Effects of Hyperglycemia on Glucose Transporters of the Muscle: Use of the Renal Glucose Reabsorption Inhibitor Phlorizin To Control Glycemia

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ABSTRACT

Individuals with non-insulin dependent or insulin-dependent diabetes mellitus present insulin resistance in peripheral tissues. This is reflected in a subnormal whole body insulin-dependent glucose utilization, largely dependent on skeletal muscle. Glucose transport across the cell membrane of this tissue is rate limiting in the utilization of the hexose. Therefore, it is possible that a defect exists in insulin-dependent glucose transport in skeletal muscle in diabetic states. This review focuses on two questions: is there a defect at the level of glucose transporters in skeletal muscle of diabetic animal models, and is this a consequence of abnormal insulin or glucose levels? The latter question arises from the fact that these parameters usually vary inversely to each other. Glucose transport into skeletal muscle occurs by two membrane proteins, the GLUT1 and GLUT4 gene products. By subcellular fractionation and Western blotting with isoform-specific antibodies, it was determined that isolated plasma membranes (PM) contain GLUT4 and GLUT1 proteins at a molar ratio of 3.5:1 and that an intracellular fraction (Internal membranes; IM) different from sarcoplasmic reticulum contains only GLUT4 transporters. The IM furnishes transporters to the PM in response to insulin. Both transporter isoforms bind cytochalasin B in a D-glucose-protectable fashion. In streptozocin-induced diabetes of the rat with normal fasting insulin levels and marked hyperglycemia, the number of cytochalasin B-binding sites and of GLUT4 proteins diminishes in the PM whereas the GLUT1 proteins increase to a new ratio of about 1.5:1 GLUT1:GLUT4. In the IM, the levels of GLUT4 protein drop, as does the cellular GLUT4 mRNA. To investigate if these changes are associated with hyperglycemia, glucose levels were corrected back to normal values for a 24-h period with sc injections of phlorizin to block proximal tubule glucose reabsorption. This treatment restored cytochalasin B binding, restored GLUT4 and GLUT1 values back to normal levels in the PM, and partly restored cytochalasin B binding but not GLUT4 levels in the IM, consistent with only a partial recovery of GLUT4 mRNA. It is concluded that GLUT4 protein in the PM correlates inversely whereas GLUT1 protein correlates directly with glycemia. It is proposed that the decrease in GLUT4 levels is a protective mechanism, sparing skeletal muscle from gaining glucose and experiencing diabetic complications, albeit at the expense of becoming insulin resistant. A hypothesis is offered suggesting that, if GLUT1 transporters are elevated in other tissues (not expressing GLUT4) as they are in muscle in the diabetic state, then these tissues may gain glucose and experience diabetic complications. This may be exacerbated in tissues expressing the high Km GLUT2 transporter, such as the kidney, thus contributing to the genesis of diabetic nephropathy.

Key Words: Glucose uptake, diabetes, insulin action, glucose transport, insulin resistance

Non-insulin dependent diabetes mellitus (NIDDM) affects between 5 and 10% of the North American population. Recently, several factors involved in the pathogenesis of this disorder have been the subject of intense investigation using new methodologies. This disease is characterized by hyperglycemia, insulin resistance, and defects in insulin secretion. The hyperinsulinemia seen in NIDDM is usually related to obesity (1). Hyperglycemia and insulin resistance are also common features of insulin-dependent diabetes mellitus (IDDM) (see reference 2). Because individuals with IDDM are treated with peripheral sc insulin injections, peripheral hyperinsulinemia is a common feature of this type of diabetes.
The metabolic insulin resistance is manifested both in the liver as subnormal inhibition by the hormone of hepatic glucose production (5) and in the periphery as subnormal insulin-dependent metabolic glucose clearance and oxidation (4, 5). The origin of insulin resistance, hepatic or peripheral, has been a matter of debate, as has been the question of whether it is primary or secondary to diabetes mellitus. Whereas in IDDM, insulin resistance is secondary to pancreatic β-cell autoimmune necrosis, the cause of NIDDM may be genetically heterogeneous. Defects in glucose-stimulated insulin secretion and in hepatic and peripheral insulin resistance have been postulated to be of primary biologic importance (1). In IDDM in particular, but also in NIDDM, insulin resistance is largely reversible upon the restoration of glycemic control and the normalization of body weight. This had led to the belief that an important component of the diminished glucose utilization and insulin resistance that accompany the diabetic state is acquired and related to a preceding alteration.

A compelling line of evidence implicates the circulating blood glucose level as an important determinant of the degree of diabetic metabolic defects, i.e., diminished glucose utilization and insulin resistance. Glycemia has also been strongly associated directly with the severity of the well-known complications that ensue from the chronic diabetic state, primarily, retinopathy and lens cataracts, nephropathy, and neuropathy. (6). This review focuses on the role of hyperglycemia in some of the metabolic derangements of diabetes mellitus, with emphasis on the glucose transport system of the peripheral insulin-responsive tissues, muscle, and fat. The role of hyperglycemia in the complications of diabetes has been widely reviewed in other sources (6) and will not be a primary subject of this review, although a unifying hypothesis is offered towards the end. Because, by mass, the muscle contributes to glycemic control to a larger extent than fat and because it constitutes the largest insulin-responsive tissue, the results discussed will center on the effects of glycemia on the muscle glucose transport system.

ROLE OF SKELETAL MUSCLE IN THE MAINTENANCE OF GLYCEMIA

Circulating plasma glucose levels are determined by intestinal glucose absorption, hepatic glucose uptake/release, renal glucose reabsorption, and peripheral glucose uptake. Of the peripheral tissues, brain is the main glucose consumer in the postabsorptive state, whereas skeletal muscle constitutes the main glucose consumer in the fed state. Muscle also has the ability to use a wide variety of fuels in addition to glucose. These include circulating free fatty acids (7), as well as glycerogen (8) and triglycerides (9). Therefore, it would appear that because muscle has other options for energy production, it might not regulate its glucose utilization very precisely. However, muscle has the unique role of accommodating the majority of glucose dumped into the circulation after a meal and therefore must be able to adjust its metabolic machinery rapidly to meet such demand. In the resting state, free fatty acids constitute the predominant fuel for skeletal muscle and blood glucose plays a relatively minor role (10). However, in the fed state, under the influence of insulin, the rate of skeletal muscle glucose uptake increases by 5- to 10-fold and actually accounts for 80 to 90% of the whole-body glucose disposal. It is apparent, therefore, that even small perturbations to the glucose uptake system in skeletal muscle, particularly during the fed state, could have serious implications to the overall glucose homeostasis of the body. If a 10% reduction in the fed-state glucose uptake capacity of muscle occurred, it would result in a 100% increase in the amount of glucose presented to other tissues of the body.

Glucose transport is the rate-limiting step in glucose utilization by muscle under most metabolic conditions in vivo and in vitro (11–13), although further metabolism of the hexose, primarily its phosphorylation by hexokinase, also appears to play an important kinetic role, especially in states of very high glucose uptake such as hyperglycemic hyperinsulinemia (13). Evidence suggesting that glucose transport is rate limiting over phosphorylation in muscle glucose utilization under physiologic conditions is that there is no accumulation of free glucose in the muscle fiber (14).

THE MUSCLE GLUCOSE TRANSPORT SYSTEM

Unlike the Na+ gradient–coupled glucose reabsorption in kidney tubule cells, glucose transport across the plasma membrane (PM) of skeletal muscle is accomplished by facilitative passive diffusion (14). This process is catalyzed by integral membrane proteins that are members of a multifamily of gene products (15). The biochemical function of these membrane proteins is to transport glucose across membranes along its concentration gradient. The nomenclature of these gene products obeys their chronologic discovery. The distinct isoforms, which are all products of genes localized in different chromosomes, are also differentially expressed in tissues (see references 15–17 for reviews). In brief, GLUT1 (18,19) is most abundant in the blood brain barrier microvessels of rodents and ruminants (20), and in the human it is a major protein of the erythrocyte membrane (see reference 17). Its expression in other tissues is comparatively lower than that in the above two cell types. GLUT2 is a gene product expressed in tissues specialized in glucose export to the circulation, such as the liver, kidney, and small intestine, and is also present in the pancreatic β cell, presum-
ably fulfilling the role of being a part of the glucose sensor (17,21). GLUT3, first cloned from a fetal human muscle cDNA library, is present in neuronal cells of the rat and in low levels in several tissues in the human (22,23). GLUT4, primarily expressed in muscle and fat (24,25), has been dubbed the muscle/fat transporter or the "insulin-responsive glucose transporter." Although all four transporter gene products, when expressed, perform facilitative glucose transport, there are differences in their \(K_m\) values, with GLUT1 and GLUT2 having lower affinities for glucose \((K_m, 10 \text{ to } 40 \text{ mM})\) than GLUT3 and GLUT4 \((K_m, 1 \text{ to } 5 \text{ mM})\), however their turnover numbers have not been determined because of a lack of measurements of the molar amount of each transporter protein expressed at the plasma membrane. Differences in the cellular environment and intracellular glucose may also alter the kinetics of glucose uptake by each transporter isoform.

The two glucose transporter isoforms detected to date in mature rat skeletal muscle are the GLUT1 and GLUT4 products, whereas in human skeletal muscle, GLUT1, GLUT4, GLUT5 and perhaps, GLUT3 are expressed (so far, GLUT3 mRNA—but not GLUT3 protein—has been reported in this tissue). GLUT1 protein is present in homogenates of skeletal muscle, and GLUT1 mRNA is detectable in total RNA extracts from this tissue, but recent debate has centered on whether it is cytoplologically located to the muscle fiber per se or to ancillary tissues copurified with muscle extracts, such as endothelial, vascular, and neuronal cells. Immunocytochemical studies have revealed that, indeed, the perineurial sheath surrounding the innervating axons contains large levels of immunoreactive GLUT1 protein (26-28), with no detectable levels in the neighboring endothelial or vascular cells. However, immunocytochemical evidence also demonstrates the presence of low levels of GLUT1 proteins in the muscle fiber itself, primarily on the muscle cell periphery (27,28). Hence, GLUT1 appears to be a bona fide glucose transporter of the skeletal muscle fiber.

GLUT4, in contrast, is abundantly localized to the interior of the muscle fiber, although in the resting muscle isolated from fed and fasted animals it is also found on the cell surface (28,29). On an ultrastructural level, this intracellular localization has been ascribed to elements coinciding with the site of the cisternae of the sarcoplasmic reticulum (30), although more recent studies have disputed this localization on the basis of fixation artifacts and have instead demonstrated abundant localization of GLUT4 proteins in vesicular structures underneath the plasma membrane, in mitochondria-rich areas (29). The latter localization is more in keeping with ultrastructural observations made in rat brown fat cells (31) and in rat heart cells (32), as well as with biochemical studies based on subcellular fractionation of skeletal muscle itself (33,34). Our laboratory has demonstrated that biochemical fractionation of skeletal muscle membranes allows one to separate the sarcoplasmic reticulum vesicles from a GLUT4 protein-rich intracellular organelle (internal membranes; IM); in contrast, the sarcoplasmic reticulum fraction lacks this protein (33-35). The IM, which is devoid of GLUT1 protein or plasma membrane markers, may correspond to the submembrane vesicular structures rich in GLUT4 proteins detected by immunocytochemistry. In isolated PM from skeletal muscle of resting normal rats, the molar ratio of GLUT4:GLUT1 protein was found to be around 3.5:1, indicating that GLUT4 is the predominant isoform present at the cell surface. However, the relative contribution of each transporter isoform to transport activity cannot be determined with present technologies.

**ACUTE REGULATION OF GLUCOSE TRANSPORTERS**

The differential spatial distribution of the GLUT1 and GLUT4 glucose transporters in muscle cells is key to their role in glucose uptake activity. It is presumed that in the basal state the GLUT1 and GLUT4 transporters present in the PM are responsible for glucose uptake. Upon insulin binding to its receptor, the abundance of GLUT4 glucose transporters diminishes in the intracellular organelle IM and augments in the PM, as reflected by Western blot analysis of subcellular membrane fractions (36-38). This suggests that the hormone causes a rapid recruitment of GLUT4 glucose transporters in skeletal muscle, akin to that observed in isolated fat cells exposed to insulin in vitro. This response of muscle is detected whether insulin is given during muscle perfusion or by iv injection in the live rat (37), suggesting that it is an integral part of the hormonal response in vivo.

By analogy to the effects of insulin, it has been postulated that other stimuli that augment or diminish glucose influx may also act through changes in the number of glucose transporters operating on the cell surface. Indeed, acute exercise has recently been demonstrated to elevate glucose influx by increasing transporter number in the plasma membrane (37,39). Conversely, stimuli which decrease glucose uptake by muscle could in principle down-regulate the transporter number, although such information is presently only available for cell lines exposed for prolonged times to high glucose concentrations (40). In addition to the regulation of glucose transport by changes in transporter availability at the cell surface, it has been postulated that insulin and other factors may control glucose influx through changes in the intrinsic activity of the individual transporters pres-
ent in the membrane. This possibility is exciting and indeed changes in intrinsic activity have been postulated in situations where the number of transporters at the cell surface does not account for the change in transport activity (41). However, the molecular mechanisms involved have so far alluded strict experimental scrutiny, and coverage of this topic is beyond the scope of this review. **IMPLICATIONS OF A DEFECT IN THE GLUCOSE TRANSPORT SYSTEM IN DIABETES**

Because glucose transport across the cell membrane is a key step in glucose utilization, it has been surmised that alterations in transmembrane glucose transport may be an essential part of the defect in glucose utilization seen in the diabetic state (42). Indeed, Yki-Jarvinen (43) and Rossetti et al. (44) have recently demonstrated that in both IDDM and NIDDM muscle, the insulin-resistant state is accompanied by decreases in glucose-6-phosphate and in glucose-derived glycogen, suggesting that a step before glucose-6-phosphate accumulation (i.e., glucose transport and/or hexokinase activity) is responsible for the defect. This suggests that the glucose transport system may not only be a key regulated step, as described in a previous section, but may also be a step defective in diabetes.

Recently, several groups have closely examined the metabolic consequences of hyperglycemia on elements of the glucose transport system, i.e., the expression of the GLUT1 and GLUT4 gene products as mRNA levels and translated protein amounts. Our group has carried these investigations further by examining the subcellular distribution of glucose transporters in hyperglycemic states, in an effort to delineate whether defects in intracellular sorting of transporters participate in the generation of the abnormality in glucose transport. Specifically, our attention has focused on the number of glucose transporters present in the plasma membrane under diverse metabolic states, using the subcellular fractionation approach. These studies will be discussed in the following sections.

**EXPERIMENTAL PROTOCOLS TO STUDY THE RELATIONSHIP BETWEEN GLYCEMIA AND THE GLUCOSE TRANSPORT SYSTEM**

**Use of Phlorizin To Generate a State of Euglycemia**

In the study of the effect of circulating glucose levels on the glucose transport system, it has been a major task to differentiate the potential effects of glycemia alone, independent of the levels of circulating insulin or other hormone/metabolites. Recently, however, strategies have been designed to isolate glycemia as a single variable. This is not easily done in vivo experimental models, because alterations in glycemia are obligatorily linked to changes in insulin secretion, counterregulatory hormone production, and liberation of free fatty acids. The most commonly used strategy has been the use of the drug phlorizin, which blocks renal glucose reabsorption by virtue of its specific inhibition of the luminal Na+-dependent glucose cotransporter (see reference 45).

Phlorizin or 4,6-dihydroxy-2-β-d-glucosid-β-4-(p-hydroxyphenyl)propiophenone, also known as phlorin-2'-β-glucoside, is isolated from root, bark, and leaves of several Rosaceae species. It selectively blocks Na+-dependent renal and intestinal Na+-dependent glucose uptake by competing with glucose for its binding site, with a \( K_i \) value of 5 to 10 \( \mu \)M, identical to its binding constant to brush border membranes (45). Binding of phlorizin to the Na+-dependent glucose transporter derives its specificity from the glucose moiety of the ligand. Indeed, the aglucon of phlorizin, phloretin, is a poor inhibitor of the Na+-dependent luminal transport of glucose. Because of its high affinity and specificity for the Na+-dependent glucose transporter, phlorizin has been used to label and identify the purified Na+-dependent glucose transporters of kidney (46,47) and small intestine (48,49). Its role in in vivo studies is assumed to depend on the inhibition of Na+-dependent glucose reabsorption by the proximal tubule, although action on intestinal glucose absorption cannot be ruled out. It must be pointed out that for metabolic studies in the living rat, the drug is administered either through injections or slow delivery from implanted mini-pumps. It is assumed that phlorizin reaches the luminal membranes of the proximal tubule through pinocytosis or filtration, because the drug does not cross membranes easily.

**Effects of Phlorizin on Glucose Uptake/Clearance In Vivo**

Phlorizin has been used to test the effects of hyperglycemia on muscle glucose uptake in vivo. To perform such measurements, the glucose clamp technique was used, which allows one to determine the amount of glucose removed from the circulation by the tissues without changing glycemic levels during the measuring period. Glucose uptake under such conditions reflects the ability of the transport system to remove glucose from the circulation but is also dependent on the mass effect of the prevailing glycemia on the uptake process itself. By dividing the value of the removal of glucose from the circulation into muscle by the value of glycemia, the term metabolic glucose clearance is obtained. Hence, glucose clearance is an indirect measurement of glucose transport activity. The problem with measurements of metabolic glucose clearance is that even when they are performed during constant insulin infusion, glu-
cose clearance decreases as a function of glucose concentration. This result has been explained by the fact that the brain shows relatively constant glucose uptake in normoglycemia and hyperglycemia, whereas other tissues have glucose transport systems that take up glucose at a rate proportional to its concentration in the plasma (50, 51). We speculate that the lack of glucose regulation of glucose uptake into brain indicates that the brain glucose transporter (GLUT3) is already saturated at normoglycemia. In order to obtain a better insight into the role of hyperglycemia on glucose uptake in various tissues, it is necessary to subtract the glucose uptake by the brain from the total glucose uptake, which for practical purposes has been assumed to equal 1 mg/kg/min.

Gauthier et al. (52) first showed that the administration of phlorizin caused a dose-dependent drop in glycemia below normal fasting levels in nondiabetic dogs. Subsequently, Starke et al. (53), Lussier et al. (54), and Hetenyi et al. (55) postulated that such a strategy could correct glycemia in alloxan-diabetic dogs. Figure 1A shows that, indeed, phlorizin treatment rapidly normalized glycemia in such diabetic animals. Figure 1B illustrates the effects of glycemic normalization with phlorizin on the whole-body glucose clearance rate corrected for glycosuria in the same alloxan-diabetic dogs. Metabolic glucose clearance was elevated in glycemia-corrected diabetic animals. When glucose utilization and metabolic glucose clearance were calculated (after the glucose uptake by the brain is subtracted), it was noted that metabolic clearance increased by about 50%. In other studies in which glycemia was corrected for 2 days in phlorizin-treated, alloxan-diabetic dogs, the metabolic glucose clearance increased further, although still remaining subnormal presumably because of the substantial hypoinsulinemia. These results suggest that even with low insulin levels, diabetic animals exhibited a reversible glucose transport defect that was caused by the presence of hyperglycemia itself.

The above results were obtained with short-time normalization of glycemia. It is possible that more prolonged restoration of normoglycemia may not hold the recovery of metabolic glucose clearance. To answer this possibility, Rossetti et al. (56) studied the effects of hyperglycemia on peripheral glucose metabolism in rats. Those authors used partial pancreatectomy to create a state of fasting hyperglycemia with normal fasting insulinemia. Glycemia was then corrected with continuous phlorizin delivery through implanted minipumps. Using the euglycemic-hyperinsulinemic clamp technique to measure whole-body glucose utilization, they demonstrated that insulin-mediated glucose metabolism was reduced by approximately 30% in diabetic rats (Figure 2A). Phlorizin treatment for 14 days completely normalized insulin sensitivity of these diabetic rats but had no effect on insulin action in controls. Furthermore, when phlorizin treatment was discontinued, insulin resistance reemerged in the diabetic rats. These data suggested that correction of hyperglycemia with phlorizin without change in insulin levels normalizes insulin sensitivity. Figure 2B illustrates the relationship found between various plasma glucose levels and insulin-mediated glucose uptake. The linear inverse relationship strongly suggests that the insulin-stimulated component of whole-body glucose utilization is regulated by glycemia.

The whole-body glucose uptake measurements presented above and illustrated in Figures 1 and 2 reflect glucose uptake by all tissues except brain. It was therefore necessary to find out more directly whether glucose transport into skeletal muscle can also respond to normalization of glycemia. In the insulinized state, it could be expected that skeletal muscle participates largely in the restoration of glucose uptake, because this tissue normally accounts for about 90% of the insulin-stimulated glucose uptake in vivo. However, the participation of glucose uptake in the absence of insulin is not as dominant over other tissues, and in no instance had the effect of glycemia on the glucose transport system of skeletal muscle been studied directly. We therefore undertook to investigate the effects of glycemia on the levels and subcellular distribution of the glucose transporters of skeletal muscle.

Figure 1. Effect of an infusion of phlorizin (50 μg/kg/min) on the metabolic clearance rate (A) and glucose concentration in plasma (B) in insulin-deprived, alloxan-diabetic dogs. Results adapted from reference 54.

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The initial requirement was to establish an animal model of hyperglycemia with minimal alterations in insulinemia, in order to prevent large differences in insulin levels between experimental groups. This was achieved with doses of streptozocin previously determined to cause limited β-cell death. The protocol used has enabled us to generate diabetic (hyperglycemic) rats with normal fasting insulin levels (57,58). Clearly, because the streptozocin-diabetic animals have normal fasting insulin levels, they must mount an insufficient insulin response upon feeding, leading to hyperglycemia. Subgroups of both diabetic and control rats were then exposed 5 days after the streptozocin injection to phlorizin injections (0.4 g/kg wt sc administered in propyleneglycol) to restore glycemia to control levels, and this treatment continued for 3 days. Thus, in the design of this study, the restoration of normoglycemia was of much shorter duration than in the experiments of Rossetti et al. (56).

At the end of 7 days, the rats were fasted overnight, so that a 24 h-period of normal glucose and insulin levels was achieved. Rats were then killed in the fasted state, and values of circulating insulin, glucose, free fatty acids, and glucagon were determined (Table 1). Subgroup 1 (untreated controls) represents the baseline reference metabolic parameters, subgroup 2 (streptozocin-treated diabetic rats) displayed hyperglycemia but no other observable differences relative to controls, and subgroup 3 (streptozocin-diabetic rats treated with phlorizin) was metabolically identical to subgroup 1 rats except that they had previously been hyperglycemic. Subgroup 4 (non-diabetic rats treated with phlorizin) is discussed later in this review.

Skeletal muscle removed upon death was used to prepare subcellular fractions of skeletal muscle, following a procedure developed in our laboratory (59) to yield PM and IM. The number of glucose transporters was measured by the specific, glucose-protectable binding of cytochalasin B. This is a selective ligand used to count the number of glucose transporters when the D-glucose-protectable component of binding is measured. Cytochalasin B binds to the GLUT1 and GLUT4 members of the facilitative glucose transporter family (60,61) and hence reflects the total number of glucose transporters present in a membrane fraction, irrespective of transporter isoform. Figure 3 illustrates the D-glucose-protectable binding of cytochalasin B per milligram of protein to isolated PM and IM of muscle. The cytochalasin B-binding sites in the PM can be considered to reflect the number of transporters actually available for glucose influx into the fiber, and the IM represent the reserve transporters that may determine in part the ability of insulin or other stimuli to elicit rapid recruitment of transporters. In the PM of diabetic animals, there was a decrease in cytochalasin B binding, which was completely reversed upon normalization of blood glucose with phlorizin. In the IM fraction of diabetic rats, there was also a reduction in
cytochalasin B–binding activity and a complete reversal with phlorizin treatment.

The measurements of cytochalasin B binding reflect the total number of glucose transporters that bind this ligand, and hence, this information is useful in order to relate transport activity to transporter number. On the other hand, because each isoform is a different gene product, information on the content of the specific isoforms is informative regarding the mechanism underlying the regulation of transporter number. Membrane fractions were reexamined for effects of glycemia on individual transporter isoforms (58). In diabetic rat muscle, GLUT4 transporters decreased in both the PM and the IM. Upon phlorizin treatment, GLUT4 transporter content recovered completely in the PM and partly in the IM. GLUT1 transporters on the other hand were found to be much less abundant in this tissue and were only detectable in the PM. In this fraction, GLUT1 transporter number increased with diabetes and decreased back to control values with phlorizin treatment. These results further support the concept that GLUT4 is quantitatively the predominant transporter in skeletal muscle (see above), because the changes seen in GLUT4 levels in the PM were paralleled by the changes in cytochalasin B binding. Furthermore, the lack of recovery of GLUT4 transporter number in the IM in the face of the complete recovery of cytochalasin B binding and the absence of GLUT1 transporters from this fraction suggests the exciting possibility that a third type of transporter may coexist in this fraction and may be under inverse regulation by glucose.

It is interesting to note that the effects of glycemia were opposite for the two transporter isoforms. This may suggest that tissues containing only GLUT1 may experience up-regulation of glucose influx in response to hyperglycemia, whereas the muscle experiences down-regulation of glucose influx because GLUT4 diminishes and this is the major glucose transporter isoform in this tissue, even in the PM (see above).

Effects of Glycemia on GLUT4 mRNA

Overall, the above results indicate that hyperglycemia lowers the net amount of glucose transporters and that a major change is experienced at the level of the plasma membrane. A plausible explanation for this phenomenon is that hyperglycemia causes glucose transporters to endocytose from the plasma membrane to an intracellular site. However, if this were the full extent of the response to glycemia, the intracellular fraction containing glucose transporters would have been expected to gain transporters. Instead, this fraction also experienced a reduction in glucose transporter content. It was therefore hypothesized that this pool could be further depleted through either increased transporter degradation or diminished transporter synthesis. The latter possibility was confirmed by observations of reduced levels of GLUT4 mRNA in the diabetic animals (Figure 4) (58). Similar reductions have been previously reported for skeletal muscle of other models of animal diabetes, mainly the partially pancreatectomized rat and the streptozocin-generated hypoinsulinemic diabetic rat (62,63). Importantly, our findings show that the restoration of glycemia by the in vivo phlorizin protocol resulted in a partial restoration of the GLUT4 mRNA levels. This indicates that either the transcription of the GLUT4 gene or the stability of its transcript is at least in part susceptible to regulation by glycemic levels. The incomplete restoration of GLUT4 mRNA may explain the partial restoration of GLUT4 glucose transporter content in the IM fraction of phlorizin-treated diabetic rats described in Figure 3.

Taken together, the observations shown in Figures

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**TABLE 1. Metabolic parameters of streptozocin-diabetic rats with and without phlorizin treatment**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + Phlorizin</th>
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<tr>
<td>Weight</td>
<td>Day 8 fasted (g)</td>
<td>309 ± 11</td>
<td>293 ± 5</td>
</tr>
<tr>
<td>Glycemia</td>
<td>Day 7 before fasting (mM)</td>
<td>5.4 ± 0.3</td>
<td>&gt;22</td>
</tr>
<tr>
<td>Glycemia</td>
<td>Day 8 fasted (mM)</td>
<td>5.5 ± 0.5</td>
<td>19.6 ± 1.6</td>
</tr>
<tr>
<td>Glycosuria</td>
<td>Day 8 fasted (g/L)</td>
<td>0.24 ± 0.2</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>Urine vol.</td>
<td>Day 8 fasted (ml/day)</td>
<td>26 ± 5</td>
<td>160 ± 15</td>
</tr>
<tr>
<td>Insulin</td>
<td>Day 8 fasted (μU/ml)</td>
<td>10.1 ± 1.0</td>
<td>9.1 ± 1.1</td>
</tr>
<tr>
<td>FFA</td>
<td>Day 8 fasted (μEq/L)</td>
<td>661 ± 55</td>
<td>624 ± 71</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Day 8 fasted (pg/ml)</td>
<td>250 ± 41</td>
<td>314 ± 72</td>
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* FFA, free fatty acids. Rats were injected on day 1 with saline (controls) or with streptozocin (diabetic) as indicated in the Text. On day 4, a subgroup of diabetic animals was subjected to phlorizin treatment as indicated in the Text (Diabetic + Phlorizin). On day 7, all animals were deprived of food for 24 h and were killed on day 8. Total number of animals: 10 controls, 6 diabetic, 6 diabetic + phlorizin. Ketosis was not observed in any group.
3 and 4 suggest that the regulation of glucose transporters in the plasma membrane may be strictly linked to glycemic values and may be regulated within the time of restoration of glycemia analyzed in this study (24 h). It is possible that postprandial glucose levels could have also improved with the 3-day phlorizin regimen, although such values were not measured. On the other hand, the levels of transporters present in intracellular stores may be regulated by glycemia but this regulation may involve a tardier response, which may include regulation of gene expression of glucose transporters. Additional regulation at the level of glucose transporter degradation cannot be ruled out.

It must be emphasized that the regulatory scheme discussed above appears to apply to skeletal muscle specifically. Similar studies carried out recently by Rossetti, Kahn and colleagues (63) show that although whole-body glucose uptake is restored by phlorizin treatment (3 wk) of depancreatized rats, this was not accompanied by the restoration of glucose transporter levels in membranes isolated from adipocytes. GLUT4 mRNA levels were also not restored in fat cells from hypoinsulinemic streptozocin-induced rats treated with phlorizin for several days (64). It is possible that either differences in the experimental protocols as they refer to the type of diabetic animal model and phlorizin regimen or tissue-specific responses are responsible for the discordant variation of muscle and fat cell glucose transporters with glycemia. It should not be totally unexpected to obtain opposite responses in both tissues because their metabolic functions are very different, fat being primarily a storage site for high-energy compounds and skeletal muscle being mainly responsible for the maintenance of antigravity forces and physical activity. Moreover, it has been proposed that the nutritional status and clinical conditions affect both cell type differently (65,66) and that glucose uptake may be impaired in muscle but elevated in adipocytes in states of obesity, resulting in muscle insulin resist-
To extrapolate the implications of these observations to human states of diabetes and obesity is beyond the scope of this review. However, it must be indicated that, in apparent contrast to the observations made in diabetic animal models, GLUT4 mRNA and the total level of GLUT4 transporter proteins do not appear to be significantly abnormal in the muscle of NIDDM individuals unless they are morbidly obese. These levels are distinctively low, however, in diabetic human fat cells. The negative results in muscle are not necessarily in conflict with the observations described above of diabetic rats, because the number of glucose transporters in human samples was measured in total membrane extracts rather than in purified membrane fractions, and furthermore, a high interindividual variation was noticed. It is possible that, when techniques are applicable to isolate pure PM from human muscle biopsies, correlations between glycemia and cell surface glucose transporter number may become apparent. This possibility remains open for future investigation.

The molecular basis for the effects of glucose on intracellular traffic of transporters and transporter biosynthesis remains one of the most intriguing questions in metabolic regulation. It is expected that a metabolite(s) of glucose may allosterically control some of the elements participating in these pathways, but research in this area is still outstanding. Recently, Marshall and coworkers advanced the interesting possibility that the regulation of elements of the glucose transport system may be exerted at least in part by changes in levels of intracellular glucosamine-6-phosphate in fat cells through the induction of glutamine/fructose-6P amidotransferase. Whether this regulation operates in skeletal muscle remains to be determined.

**TABLE 2. Metabolic parameters of phlorizin-treated nondiabetic and diabetic rats**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<td>309 ± 11</td>
<td>305 ± 10</td>
<td>293 ± 5</td>
<td>287 ± 6</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>5.5 ± 0.5</td>
<td>8.6 ± 2.8</td>
<td>19.6 ± 1.6</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>Insulin (μU/mL)</td>
<td>10.1 ± 1.0</td>
<td>10.3 ± 1.2</td>
<td>9.1 ± 1.1</td>
<td>7.5 ± 0.6</td>
</tr>
</tbody>
</table>

*Control and diabetic animals (see Table 1) were subdivided, and subgroups were treated on day 4 with phlorizin. On day 7, a 24-h fasting period was initiated. Insulin and glucose levels were determined in trunk blood at the end of this period.*
POTENTIAL ROLE OF THE GLUCOSE PROTECTING EFFECT IN MUSCLE AND ITS IMPLICATION FOR TISSUES WHICH DEVELOP LONG-TERM DIABETIC COMPLICATIONS

Advanced Glycosylation Endproducts and the Polyol Pathway

In addition to the metabolic alterations of NIDDM described above, i.e., diminished glucose influx into tissues and insulin resistance, the disease is characterized by long-term consequences that often take many years to become clinically manifest. In the early stages, patients experience mainly diminished glucose uptake in response to insulin and polyuria subsequent to elevations in plasma glucose above the renal threshold. However, with time, the risk of developing retinopathy, nephropathy, and neuropathy increases dramatically and these defects are largely irreversible. Epidemiologic evidence demonstrates that the frequency and temporal course of the appearance of such complications have a strong positive correlation with fasting plasma glucose levels (6).

The biochemical mechanisms by which plasma glucose levels translate into diabetic complications have been the subject of intense investigation and include the accumulation of free glucose and its derivatives sorbitol, fructose, and glucose-6-phosphate, mostly in tissues susceptible to diabetic complications. The effects of intracellular glucose, glucose-6-phosphate, and fructose may arise from the nonenzymatic glycosylation (glycation) or proteins essential for the maintenance of retinal and perhaps renal and neuronal function (72). Recently, it was demonstrated that glycation by glucose-6-phosphate and by fructose is more efficient than that involving glucose per se (73,74). The specific proteins glycated have not been fully identified, but a large body of research has demonstrated significant glycation of over 20 different proteins in several tissues from diabetic humans and animals (see reference 75 and references within).

Glycation and its continuation into the generation of advanced glycosylation endproducts are thought to be a major culprit of diabetic complications, and the clinical use of drugs that interfere with such a reaction is receiving increasing attention. In particular, drugs such as aminoguanidine, which prevents the progression from glycation to advanced glycosylation products, appear promising (76,77).

The effects of high levels of sorbitol, although less understood at a biochemical level, also show a strong association with diabetic complications such as nephropathy (78). The potential usefulness of inhibitors of aldose reductase such as sorbinyl is currently...
controversial, but in vitro, these agents reverse several of the chemical and functional consequences of cellular exposure to high glucose (78). A subnormal level of myoinositol often accompanies the elevated levels of sorbitol in diabetes (78,79). Again, the function of this metabolite in the development of complications is largely unknown but it has been postulated that changes in this polyol are critical in pathways involved in signal transduction of several hormones and neurotransmitters (78,79) that are deemed essential for normal renal and neuronal function.

Mechanisms Sparing Skeletal Muscle From Glucose Complications

Surprisingly, skeletal muscle does not develop major clinical complications in the course of sustained diabetes. This raises the possibility that this tissue may have protective mechanisms that prevent the buildup of harmful glucose derivatives. Of course, an important inherent mechanism of muscle that could partake in this protective action is the fact that glucose transport is rate limiting in glucose utilization by muscle, with the consequence that glucose does not build up in the muscle sarcoplasm (14). It could be envisaged that an increase in glycemia would bring about a glucose gain by the muscle fiber, by virtue of the mass action of glucose. The mass action of glucose dictates that more glucose enters the muscle at higher levels of glycemia. This applies to all tissues, provided that glucose transporters are not saturated at normoglycemic values. The former is the case for GLUT1 and GLUT2 glucose transporters, both of which have Km values well above 5 mM (80). Hence, tissues with GLUT1 and particularly GLUT2 are especially susceptible to gain glucose in the face of hyperglycemia. We would further speculate that hyperglycemia may actually increase the amount of GLUT1 transporters in the plasma membrane of complication-prone tissues, if this isoform responds in those tissues in a similar way to that noted in skeletal muscle (Figure 3). This would further increase glucose influx into these tissues in the diabetic state. Indeed, chronic hyperglycemia has been recently found to increase the density of glucose transporters in rat enterocytes (81) and to elevate the concentration of GLUT1 transporters in human erythrocyte membranes (82). Consistent with this hypothesis, glycation of proteins has been found to occur in tissues that express the GLUT2 transporter such as liver and kidney (83) but a correlation with the presence of this isoform has not yet been established. In contrast to diabetic muscle, glucose-6-phosphate increases in diabetic liver (84). The latter tissue, however, does not show diabetic complications such as those of kidney, likely because it funnels glucose and glucose-6-phosphate into fat. The price paid is a fatty liver.

On the other hand, skeletal muscle does not express GLUT2 transporters and GLUT1 transporters constitute only a small fraction of the glucose transporters present at the cell surface (vide supra). Even when GLUT1 levels rose in the hyperglycemic state, this increase was less than the reduction in GLUT4 transporters, as evidenced by the net drop in the number of all glucose transporters of the PM measured as cytochalasin B-binding sites (Figure 3). This is in keeping with the demonstration that the main glucose transporter of muscle PM, in the presence or absence of insulin, is GLUT4. This isoform is expected to be almost saturated at 5 mM glucose, because Km values for this transporter have been reported to be around 1 to 2 mM (80). Hence, skeletal muscle is inherently protected from gaining glucose to the same extent as tissues such as the kidney. Even in the diabetic rat, the calculated molar ratio of GLUT4:GLUT1 in skeletal muscle PM is about 1.5:1.

However, the diabetic state does pose an impairment in glucose handling by skeletal muscle. As presented above, chronic exposure to high glucose levels provokes an adaptive response in this tissue, characterized by diminishing GLUT4 transporters at the PM. This results in a reduction in the number of the main glucose transporters operating on the cell surface and would curb glucose influx. We speculate that transporters removed from the cell surface may be routed to an intracellular pool. However, this pool does not gain GLUT4 transporters and in fact experiences a decrease of these in the diabetic animal, possibly because of diminished transcription of the GLUT4 gene (Figure 4). This latter response, which may also be part of the protective mechanism, is an undesirable consequence in that, by diminishing the intracellular pool of glucose transporters, insulin resistance may ensue from insufficient availability of glucose transporters for recruitment to the cell surface. Consistent with this action, glucose-6-phosphate is diminished (43, 44). This scenario is illustrated in Figure 7.

In conclusion, we speculate that skeletal muscle is protected from the diabetic complications of hyperglycemia by the complement of glucose transporter isoforms expressed in this tissue, mainly GLUT4, which is not capable of elevating glucose uptake by glucose mass action. In addition, muscle appears to respond to hyperglycemia with a reduction in the number of glucose transporters operating at the cell surface. This may spare the muscle from gaining excessive glucose and experiencing reactions via glycation or the polyol pathway but may render the muscle insulin resistant. Moreover, by diminishing glucose influx into this, the largest glucose-consuming tissue in the fed state, the protective mechanisms of muscle may actually contribute to the hyperglycemic state, thus accelerating or intensifying the pathologic consequences in complication-susceptible tissues such as eye, brain, and kidney.
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