of GSK3 and dephosphorylation of GS at site 3a+3b. In these individuals, however, a markedly reduced insulin clearance seems to partially rescue insulin signaling to glucose transport and GS.

Adiponectin is thought to improve insulin sensitivity primarily by increasing lipid oxidation through activation of the enzyme AMPK, and possibly via cross-talking of adiponectin with insulin signaling, and hence glucose transport and glycogen synthesis. We demonstrated a strong correlation between plasma adiponectin and insulin action on glucose disposal and glycogen synthesis in obesity, type 2 diabetes and PCOS. In individuals with inherited insulin resistance, plasma adiponectin was normal, but the correlation of adiponectin with insulin-stimulated glucose uptake and glycogen synthesis was at least equally strong. Moreover, we found a correlation between plasma adiponectin and insulin activation of GS. This result is supported by a number of recent studies of animal models and muscle cell lines, which have shown that adiponectin augments insulin action on enzymes in the insulin signaling cascade. In contrast, we observed no differences in the abundance or activity of AMPK in obesity, type 2 diabetes, PCOS or inherited insulin resistance. This indicates that reduced insulin sensitivity in these conditions is not mediated via abnormal AMPK activity.

The results from these studies demonstrate that the well-established abnormalities in insulin action on glucose uptake and glycogen synthesis are reflected by defects in insulin signaling to these cellular processes in type 2 diabetes, obesity, and PCOS, and as expected also in inherited insulin resistance caused by a mutation in *INSR*. In common metabolic disorders, low plasma adiponectin may contribute to insulin resistance and defects in insulin signaling, whereas in inherited insulin resistance a normal plasma adiponectin and reduced insulin clearance could contribute to maintain a sufficient activation of the insulin signaling cascade. The insight gained from these studies have improved our understanding of the molecular mechanisms underlying insulin resistance in skeletal muscle of humans, and can form the basis for further studies, which can lead to the development of treatment that more directly targets insulin resistance, and hence reduce the risk of type 2 diabetes and cardiovascular disease.

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