REGULATION OF THE GLUCOSE TRANSPORT PATHWAY IN ADIPOSE TISSUE OF HORSES WITH INSULIN RESISTANCE

Undergraduate Thesis as Required for Graduation with Distinction

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Honors Research Project
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**Common Abbreviations**

GLUT: Glucose Transporter

AS160: Akt Substrate 160

IS: Insulin sensitive

IR: Insulin resistant

SM: Skeletal Muscle

AT: Adipose Tissue

TH: Tailhead (adipose tissue)

NL: Nuchal ligament (adipose tissue)

MES: Mesenteric (adipose tissue)

RP: Retroperitoneal (adipose tissue)

OM: Omental (adipose tissue)

SC: Subcutaneous (adipose tissue)

VIS: Visceral (adipose tissue)

CASQ: Calsequestrin
ABSTRACT:

Although the importance of adipose tissue (AT) glucose transport in regulating systemic insulin sensitivity is becoming increasingly evident and insulin resistance (IR) has been widely recognized, the underlying alterations in glucose transport and its contributions to IR are still not well understood. The purpose of the present study was to determine the early pathological changes in glucose transport by characterizing alterations in glucose transporters (GLUTs) in visceral and subcutaneous adipose depots in a novel equine model of naturally occurring compensated IR. AT biopsies were collected from horses, which were classified as insulin-sensitive (IS) or compensated IR based on the results of an insulin-modified frequently sampled intravenous glucose tolerance test. Protein expression of GLUT-4 (major isoform) and GLUT-12 (a newly discovered isoform) were measured by Western blotting in visceral and subcutaneous adipose depots, along with AS160 (a potential key protein in GLUT cell-surface trafficking). Using a biotinylated bis-mannose photolabel technique, active cell surface GLUT-4 and-12 content was also quantified to estimate GLUT trafficking. Omental AT had the highest total GLUT-4 and GLUT-12 content compared to other sites during the IS state. IR was associated with a significantly reduced total GLUT-4, but not GLUT-12, content in omental AT, without a change in content in the other adipose sites. In addition, active cell surface GLUT-4, but not -12, was lower in subcutaneous and visceral AT of IR compared to IS horses. Impairment in GLUT-4 trafficking occurred independently of any changes in AS160 phosphorlyation between groups. The data suggests that GLUT-4, but not -12, is a pathogenic factor in AT during naturally occurring compensated IR, despite normal AS160 activation.
BACKGROUND:

Diabetes mellitus has been known in humans since the early 20th century, and currently, approximately 171 million people worldwide (2.8% of the global population) are afflicted with the disease. By the year 2030, that number is expected to more than double, leading the Centers for Disease Control to label the disease as an epidemic. In particular, the vast majority of diabetes mellitus cases are Type II Diabetes mellitus (T2DM). T2DM is characterized by a lack of insulin action (i.e., insulin resistance), which causes hyperglycemia due to decreased glucose uptake into insulin-sensitive cells (i.e., muscle and adipose tissue), which if left untreated, can lead to multi-system organ dysfunction and potentially failure. In more recent years, insulin resistance and obesity (both characteristic of T2DM) have been linked with hypertension and dyslipidemia in a disease phenotype known as metabolic syndrome, which is a generalized collection of risk factors for cardiovascular disease in humans. Additionally, there has been an increase in clinical awareness of insulin resistance and diabetes in horses, which has been rarely diagnosed or tested for in the past. While it is known that Type II diabetes results from insulin resistance, the exact mechanism and pathways by which glucose uptake into cells is decreased is not. It has been known for the last 50 years that glucose uptake is mediated by a family of glucose transporters (GLUTs). GLUT4 is the major isoform and its translocation to the cell surface is mediated by both an insulin- and a contraction-regulated pathway. As such, we quantified the amount of the primary glucose transporter, GLUT-4, in adipose tissue in both insulin-sensitive and insulin-resistant horses. Additionally, in an attempt to further investigate the mechanisms associated with decreased glucose uptake, the translocation of GLUT-4 from an intracellular pool (inactive site) to the cell surface (active site), the rate-limiting step for glucose uptake, was also studied. Also, GLUT-12, a newly discovered GLUT protein, may present as a
second insulin-sensitive GLUT; however, its functional role is not well defined in insulin-sensitive tissues, especially adipose tissue\textsuperscript{6}. With this in mind, total GLUT-12 content and activation were also quantified in the insulin-sensitive and insulin-resistant horses. Leading research in related fields has also shown that there is a link between visceral adipose sites and the development of insulin resistance and cardiovascular disease in humans\textsuperscript{5}. Studies have shown that human omental adipocytes have higher glucose uptake rates than subcutaneous sites, resulting in more glucose transporter expression, and that visceral sites are more susceptible to glucocorticoid-induced insulin resistance than subcutaneous sites\textsuperscript{5}. We investigated this potential pathophysiologic process in our equine model by quantifying total and active amounts of glucose transporters in subcutaneous adipose tissues compared to visceral adipose tissues. Finally, the Akt Substrate Protein with a molecular weight of 160 kDa (AS 160) has recently been identified as a key player in both the insulin- and contraction-regulated pathways of glucose transporters. AS160 is the most distally known signaling protein involved in GLUT-4 translocation. As such, the activity (or rather the lack thereof) of AS160 could be a potentially pathogenic factor for the development of insulin resistance and eventually diabetes.

\textbf{MATERIALS \& METHODS:}

\textit{IN VIVO:}

The effect of insulin resistance on the regulation of glucose transport in adipose tissue was examined in an integrative physiologic study, using horses with \textit{naturally occurring} insulin resistance. In collaboration with the Ohio State University College of Veterinary Medicine, five insulin-resistant and five insulin-sensitive (control) light breed mares, housed at Finley Research
Farm, were sampled from the research herd and used for this study. The horses were classified as either insulin-resistant or insulin-sensitive based on the results of an insulin-modified frequently sampled intravenous glucose tolerance test. Horses chosen for inclusion in the study underwent general anesthesia at the Ohio State University Veterinary Teaching Hospital for incisional biopsy collection of visceral adipose tissue (omental, retroperitoneal and mesenteric) and subcutaneous adipose tissue (nuchal ligament and tailhead). A portion of tissue from each site (~200 mg) was photolabeled and the remaining sample was flash frozen in liquid nitrogen. Please note that all procedures were approved by the Ohio State University Institutional Animal Care and Use Committee. All tissues collected were stored at -80°C until the time of analysis.

IN VITRO, WESTERN BLOTTING:

Total GLUT (-4 & -12) and AS160 (total and phosphorylated) protein expression were measured in tissue homogenates by use of electrophoresis and subsequent western blotting. For analysis of GLUT-4 and GLUT-12, plasma membrane-enriched fractions were obtained from adipose tissue and skeletal muscle of insulin-sensitive and insulin-resistant horses, as previously described\textsuperscript{12}. Frozen tissue fractions were homogenized in homogenizing buffer (210 mM Sucrose, 40 mM NaCl, 2 mM EGTA, 30 mM Hepes, and 0.35 mg/ml PMSF, pH 7.4) using a polytron homogenizer (Biospec Products, Inc). The homogenate was then mixed with 58.3 mM sodium metaperiodate and 1.17 M KCl. Plasma membrane-enriched fractions were then recovered from muscle and fat by centrifugation (100,000 g, 70.1 Ti rotor, ultracentrifuge, Beckman Coulter). The pellets were resuspended in 10 mM Tris, 1 mM EDTA (pH 7.4), and then 16% SDS was added. Samples were then centrifuged again.
Total lysates for analysis of AS-160 and phosphorylated-AS160 were also obtained from insulin-sensitive and insulin-resistant horses. Frozen tissue fractions were homogenized in homogenizing buffer [50 mM Tris-HCl at pH 7.4, 50 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 10 uL/mL buffer protease inhibitor cocktail, and 10 uL/mL buffer phosphatase inhibitor cocktail (for phosphorylated-AS160 only) using a polytron homogenizer. Total lysates were recovered from fat by centrifugation (10,000g, 70.1 Ti Rotor, ultracentrifuge, Beckman Coulter) and by extraction of the supernatant.

Protein concentrations of the supernatant were determined spectrophotometrically (Microplate reader, Fluostar Optima, BMG Labtech) using a BCA Protein Assay Kit in conjunction with a BSA standard (Pierce, Rockford, IL). Adipose tissue fractions were then analyzed for GLUT-4, GLUT-12, and AS160 by electrophoresis and subsequent western blotting. Samples were diluted in 1:2 laemmlie sample buffer (Bio-Rad, Hercules, CA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% resolving gel for GLUT-4 and GLUT-12 and a 7% resolving gel for AS160. The protein was then transferred electrophoretically to a PVDF membrane (Millipore, Bedford, MA) using a semi-dry blotting technique. The membranes were then blocked in a non-fat dry milk solution of 10% for GLUT-4, 5% for GLUT-12, and 0.5% for AS160 (Bio-Rad, Hercules, CA) in 0.1% phosphatase-buffered saline tween (TPBS). After blocking, the membrane was incubated with a polyclonal antibody directed against the last 12 COOH-terminal amino acids of either GLUT-4, GLUT-12, or AS160. After several washes in 0.1% TPBS and one wash in phosphatase-buffered saline (PBS), the membrane was incubated for 1 hr with an anti-rabbit horseradish peroxidase (GE Healthcare, Buckinghamshire, UK). Quantitative determination of GLUT-4, GLUT-12, and
AS160 was performed by autoradiography after revealing the antibody-bound transporter protein enhanced by chemiluminescence reaction (Kirkegaard and Perry Laboratories, Maryland, USA) according to the manufacturer recommendations. The density of the bands on scanned autoradiograph was quantified using a computerized densitometry program (MediaCybernetics, Silver Spring, MD). Kaleidoscope prestained standards (Bio-Rad, Hercules, CA) were used as a molecular weight marker for each gel to confirm the molecular weight of the labeled bands. For control samples, a rat left-ventricle from a previous study within this lab was used as an internal control in GLUT-4 and GLUT-12 studies, and a pig right atrium from a previous study in this lab was used as an internal control for the AS160 studies.

Calsequestrin (CASQ2) was used as a loading control. Previously analyzed membranes were stripped in western blot stripping buffer (Pierce, Rockford, IL). Complete removal of the GLUT primary and AS160 primary, as well as secondary, was confirmed according to manufacturer recommendations. Immunoblotting was then performed as described above, using a polyclonal antibody aimed at CASQ2 (Fisher Scientific, Hanover Park, IL).

**IN VITRO, PHOTOLABELED TECHNIQUE:**

GLUT-4 translocation to the cell surface, the *rate-limiting step in glucose utilization*, was measured by a cell-surface biotinylation assay, using a *photolabeled cell-impermeant compound* in adipose tissue. *Please note all processes related to the biotinylation assay were performed by the post doctorate fellow in our lab, Dr. Amanda Waller.*
RESULTS:

IN VIVO:

There were no significant differences in body weight, body condition score, neck circumference, girth, or ultrasonographic retroperitoneal fat thickness between groups (Table 1). There were also no differences in baseline blood glucose or plasma insulin concentrations (Table 1). Insulin sensitivity was significantly lower in insulin-resistant versus insulin-sensitive horses (P=0.014, Table 1). The peripheral insulin resistance was compensated by a tendency for increased insulin response (AIRg), such that neither glucose effectiveness (Sg) nor secretory function (DI) were significantly lowered in insulin-resistant compared to insulin-sensitive horses (Table 1), thus validating the large animal model of naturally occurring insulin resistance.
Table 1. Baseline parameters in insulin sensitive and insulin resistant horses.

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<th>Insulin Resistant</th>
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<tr>
<td>Body weight (kg)</td>
<td>534.4 ± 28.8</td>
<td>510.2 ± 11.5</td>
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<tr>
<td>Body Condition Score</td>
<td>6.5 ± 0.7</td>
<td>7.0 ± 0.2</td>
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<tr>
<td>Basal [Insulin] (mIU L⁻¹)</td>
<td>16.4 ± 5.8</td>
<td>18.7 ± 4.4</td>
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<tr>
<td>Basal [Glucose] (mg dL⁻¹)</td>
<td>100.2 ± 4.75</td>
<td>101.3 ± 3.76</td>
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<tr>
<td>SI (L min⁻¹ mU⁻¹)</td>
<td>2.53 ± 0.60</td>
<td>0.62 ± 0.11 *</td>
</tr>
<tr>
<td>AIRg (mU L⁻¹ min⁻¹)</td>
<td>405.6 ± 80.0</td>
<td>983.6 ± 256.2</td>
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<tr>
<td>DI</td>
<td>874.0 ± 221.6</td>
<td>593.4 ± 83.9</td>
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<tr>
<td>Sg (min⁻¹)</td>
<td>1.67 ± 0.12</td>
<td>1.93 ± 0.28</td>
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SI: insulin sensitivity, AIRg: acute insulin response to glucose, Sg: glucose effectiveness, DI: disposition index [=SI X AIRg]. * denotes significant difference between groups (P<0.05). n=5/group.
**TOTAL GLUT CONTENT IN ADIPOSE TISSUE OF INSULIN-SENSITIVE HORSES:**

Total GLUT-4 and GLUT-12 protein content were measured in plasma membrane enriched fractions across the various adipose tissue depots (Fig. 1). Differential expression was noted across the various adipose tissue sites, with total GLUT-4 protein content being elevated in visceral (mesenteric and omental) adipose tissue compared to the corresponding subcutaneous adipose tissue sites (Fig. 1). Similarly, total GLUT-12 protein content was significantly greater in visceral adipose tissue (mesenteric, with a tendency in omental) compared to subcutaneous adipose tissue depots in the insulin-sensitive horses (Fig. 1).
Fig. 1 Differential protein expression of total GLUT-4 (white bars) and GLUT-12 (black bars) across various visceral and subcutaneous adipose tissues. Top panel: Representative Western blot of GLUT from a plasma membrane-enriched preparation of adipose tissue. Calsequestrin (CASQ) protein content was used as a loading control. Bottom panel: Mean±SE total content of GLUT protein in skeletal muscle (SM) vs. visceral [omental (OM), retroperitoneal (RP), and mesenteric (MES)] and subcutaneous [tailhead (TH) and nuchal (NL)] adipose tissue depots (n=5/group). Relative units were expressed in relation to the skeletal muscle of insulin-sensitive subjects. *: Significantly greater than the skeletal muscle control group †: Significantly greater than all subcutaneous sites within the same insulin-sensitive group, P=0.05. Please note that there is a tendency for GLUT-12 content to be higher in the omental site compared to other sites during the insulin-sensitive state.
TOTAL GLUT CONTENT IN IS vs. IR HORSES:

A comparison of total glucose transporter content in insulin-sensitive versus insulin-resistant horses showed 44% lower total GLUT-4 content in omental adipose tissue of the insulin-resistant group compared to the control insulin-sensitive group (P=0.027, Fig 2A). In contrast, there were no significant differences in total GLUT-4 protein content between insulin-sensitive and insulin-resistant horses for all other adipose tissue sites sampled. Similarly, no differences were observed in total GLUT-12 content (Fig. 2B) for any of the adipose tissue sites sampled between groups.
Fig. 2 Compensated insulin resistance (IR) induces decreases in omental total GLUT-4 protein content. Top panel: Representative Western blot of GLUT from a plasma membrane-enriched preparation of subcutaneous and visceral adipose tissues. Calsequestrin (CASQ) protein content was used as a loading control. Bottom panel: Mean±SE of total GLUT-4 (A) and GLUT-12 (B) protein content in various visceral and subcutaneous adipose tissue depots during insulin-sensitive (IS) and insulin-resistant (IR) states (n=5 per group). Relative units were expressed in relation to IS horses for each specific depot; ‡: P=0.05 vs. IS group. Please refer to Fig. 1 for legends.
GLUT TRANSLOCATION IN IS vs. IR. HORSES:

Active cell-surface GLUT content was quantified in photolabeled subcutaneous and visceral adipose tissue in order to determine whether insulin resistance corresponded to decreased total GLUT content and/or decreased GLUT translocation. An overall group effect was noted, with significantly lower (P=0.033) active cell surface GLUT-4 content in adipose tissue of insulin-resistant compared to insulin-sensitive subjects (by 63% and 70% in visceral and subcutaneous adipose sites, respectively, Fig. 3), suggesting that insulin resistance decreased GLUT-4 translocation to the cell-surface in the adipose tissue. In contrast, there was no difference in cell-surface GLUT-12 content between groups for either subcutaneous or visceral sites (P=0.454, Fig. 4).
Fig. 3 Insulin resistance decreases active cell-surface GLUT-4 in photolabeled adipose tissue. (A): Representative Western blot of cell-surface GLUT-4 during insulin-sensitive (IS) and insulin-resistant (IR) states. L: labeled fraction; UL: unlabeled fraction; TL: total lysate. (B) Mean±SE of labeled cell-surface content of active GLUT-4 in subcutaneous (SC; tailhead) and visceral (Vis; omental and mesenteric) adipose sites during insulin-sensitive (IS) and insulin-resistant (IR) states (n=2-4/group). Relative units expressed in relation to an internal positive control; *: P=0.05 vs. IS group.
Fig. 4 Insulin resistance does not alter active cell-surface GLUT-12 in photolabeled adipose tissue. (A):
Representative Western blot of cell-surface GLUT-12 in insulin-sensitive (IS) and insulin-resistant states. (B):
Mean±SE of labeled cell-surface content of active GLUT-12 in subcutaneous and visceral adipose sites (n=2-4/group). Relative units expressed in relation to an internal positive control. Please refer to Fig. 3 for legends.
AS160 ACTIVATION IN IS vs. IR HORSES:

In order to characterize potential molecular mechanisms underlying the alterations in GLUT-4 trafficking, AS160 was quantified in subcutaneous and visceral adipose tissue, and no differences in total AS160 (Fig. 5A, \( P=0.799 \)) or phosphorylated-AS160 (Fig. 5B, \( P=0.896 \)) were found between groups for each site.
Fig. 5 The decrease in active GLUT-4 content in omental tissue occurs despite normal activation of AS160 during the insulin-resistant state. Total Protein content of (A) AS160 and (B) phosphorylated AS160 in adipose tissue during insulin-sensitive (IS) and insulin-resistant (IR) states (n=5 per group). Top panel: Representative Western blot of AS160 in adipose total tissue. Bottom panel: Mean±SE of protein expression of total AS160 (A) and phosphorylated AS160 (B) in subcutaneous (SC; tailhead) and visceral (VIS; omental and mesenteric) adipose sites.
DISCUSSION:

Our data demonstrated that: 1) healthy insulin-sensitive horses exhibit differential GLUT protein expression across various visceral and subcutaneous adipose sites, with greater GLUT-4 and GLUT-12 expression noted in visceral (mesenteric and omental) adipose tissue; 2) insulin resistance decreases total omental GLUT-4, but not GLUT-12, protein content; 3) insulin resistance decreases GLUT-4, but not GLUT-12, translocation to the cell surface in photolabeled visceral and subcutaneous adipose tissue; 4) these changes occur independently of alterations in AS160 activity; 5) this large animal model of naturally occurring insulin resistance provided some novel insights into the molecular mechanisms involved in the pathogenesis of IR and may provide some novel therapeutic targets for the prediabetic and diabetic population.

TOTAL GLUT CONTENT IN ADIPOSE TISSUE OF INSULIN-SENSITIVE HORSES:

Differential GLUT protein expression was found across various visceral and subcutaneous adipose tissue depots in the healthy, insulin-sensitive subjects. Total GLUT-4 and GLUT-12 content were noted to be higher in visceral (e.g. omental and mesenteric) adipose tissue when compared against their subcutaneous counterparts, consistent with previous studies showing that visceral adipose tissue likely plays a comparatively more substantial role in systemic glucose homeostasis.

GLUT-4 CHARACTERIZATION IN ADIPOSE TISSUE OF INSULIN-RESISTANT HORSES:

GLUTs are required for the facilitated diffusion of glucose across the plasma membrane where glucose is oxidized by the cell in order to obtain high energy phosphates (i.e., ATP), which are required for cellular processes. GLUT-4 is predominantly expressed in insulin-sensitive tissues
(e.g., muscle and adipose tissue), and it is known to play a crucial role in whole-body glucose homeostasis. In our study, we quantified total GLUT-4 content in the insulin-sensitive versus insulin-resistant horses within our sampled adipose tissues. Further, we investigated the effects of insulin-resistance on the rate-limiting step of glucose uptake into insulin-sensitive tissues, GLUT-4 translocation to the cell-surface membrane. Insulin resistance decreased total GLUT-4 content in omental adipose tissue only, with no significant differences found in the other sampled adipose tissue sites. Additionally, insulin resistance resulted in decreased active GLUT-4 content at the cell surface in visceral (mesenteric and omental) and subcutaneous (tailhead) adipose tissue when compared to insulin-sensitive counterparts. While the mechanisms involved in the pathogenesis of insulin resistance remain poorly understood, our data from a large animal model exhibiting the earliest stages of preclinal diabetes suggests that decreased total omental GLUT-4 protein content and/or decreased GLUT-4 translocation may be contributory or even primary factors in the creation of the insulin resistant (and eventually diabetic) phenotype.

**GLUT-12 CHARACTERIZATION IN ADIPOSE TISSUE OF INSULIN-RESISTANT HORSES:**

GLUT-12 is a more recently identified GLUT that, according to some studies, may serve as a second insulin-sensitive transporter protein. In conjunction with our GLUT-4 studies, we examined total GLUT-12 content in the insulin-sensitive versus the insulin-resistant horses within our sampled adipose sites. We also examined active cell-surface GLUT-12 via the same photolabeling technique used to examine GLUT-4. Insulin resistance was not associated with any significant alterations in total GLUT-12 or active cell-surface protein content in any of our surveyed adipose tissue depots, suggesting that GLUT-12 is not a pathogenic factor during insulin resistance.
AS160 ACTIVITY IN ADIPOSE TISSUE OF INSULIN-RESISTANT HORSES:

AS160 is a recently discovered signaling protein in the calcium/contraction- and insulin-signaling pathways that regulates GLUT-4 translocation. It is currently the most distally known signaling protein in these pathways. In direct relation to the dysregulation of GLUT-4 translocation in the sampled adipose tissues, AS160 was proposed as a potential molecular mechanism for this decreased GLUT-4 activation. In theory, decreased phosphorylation (and thus activation) of AS160 could have been responsible for the decreased GLUT-4 activation, as AS160 activation is known to be associated with GLUT-4 translocation to the cell-surface. Our results, however, indicated no changes in the protein content of either total or phosphorylated (active) AS160 between insulin-sensitive and insulin-resistant horses in the adipose tissues sampled, denoting that the changes in total omental GLUT-4 content and adipose GLUT-4 translocation were occurring independently of any changes in AS160 activity.

NOVEL, LARGE ANIMAL MODEL OF NATURALLY OCCURRING INSULIN-RESISTANCE:

Our equine subjects served as excellent large animal models of insulin resistance. This is a novel example of naturally occurring insulin resistance versus past models which have relied upon artificially induced insulin resistance through transgenicity or a combination of diet and pharmaceutical compounds. Additionally, the equine animal model allowed for easier tissue collection, with much larger tissues to biopsy, and these animals provided a more translatable research model, as horses thermoregulate (i.e. sweating and shivering) more in accordance with humans, whereas rodents typically rely on brown adipose tissue lipolysis. Furthermore, our equine model only exhibits the physiological characteristics of the prediabetic or insulin resistant state, whereas rodent models typically display early onset hyperglycemia, an inappropriate
physiological attribute for characterizing insulin resistance. With all this in mind, this equine model of naturally occurring insulin resistance provided novel insights into the molecular mechanisms involved in the pathogenesis of insulin resistance and may yet provide some novel therapeutic targets for prediabetic or diabetic patients.

**CONCLUSION:**

In summary, naturally occurring compensated insulin resistance in this large animal model is characterized by selective impairment of the glucose transport pathways, namely that active cell-surface GLUT-4 content was lower in adipose tissue (visceral and subcutaneous) depots of the insulin-resistant compared to the insulin-sensitive group, while total GLUT-4 content was only noted to be reduced in omental adipose tissue. In addition, GLUT-12, one of the most recently discovered GLUT isoforms, does not appear to be a pathogenic factor in our model of the early stages of insulin resistance, since neither its total nor active content were affected in any of the sampled adipose depots in the insulin-resistant state. It is concluded that adipose tissue GLUT-4, but not GLUT-12, particularly in the omental site, contributes to the pathogenesis of naturally occurring compensated insulin resistance.

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Works Cited


