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The effect of training on GLUT expression

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Pre ambule

The corona crisis did not have any effect on my thesis.

Foreword

Writing this thesis has been a new experience for me. Training and physiology have had my interest for many years now, but looking at it with a scientific view is new. This thesis has taught me how to read, summarize and interpreted scientific articles.

I could not have done this without the help of Lorie de Maré, Carmen Vidal Moreno de Vega and Prof. dr. Catherine Delesalle. I would like to thank them for their help, corrections and advice. I would also like to thank my parents and friends for their unconditional support.

List of abbreviations

AMPK: Adenosine monophosphate-activated protein kinase
AS160: Phosphorylated Akt substrate of 160 kDa
ATP: Adenosine triphosphate
ATPase: Adenosine triphosphatase
EMS: Equine metabolic syndrome
GAP: GTPase-activating protein
GLUT: Glucose transporter
GS: Glycogen synthase
GSK3: Glycogen synthase kinase 3
HKII: Hexokinase II
HIT: High intensity training
ICDH: Isocitrate dehydrogenase
IR: Insulin resistance
IRS: Insulin responsive substrates
IS: Insulin sensitive
LDH: Lactate dehydrogenase
LIT: Low intensity training
MHC: Myosin heavy chain
mTORC2: Mammal target of rapamycin complexed with Rictor
NADH: Nicotinamid-Adenin-Dinucleotid Hydrid
PDK1: Phosphoinositide-dependent protein kinase 1
PKB: Protein kinase B
PKC: Protein kinase C
PI3K: Phosphatidylinositol 3-kinase
PIPD 1 & 2: Phosphatidylinositol dependent protein kinase 1 and 2
SLC2: Solute carrier family 2
SNARE: soluble N-ethylmaleimide-sensitive factor-attachment protein receptors
VAMP2: Vesicle associated membrane protein 2

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Abstract

Glucose is a key molecule in mammals. Glucose will be used as an energy source and converted into glycogen and adenosine triphosphate (ATP). Glucose transporters (GLUTs) are important for getting glucose into the cell. There are different isoforms of GLUT. Not all of these isoforms are present in muscle tissue. GLUT4 is one of the most important isoforms present in muscle tissue and adipose tissue. GLUT4 is able to translocate and their expression is able to change mainly due to muscle contraction or an insulin stimulus. GLUT4 translocation has an effect on the glucose uptake rate of the cell. The translocation pathways and signaling cascade of GLUT4 are not completely clear yet. Concentric and isometric contractions lead to an increase in GLUT4 content in the muscle cell. Eccentric concentrations lead to a decrease in GLUT4 content. High intensity training (HIT) and low intensity training (LIT) can lead to the same level of GLUT4 expression, since there is a maximum level of GLUT4 expression in muscle cells. Not all animal species have the same glucose transporter isoforms and react the same to exercise training. Horses show the least increase in GLUT4 content after training. Detraining causes a quick decrease in GLUT4 content. Training to increase GLUT4 expression can be useful in the therapy of certain pathologies, like diabetes mellitus type 2 and equine metabolic syndrome (EMS). Both these diseases are characterized by insulin resistance. More GLUT4 expression will lead to a better insulin sensitivity.

Samenvatting

Glucose is een zeer belangrijke molecule voor zoogdieren. Glucose kan gebruikt worden als energiebron en omgezet worden in glycogeen en adenosine trifosfaat (ATP). Glucose transporters (GLUTs) zijn belangrijk om glucose in de cel te brengen. Er zijn verschillende isovormen van GLUT. Niet alle isovormen zijn aanwezig in spierweefsel. GLUT4 is een van de meest belangrijke isovormen die aanwezig is in spierweefsel en in vetweefsel. GLUT4 is in staat om te transloceren en zijn expressie is kan vooral veranderen door spiercontracties of een stimulus van insuline. GLUT4 translocatie heeft een effect op de glucose opname snelheid van de cel. De translocatie pathways en signaal cascade van GLUT4 zijn nog niet helemaal duidelijk. Concentrische en isometrische contracties leiden tot een verhoging van de hoeveelheid GLUT4 in de spiercel. Excentrische contracties leiden tot een verlaging van de hoeveelheid GLUT4. High intensity training (HIT) en low intensity training (LIT) kunnen beide tot hetzelfde level van GLUT4 expressie leiden, aangezien er een maximaal level van GLUT4 expressie is in de spiercellen. Niet alle dieren hebben dezelfde glucose transporters isovormen en reageren hetzelfde op oefentraining. Paarden laten de laagste toename zien in GLUT4 hoeveelheid na training. Stoppen met trainen zorgt voor een snelle daling van de GLUT4 hoeveelheid. Training om de GLUT4 expressie te verbeteren kan bruikbaar zijn in de therapie van bepaalde pathologieën, zoals diabetes mellitus type 2 en equine metabool syndroom (EMS). Beide ziektes worden gekarakteriseerd door insuline resistentie. Meer GLUT4 expressie zal zorgen voor een betere insuline sensitiviteit.

Introduction

Animals and plants need especially glucose as an essential energy source to produce Adenosine triphosphate (ATP). Food can provide glucose to the body; however, the body can produce glucose as well via certain pathways like the gluconeogenesis. The gluconeogenesis takes place in the liver and produces glucose from non-carbohydrate sources. Once glucose is uptaken or produced, the glucose molecules will be transported in the bloodstream, but they

need to find a way to get into the cells. Glucose uptake into the cells is the rate-limiting step, especially in insulin-sensitive tissue such as striated muscle tissue and adipose tissue. The blood glucose level is maintained within a narrow range in all mammalian cells. Glucose passes the cell membrane by passive facilitative transport since the lipid bilayer is impermeable to glucose (Navale and Paranjape., 2016). Sjaastad et al. (2010) mentions "glucose molecules do not readily penetrate cell membranes and must be transported into cells by special transport proteins. These transport molecules are called GLUT molecules, an abbreviation for Glucose transport molecules". These GLUTs are considered as the key regulators in the glucose homeostasis as there are in every cell type one or more GLUT-isoforms. So far known, there are fourteen different GLUT molecules, GLUT 1 - GLUT 14, each located in different cell types.

Once glucose resides in the cell, it gets broken down in the glycolysis to produce ATP and reduced coenzyme (NADH, an abbreviation of Nicotinamid-Adenin-Dinucleotid Hydrid) (Sjaastad et al, 2010). The dephosphorylation process of ATP causes energy release that can be used for cell metabolism and muscle contraction (Sjaastad et al., 2010). During activity, the need of ATP increases exponentially for muscle contraction. When all the ATP stored in the muscle cells is used, there are several ways to regain ATP such as oxidative phosphorylation and the creatine phosphate pathway (Hopkins, 2006).
Once glucose is transported into the muscle cell by GLUT molecules, glucose can be used in the glycolysis when ATP is required, or it can be transformed to glycogen. Glycogen is stored in the muscle cell and will be used for ATP production when activity increases (Sjaastad et al, 2010).

Research showed that there are basal GLUTs and GLUTs that translocate from an intracellular pool to the cell membrane to increase glucose uptake into the cell when needed. This process is called translocation (Mueckler and Thorens, 2013). GLUT1 for example is located at the cell surface as a basal transporter and is considered to be the primary transporter in many cell types for basal glucose uptake. In contrast, GLUT isoforms that are stored intracellular need a stimulus to translocate to the cell membrane (Ashrafian and Bogle, 2004).
Insulin is a hormone excreted by the pancreas that regulates the blood glucose levels. The moment glucose levels increase in the blood stream, insulin will be secreted to stimulate the glucose uptake in muscle and fat tissue. It is known that insulin stimulates the exocytosis of GLUT molecules to the cell membrane. The amount of glucose transported into the muscle cells depends on the concentration of GLUT molecules on the membrane of the cell (Bouman et al.,2008). Recent studies showed that GLUT4, 8 and 12 are predominantly expressed in insulin-sensitive tissue such as striated muscle and adipose tissue (Stuart et al., 2009; Lacombe, 2014; Coudert et al., 2015).

Training will improve the properties of the muscles in order to enhance the performance capacity. There are different kinds of training with different intensities and duration, like endurance training (aerobic) and strength training (anaerobic). During endurance training, the muscle fibers will develop more mitochondria and capillaries. This will lead to an increased capacity for oxidative phosphorylation. In this way the muscle is able to keep up the activity over longer periods without tiring (Sjaastad et al., 2010). Strength training mainly focusses on increasing the synthesis of actin and myosin, particularly in the fast glycolytic fibers. The maximum contractile force of the muscle will increase and hypertrophy of the muscle will occur. A combination of both training methods is preferred, because the stamina of the muscle does not improve by strength training. Glucose is needed to maintain muscle function and must be able to flow into the muscle cells more easily during training than during resting conditions.

This is possibly achieved by an increase of GLUTs in the cell membrane to allow a higher concentration of glucose to enter (Sjaastad et al., 2010).

Some diseases have a link to insulin resistance. Type 2 diabetes in humans is a condition where the body is in an insulin resistant state. This condition goes hand in hand with glucometabolic impairment and systemic subclinical inflammatory processes. Physical activity seems to have a positive influence on the uptake of glucose. Type 2 diabetes patients have the normal amount of GLUT4, however because of the insulin resistant state, the body is not able to induce the insulin signaling. Physical activity can induce insulin signaling pathways, which facilitate GLUT 4 expression and translocation (Röhling et al.,2016).
Equine metabolic syndrome (EMS) is a quite common disorder in horses and comparable to insulin resistance in humans. These animals are characterized by a high body condition score and are prone to develop laminitis, which is a life threatening disease in horses. The horse's hoof is made out of digital lamellae, among other structures. These lamellae contain lots of GLUT1 transporters. This indicates a high metabolism in the digital lamellae (Lacombe et al., 2014). Insulin resistance means cells are not able to stimulate glucose disposal into insulin-sensitive tissues. When a horse is in an insulin resistant state, the functional capability of the insulin- responsive GLUTs is impaired in muscle and adipose tissue (Lacombe et al, 2014). A good diet to make the horses lose weight is a key factor in the treatment, but regular low intensity exercise has additional benefits for glucose uptake and insulin sensitivity (Bamford et al.,2019).

This study will focus on the effect of training on GLUT expression because there are several insulin-dependent GLUTs present in skeletal muscle and adipose tissue that can possibly alter performance capacity, however the underlying pathways are not fully clarified yet. The questions this study will try to answer, are: Does training increase the membrane fraction of the GLUTs? Which GLUT isoforms will increase and how fast? Does the type of training affect the GLUT molecules? Are there any links between increasing the concentration of GLUT molecules and certain pathologies? Will the concentration of GLUT molecules decrease again when the training is stopped? Most of the studies investigated GLUT expression in humans and rodents. However, more and more studies are interested in the GLUT expression in other animals as well such as horses. This study will try to see if there are any similarities or big differences between human and animal GLUT expression.

1. Important substances

1.1 Glucose

Glucose is one of the most important molecules in mammals. Glucose is a carbohydrate molecule, a monosaccharide, also referred to as $C_6H_{12}O_6$. It is a non-phosphorylated sugar. Glucose is more than just an energy source, it plays a role in the glucose and energy homeostasis of the body as a signaling molecule and participates in specific gene transcription, enzyme activity, and hormone secretion (Thorens and Mueckler, 2010).
Not all mammals have access to glucose in the same way. For instance, monogastric mammals absorb glucose in the small intestine, while adult ruminants ferment carbohydrates in the rumen with propionate, a precursor of glucose. Young ruminants still have enteral absorption, because the rumen is not fully developed yet. Adult ruminants depend on the endogenous gluconeogenesis for their glucose supply (Duehlmeier et al., 2007).

In a basal state, 20% of the whole-body glucose disposal is through skeletal muscle. This amount increases to about 75-95% in a hyperinsulinaemic state, where most of the glucose will be transformed into glycogen (Mueckler, 1994).
Glucose is the key molecule in the glycolysis. During this process glucose gets broken down to ATP and reduced nicotinamide adenine dinucleotide (NADH) (Sjaastad et al, 2010). One of the three phosphate groups of ATP can be transferred to another molecule and when this happens, energy is released and can be used for other reactions in cells (Sjaastad et al., 2010).

Glucose is transported into the muscle cell by glucose transporters. Once glucose enters the cell, hexokinase II (HKII) will catalyze the phosphorylation of glucose to glucose-6-phospate. The phosphorylation of glucose is the first step of the glycolysis. Glucose can be used in the glycolysis when ATP is required, but also be transformed to glycogen, which serves as storage form of carbohydrates. Glycogen is stored in the liver and skeletal muscle cells and will be broken down when needed. The result of breaking down glycogen is a molecule called glucose-l-phosphate, which will be utilized for ATP production (Nelson and Cox, 2008). Glycogen can be seen as an emergency supply for energy in the muscles. In a “fight” of “flight” situation glycogen is the first energy source used (Jensen et al., 2011).
Glucose homeostasis is maintained by the body both in situations of food availability and scarcity through the action of insulin and glucagon.

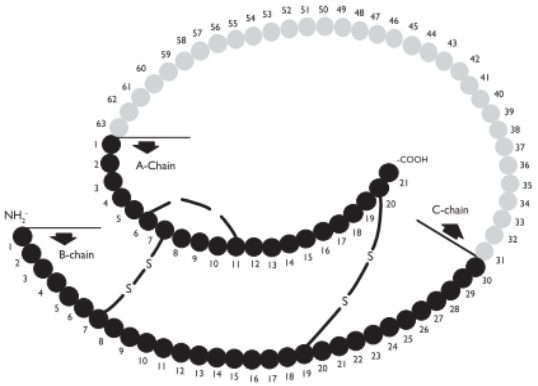

Figure 1. The conformation of an insulin molecule (Joshi et al., 2007)

1.2 Insulin

Insulin is a hormone secreted by the endocrine part of the pancreas. It is a protein consisting of 2 main polypeptide chains (A and B). There is a third chain, C-chain, that is responsible for connecting the A and B chains (Fig. 1) (Joshi et al., 2007). First, preproinsulin is formed in the beta cells and then it moves to the cisternal space of the rough endoplasmatic reticulum, where proteolytic enzymes will cleave it into proinsulin. The Golgi apparatus will be the place where proinsulin is released in vesicles. Prohormone convertase 2 and 3 and carboxypeptidase H will help with the conversion of proinsulin into insulin in maturing granules. The translocation of these granules from their intracellular location to the beta cell surface will be facilitated by microtubules and microfilaments (Joshi et al., 2007; Tripathy et al., 2012). One of the factors stimulating the secretion of insulin is glucose. Glucose transporters take up glucose in the beta cells. Glucokinase will oxidize glucose and act as a glucose sensor (Joshi et al., 2007). When glucose is above a certain threshold, insulin will be secreted. This threshold may vary depending on factors like obesity, normal diet or food deprivation (Chen, Tassava and Romsos, 1993). The beta cell membrane is at a negative potential when glucose concentrations are under the threshold. K^+ efflux is responsible for the negative potential and keeps voltage-

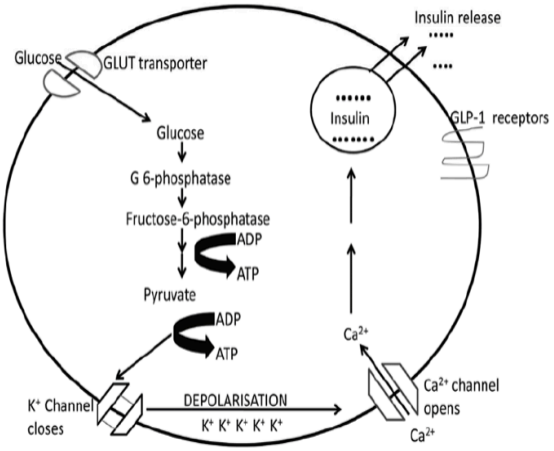

Fig 2. The pathway of insulin secretion due to glucose with the glycolysis, closing of ATP sensitive potassium channels and depolarization of the membrane leading to the opening of Calcium channels. Calcium influx triggers exocytosis of insulin granules (Gunton and Girgis, 2012)

gated Ca^{2+} channels closed (Joshi et al., 2007). ATP concentrations will rise in the beta cells after a rise in plasma glucose and upregulation of the glucose uptake and metabolism. ATP-sensitive potassium channels (K_{ATP}) will close and the negative potential of the membrane will change into a membrane depolarization (Fig. 2).
The voltage gated Ca^{2+} channels open up and Ca^{2+} will flow into the cell. This will trigger exocytosis of the insulin granules (Joshi et al., 2007). The secretion of insulin is biphasic, initially fast and followed by a slower release (Wilcox., 2005). The first phase is a high peak, coming from granules that are ready to be released. The insulin concentration drops again and the second phase starts, where the granules need to be prepped before they can be released (Bratanova-Tochkova et al., 2002).

2. Glucose transporters

2.1 GLUTs, what are they?

Glucose is transferred into the eukaryotic cell, through the lipid bilayer, by a glucose transporter, also known as GLUT. So far fourteen GLUTs are known to exist in the human body (Thorens and Mueckler, 2010; Simmons, 2017). GLUTs are integral membrane proteins containing twelve transmembrane segments, a site of N-linked glycosylation, a central cytoplasmatic linker domain and N and C termini positioned in the cytoplasm. GLUTs are encoded by the solute carrier family 2 (*SLC2*) of genes and are part of the major facilitator superfamily (Mueckler and Thorens, 2013). There are three classes of GLUTs. Class I: GLUT1-4 and GLUT14; Class II: GLUT5, 7 , 9 and 11; Class III: GLUT6, 8, 10 ,12 and 13 (Coudert et al., 2015). They are categorized based on sequence similarity (Mueckler and Thorens, 2013). GLUT1, 4, 8, 11 and 12 can be found in skeletal muscle tissue and are especially investigated to enhance exercise performance capacity (Navale and Paranjape, 2016). The GLUTs discussed in this study are all facilitative transporters. This means the GLUTs help glucose to enter the cell when the extracellular glucose concentration is higher than the intracellular glucose concentration. This facilitated diffusion is a passive form of transportation that does not require energy (Sjaastad et al., 2010). While GLUT4 is especially investigated, little is known about GLUT8, 11 and 12 (Thorens and Mueckler, 2010). GLUT4 is able to translocate and the rate of translocation changes due to exercise for instance. GLUT8, 11 and 12 are also present in muscle tissue, but the change in expression is small compared to GLUT4. GLUT5 transports fructose instead of glucose (Duehlmeier et al., 2007). The concentration of GLUT1 in muscle tissue is low, and they do not translocate (Richter and Hargreaves, 2013).

Not all animal species seem to have all GLUTs. Chickens for instance have GLUT1, 2, 3, 8 and 9, but lack GLUT4. GLUT12 is noted as a possible insulin sensitive glucose transporter in chickens (Coudert et al., 2015). Pigs have in their muscle tissue a high amount of GLUT4 and at a lower level, GLUT1, 3, 5, 8, 10 and 11 (Aschenbach et al., 2009). The genome of the mouse lacks the genes for GLUT11 and GLUT 14, namely *SLC2A11* and *SLC2A14* respectively. Their function is maybe taken over by another member of the SLC2 gene family (Scheepers et al., 2005).

2.2 GLUT4

The predominant substrates of GLUT4 are glucose and glucosamine (Mueckler and Thorens, 2013). The half-life of GLUT4 is about 48 hours (Foley et al., 2011). GLUT4 is an insulin responsive glucose transporter and is mostly located in skeletal and cardiac muscle tissue and adipocytes. About 90% of GLUT4 is located intracellularly, while 10% is present in the cell membrane of unstimulated cells (Thong et al., 2005). GLUT4 is able to translocate if there is a

signal resulting from muscle contraction or if insulin receptors send a stimulus. (Ashrafian and Bogle, 2004; Mueckler and Thorens, 2013). During low insulin levels GLUT4 can be found in intracellular membrane compartments, but after a meal, insulin levels will rise and GLUT4 will translocate to the plasma membrane (fig. 3). Because of this translocating there will be an increase in glucose uptake and metabolism (Mueckler and Thorens, 2013). There is a correlation between glucose uptake and GLUT4 expression in muscle tissue. Experiments have been done in humans and rats that show an increase in GLUT4 content in the plasma membrane after a stimulus of insulin or muscle contraction (Richter and Hargreaves, 2013). The increase in glucose transport is higher than just the increase in GLUT4, which suggests an increase in intrinsic GLUT4 activity as well. It must be noted that neuroendocrine activity, which is present when exercising, might be responsible for this change in intrinsic GLUT4 activity as well (Kristiansen et al., 1996). Hansen et al. did not find a significant difference between the rise in GLUT4 translocation and the increase in glucose transport activity. This result seems to mean that the intrinsic activity of GLUT4 did not change (Hansen et al., 1998). There is still a lot unknown about the intrinsic activity of GLUT4, so at this point researchers agreed that the rise in glucose uptake is caused by GLUT4 translocation, rather than increased its intrinsic activity (Richter and Hargreaves, 2013).

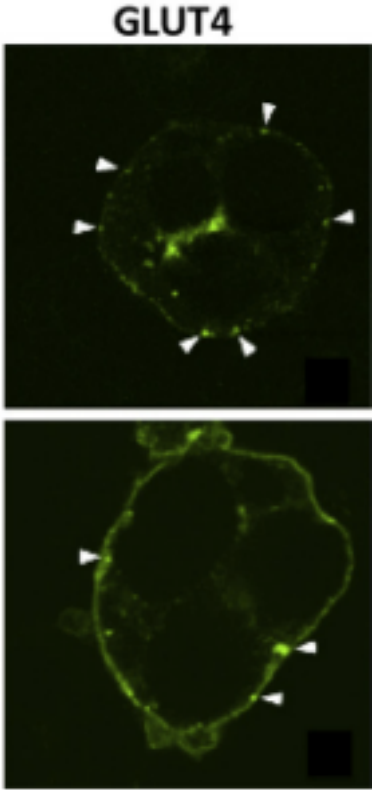

Fig. 3: Translocation of GLUT4 from intracellular to the plasma membrane of the cell after stimulation with insulin. The photo at the top shows the GLUT4 intracellular, the bottom picture shows GLUT4 in the cell membrane after translocation. (Mueckler and Thorens, 2013)

2.3 GLUT8

The highest concentration of GLUT8 can be found in testis tissue. A smaller amount is present in spleen, heart muscle, skeletal muscle, prostate, brain and small intestine tissue. Human and mouse *SLC2A8,* this is the gene encoding GLUT8, are very similar, 85,2% of the nucleotides of *SLC2A8* are the same between these two species (Doege et al., 2000). GLUT8 and GLUT4 show a glucose transport activity similar to each other (Doege et al., 2000). Besides glucose, GLUT8 transports fructose and galactose (Simmons, 2017).

2.4 GLUT11

GLUT11 is present in human heart and skeletal muscle tissue (Simmons, 2017). D-glucose is transported across the cell membrane through this glucose transporter. It is remarkable the fact that fructose inhibits the activity of GLUT11. GLUT11 has a high affinity for fructose, just like GLUT5 (Doege et al., 2001). Rat tissues do not contain the same amount of GLUT11 as humans. Rat heart muscle tissue possesses GLUT11, but the concentration of GLUT11 in skeletal muscle tissue is significantly lower than in humans (Doege et al., 2001). GLUT11 is mainly present in slow twitch muscle fibers in humans (Scheepers et al., 2005).

2.5 GLUT12

GLUT12 can also be found in heart and skeletal muscle (Simmons, 2017). In human skeletal muscle cells, GLUT12 can be stimulated by insulin to translocate from intracellular membrane compartments to the plasma membrane (Stuart et al., 2009). Chicken GLUT12 have a 71% similarity with human GLUT12. Human GLUT12 has the ability to internalize. Due to a difference in conformation, chicken GLUT12 does not have that ability (Coudert et al., 2015).

3. GLUT4 translocation pathways

GLUT4 translocates from an intracellular compartment to the cell surface, plasma membrane and transverse tubules, in the presence of a stimulus of insulin (Rodnick et al., 1992, Jensen et al., 2011). Muscle contractions also lead to a higher GLUT4 content in the sarcolemma (Richter and Hargreaves, 2013). GLUT4 is continuously recycled while moving from and towards the plasma membrane. The uptake back into the cell, away from the plasma membrane, is called internalization (Foley et al., 2011). There are two intracellular pools of GLUT4 (Richter and Hargreaves, 2013). Coderre et al. found out that those intracellular pools react to different stimuli. One reacts to insulin, the other one to exercise (Coderre et al., 1995).

Clathrin-mediated endocytosis and cholesterol dependent endocytosis are the pathways through which GLUT4 internalization from the plasma membrane takes place (Foley et al., 2011). Along with endocytosis, exocytosis is also an important process in the recycling of GLUT4. In rat muscle cells, the internalization rate of GLUT4 is not regulated by insulin, but the exocytosis rate of GLUT 4 is (Foley et al., 2011). Fazakerley et al. found different results in L6 myoblasts, which say endocytosis of GLUT4 is slower after a stimulus of insulin (Fazakerley et al., 2010). The internalization process was not the same in these studies, which might explain the difference in outcome. More tests were done in a different manner by Fazakerley et al. showing inhibition of endocytosis as a result to a stimulus of insulin (Fazakerley et al., 2010). Endocytosis rate of GLUT4 declines by stimuli that change the oxidative metabolism, hyperosmolarity, and muscle cell depolarisation (Foley et al., 2011). Increased exocytosis leads to a higher GLUT4 concentration in the membrane. Decreased endocytosis contributes to that higher concentration. The mitochondrial uncoupler 2,4-dinitrophenol inhibits endocytosis, which leads to a higher GLUT4 membrane concentration. Exercise and muscle contraction will give the same results (Fazakerley et al., 2010).

Exercise does not only influence the translocation of GLUT4, but also the translocation of vesicle associated membrane protein 2 (VAMP2). VAMP2 plays a role in the docking and fusion of synaptic vesicles associated with neurotransmitter release (Kristiansen et al., 1996). There might be a pathway that is the same for GLUT4 vesicles transport and neurotransmitter release (Kristiansen et al., 1996). VAMP2 is one of the soluble N-ethylmaleimide-sensitive factor-attachment protein receptors (SNARE proteins). SNARE proteins can be divided in vesicle SNAREs, which are SNARE proteins inside the GLUT4 vesicles, and target SNAREs, which are proteins located in the cell membrane (Richter and Hargreaves, 2013).
Membrane ruffles are the place where some of the GLUT4 vesicles insert into the cell membrane. These membrane ruffles are caused by insulin, which induces remodelling of actin filament just beneath the cell membrane (Tong et al., 2001).

After insulin granules are released from the pancreas to a glucose stimulus, insulin binds to insulin receptors on skeletal muscle cells, among others. This will lead to tyrosine kinase activity, followed by phosphorylation of the insulin responsive substrates (IRS) (Wilcox., 2005).

The pathway is shown in figure 4. IRS1 is most common in skeletal muscle tissue. These phosphorylated IRS1 can bind phosphatidylinositol 3-kinase (PI3K) (Wilcox., 2005), which is an important enzyme in the translocation of GLUT4 (Röhling et al., 2016).

PI3K acts through Akt/protein kinase B (PKB), protein kinase C (PKC) and phosphatidylinositol dependent protein kinase 1 and 2 (PIPD1 & 2) (Wilcox., 2005). PI3K has an effect on the cellular glucose uptake. Some studies suggest PI3K leads to an activation of glycogen synthase through PKB, which is a part of the signaling cascade (Cross et al, 1995; Hurel et al., 1996; Shepherd et al., 1997).

The activation of PI3K catalyzes the formation of phosphatidylinositol 3,4,5-triphosphate. This consequently leads to the recruitment of phosphoinositide-dependent protein kinase 1 (PDK1) and PKB in the phospholipid layer of the cell membrane (Jensen et al., 2011). PDK1 phosphorylates PKB at threonine 308, which leads to activation of PKB. PKB needs to be phosphorylated at serine 473 as well to lead to full activation of PKB. The mammal target of rapamycin complexed with Rictor (mTORC2) is responsible for the phosphorylation of PKB (Alessi and Cohen, 1998). AS160, also known as TBC1D1, is the phosphorylated Akt substrate of 160 kDa (Jensen et al., 2011). This will eventually lead to GLUT 4 translocation by altering or suppressing its GTPase-activating protein (GAP) activity (Waller et al., 2011). PKB phosphorylates glycogen synthase kinase 3 (GSK3). GSK3 activity will be inhibited and glycogen synthase (GS) will be activated by dephosphorylation. Glycogen synthase is the enzyme responsible for the production of glycogen in the muscle tissue. Glycogen synthase can also be activated by glucose-6-phosphate (Jensen et al., 2011).

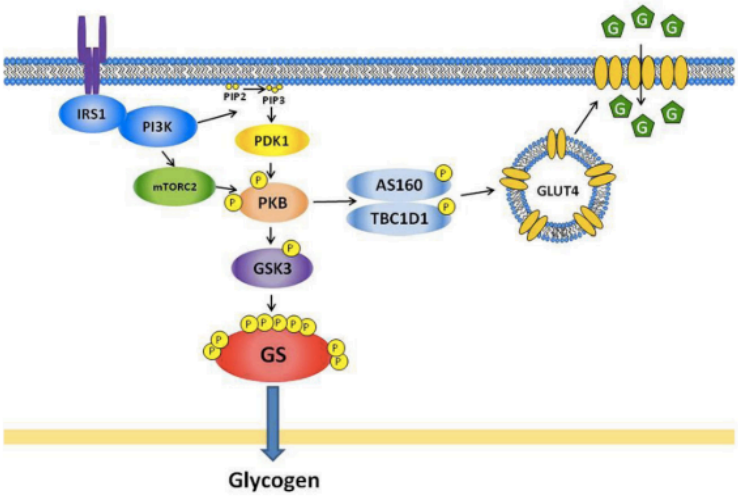

Figure 4: Signaling cascade from insulin leading to GLUT4 translocation (Jensen et al., 2011)

Adenosine monophosphate-activated protein kinase (AMPK) is one of the factors playing a role in the translocation of GLUT 4 from its intracellular location to the sarcolemma (Huber et al., 2007). Other important factors are insulin, hypoxemia and exercise (Huber et al., 2007). AMPK can be seen as the energy sensor of the cell. AMPK is activated by the contraction of muscles and it consists of 3 subunits, α-subunit, β- subunit and a γ-subunit. AMPK activity can increase enormously by binding AMP to the γ-subunit, which leads to increased AMPK phosphorylation at Thr-172 at the α-subunit. This activation will lead to a rise in glucose uptake (Richter and Hargreaves, 2013).

4. Skeletal muscle tissue

Muscle tissue can be divided into 3 different types: cardiac muscle, smooth muscle and skeletal muscle. Smooth muscle tissue can be seen in internal organs like the ones part of the digestive system and in the uterus, for instance. These muscles are controlled autonomously.

Skeletal muscles make it possible for a mammal to move and to keep a certain posture. They are connected to the bones by tendons (Sjaastad et al. 2010). Skeletal muscle tissue is composed out of muscle cells, also called muscle fibers, that have the ability to contract (Radák, 2018). In skeletal muscles not only muscle fibers are present, but also connective tissue, blood vessels and nerves (Sjaastad et al., 2010). These muscle fibers are present in all muscles, they contain thousands of myofibrils, which are composed by many sarcomeres that contain lots of myofilaments. These myofilaments are the proteins actin and myosin (Radák, 2018).

4.1 Muscle contraction

The muscle is able to contract because of the sliding filament model by Huxley (Squire et al., 2005), (fig. 5). This means actin is pulled in between two strands of myosin. During the contraction of the muscle cross bridges are formed that pull actin towards the middle of the sarcomere (Bouman et al., 2008). Myosin has a head shaped form that binds to actin. These heads can bend, so this action will result in the sliding of the actin filaments towards each other (Sjaastad et al.,2010). The muscle contraction is ATP and Ca^{2+} dependent and it relies on nerve impulses. Ca^{2+} is stored in the sarcoplasmatic reticulum in the muscle cell. Due to an action potential Ca^{2+} is released in the cytosol. When a muscle contracts the sarcomere becomes shorter, but the myofilaments stay the same length, so even though the complete muscle becomes shorter when a contraction occurs, the actin and myosin will not shorten. The myosin heads can bind to actin, but they can also bind ATP. ATP gets broken down to adenosine diphosphate (ADP) and phosphate by adenosine triphosphatase (ATPase). Energy will be released when ATP loses a phosphate group and becomes ADP. This energy will be stored as potential energy in the myosin heads, which become tilted (Sjaastad et al., 2010). Sjaastad et al. (2010) mentions “when ADP is released from myosin following a power stroke, the myosin heads bind another ATP molecule. The binding between the myosin heads and actin is then broken, and the entire process is repeated”.

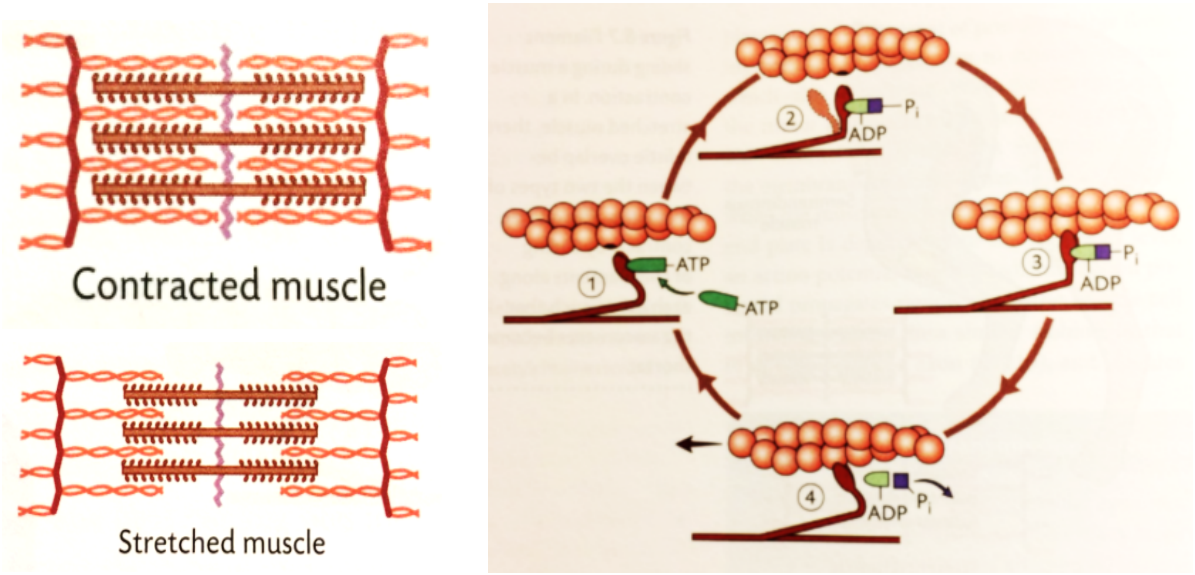

Figure 5, The sliding filament model is displayed on the left side. Actin is pulled in between the two strands of myosin, which leads to shortening of the sarcomeres. On the right side myosin heads are making cross-bridges, which release after binding of ATP. (Sjaastad et al., 2010)

An action potential is required to release the Ca^{2+} from the sarcoplasmatic reticulum. Ca^{2+} is necessary for the binding of the myosin heads to actin. When the concentration of Ca^{2+} is low in the cytosol, which is the case when the muscle fiber is relaxed, myosin will not be able to bind to actin. Troponin and tropomyosin are two proteins that block myosin from binding to actin. Tropomyosin is lying on several actin molecules and troponin is attached to tropomyosin and has the possibility to bind Ca^{2+}. When Ca^{2+} binds to troponin it causes a change in the position of tropomyosin allowing actin to be available for the binding of myosin (Sjaastad et al., 2010).

The energy metabolism of the muscle depends on ATP. This metabolism can increase dramatically when the muscle contracts, meaning the need for ATP increases. ADP can become ATP again by oxidative phosphorylation in the mitochondria. This process depends on a steady flow of oxygen, which is transported to the cells by the blood. Creatine phosphate is another important molecule in the muscle cells for the extra production of ATP when the supply is low. Creatine kinase catalyzes the reversible reaction that transforms ADP and creatine phosphate into ATP and creatine. (Sjaastad et al., 2010).

4.2 Muscle metabolism

The metabolism of the muscle tissue can be divided into aerobic and anaerobic. The aerobic pathway produces a better output in ATP and is mostly used for submaximal exercise of long duration (Westerblad et al., 2010). The anaerobic pathway is a fast way to create ATP and is mostly used for high intensity physical activity of short duration (Westerblad et al., 2010). In the anaerobic pathway, muscle glycogen gets broken down to lactate and hydrogen protons by glycogen phosphorylase. Phosphorylation makes the enzyme more active while the non-phosphorylated form is generally less active (Westerblad et al., 2010).
Oxygen is the determining factor in lactate formation. During an active state of the muscles without enough availability of oxygen, more lactate is formed (Vermeulen et al., 2017). Lactate is one of the possible products formed out of pyruvate, which is the end product of the glycolysis. During the lactate forming, lactate dehydrogenase (LDH) catalyzes the conversion of NADH into NAD^+ (Nelson and Cox, 2008). The hydrogen protons that are formed parallel with lactate cause the drop in pH (Westerblad et al., 2010).

The citrate cycle is a process to produce ATP in the presence of oxygen. Isocitrate dehydrogenase (ICDH) is an enzyme used in this cycle and can be used to measure the oxidative capacity of a muscle (Huber et al., 2007). ICDH will be high in muscle tissue when a lot of oxygen is available and usable for the muscle cells. LDH will be low in that case, because LDH is an indicator of metabolism without the presence of oxygen. For instance a heart muscle will have high concentrations of ICDH, but low concentrations of LDH (Huber et al., 2007).

4.3 Muscle fiber types

Skeletal muscles are used for lots of different activities. Some muscles are used for running, some for chewing, others for keeping the body up straight. This also means that metabolism, contractile speed and diameter will be different for these muscle fibers. Muscle tissue can be divided in four different muscle fiber types. Myosin heavy chain (MHC) isoforms are the base on which the skeletal muscles are classified (Westerblad et al., 2010). The smallest fibers are type I. They contain a lot of myoglobin, have a relative slow contraction speed and a high

oxidative capacity. Type IIa fibers are intermediate in diameter and myoglobin, have a fast contraction speed and a high oxidative capacity. Another subgroup is type IIx, which contraction speed is a bit faster than IIa, but slower than IIb. The diameter of type IIb fibers is the largest and is adapted for short bursts of high-power activity. Their myoglobin content and oxidative capacity are low, and the contraction is fast. These type IIb fibers store the highest amount of glycogen (Engelking, 2015; Hopkins, 2006).
Macroscopically there is a color difference in those types of muscle fibers. The more myoglobin the muscle fiber contains, the redder the muscle fiber will appear. For instance, type I will have a more red-looking appearance than type IIb (Hopkins, 2006).

Myoglobin is responsible for the oxidative capacity of the muscle fiber. The oxidative capacity determines whether the aerobic or anaerobic metabolic pathway will be used for producing ATP needed for muscle contraction. When the oxidative capacity is high, like in type I muscle fibers, the fibers will use the aerobic pathway. This comes with a relative slow contraction speed, but the muscle does not fatigue easily or pile up high amounts lactic acid (Engelking, 2015; Squire et al., 2005).

Muscle fibers that, like type IIb fibers, produce ATP from glucose, are called glycolytic fibers. Glycolytic fibers have an anaerobic metabolism, which means they produce ATP very fast, but less efficiently than oxidative fibers do. When muscle fibers produce ATP by means of oxidative phosphorylation, they are called oxidative fibers. Oxygen is needed for the production of ATP in this kind of fiber. These oxidative fibers have an aerobic metabolism. Glucose and fatty acids are used as energy source for the oxidative phosphorylation. These fibers are surrounded by a large network of capillaries and contain more mitochondria than glycolytic fibers. (Westerblad et al., 2010)

The slow fibers are controlled by small motor units. These type of muscle fibers are present in muscle tissue used for the posture, stabilizing joints and making small movement that are often repeated. The fast fibers are part of big motor units. They are more often used by bursts of energy and increasing muscle load. They do accumulate lactic acid and are therefore quicker to fatigue (Sjaastad et al., 2010).
Fast MHC isoforms and sarcoplasmatic reticulum Ca^{2+} isoforms consume several ATP molecules. A slow MHC isoform consumes less ATP (Westerblad et al., 2010).

Not every species or breed or specific muscle has the same ratio between the different fibers. Every muscle in the body has slow and fast fibers though all depending on the function and the demand (Engelking, 2015; Vermeulen et al., 2017).
GLUT4 expression is not the same in all muscle fiber types in rodent skeletal muscle. Type I oxidative fibers seem to express more GLUT4 than type II fiber, which are more glycolytic than type I (Richter and Hargreaves, 2013). Humans do have those differences in GLUT4 expression between muscle fiber types, but not a big a difference as in rodents (Richter and Hargreaves, 2013). Not every muscle shows those differences. M. vastus lateralis shows a difference in GLUT4 expression between the different fiber types, but m. soleus and m. triceps brachii show no significant differences in humans (Daugaard and Richter, 2004).

5. Pathologies and the influence of GLUTs

Sometimes the translocation of GLUT4 to the plasma membrane is not successful and the cells are not be able to increase the uptake of glucose. This defect is also known as peripheral insulin resistance and is part of the pathology of diabetes mellitus type 2 (Mueckler and

Thorens, 2013; Tong et al., 2001). Insulin resistance leads to symptoms like glucose intolerance and hyperinsulinemia (Garvey et al., 1998). In humans the level of GLUT4 is normal in type 2 diabetes and obesity (Garvey et al., 1998). Obese Zucker rats have a normal level of GLUT4, but the translocation of GLUT4 is impaired (King et al., 1992). Insulin resistant horses have a decreased amount of GLUT4 in the cell membrane during a basal state (Waller et al., 2011). Insulin resistance in muscle seems to be caused by defects in translocation of GLUT4 (Garvey et al., 1998). In case of obesity, there is also a disruption in the regulation of insulin (Thorens and Mueckler, 2010). The precise role of GLUT4 in insulin resistance and the primary cellular defects that lead to this state are still unknown (Thorens and Mueckler, 2010). The study of Tong et al. suggests that the cause of GLUT4 not translocating correctly towards the cell membrane is due to a defect in the remodeling of cortical actin (Tong et al., 2001).
Exercise seems to be a good way to help in the treatment of diabetes type 2. Exercise improves insulin sensitivity and blood sugar homeostasis in the body. The kind of exercise training done does not seem to matter. Both endurance and resistance training show good results (Stuart et al., 2011).

The expression of GLUT4 is also influenced by neurogenic factors. An experiment performed by Megeney et al. shows a decrease in GLUT4 expression in a denerved red gastrocnemius muscle of rats with a short nerve stump in 24 hours. Red gastrocnemius muscle is classified as type IIa muscle fiber. These results did not change in the next 24 hours. Denerved red gastrocnemius muscles with a long nerve stump only show a decrease in GLUT4 expression after 48 hours (Megeney et al., 1994). Fogt et al. also studied the effect of denervation of muscles on GLUT4 expression. Their results were different from the results of Megeney et al. They used the soleus muscles of rats instead of red gastrocnemius muscles. Soleus muscles are classified as type I muscle fibers. There was no difference in GLUT4 expression between a long or short nerve stump. In both cases GLUT4 expression decreased within 24 hours and kept declining till 96 hours after the denervation. The most likely cause of this decline is the lack of coordinated electrical activity of the muscles (Fogt et al., 1997). Neuromuscular activity seems to be an important factor for the regulation of GLUT4 expression (Fogt et al., 1997). Not only neural activity, but also neurotrophic factors seem to influence GLUT4 expression (Richter and Hargreaves, 2013).

Equine metabolic syndrome (EMS) is a condition seen in horses characterised by insulin resistance (IR), hyperinsulinemia, hypertriglyceridemia and obesity. The risk of laminitis is greater and so laminitis is often seen in horses suffering from this condition (Lacombe, 2014; Waller et al., 2011). IR can also be seen in non-obese horses (Geor and Harris, 2009). Insulin sensitivity, which means a concentration that evokes a half-maximal response, is significantly lower in horses with IR than in insulin sensitive (IS) horses. The baseline plasma insulin levels and blood glucose levels are the same in IR and IS horses (Geor and Harris, 2009; Waller et al., 2011). IR horses seem to have less active GLUT4 in the plasma membrane than IS horses, both do not respond to insulin with an increase of GLUT4 in the plasma membrane in vitro. GLUT12 is also an insulin-dependent glucose transporter, but does not show any difference in active concentration in the plasma membrane in IR or IS horses. The total GLUT4, GLUT12, total AS160 or phosphorylated AS160 content in the muscle samples were not altered by IR . (Waller et al., 2011). To increase insulin sensitivity again in IR horses, exercise and a special diet are required. This can be difficult, because many horses with IR already deal with laminitis, which is very painful and prevents them from moving (Geor and Harris, 2009).
In the study of Stewart-Hunt et al. horses were fed a starch-based diet or a fat-based diet. They completed 7 weeks of training on a treadmill, which led to an increase in middle gluteal muscle. The increase was higher in the horses that had been fed a starch-based diet. They

also found that a starch-based diet led to a decrease in insulin sensitivity, while that was not the case with the fat-based diet. Insulin sensitivity was improved after the 7 weeks of exercise (Stewart-Hunt et al., 2010).

6. Analysis methods

6.1 Western Blot

One of the techniques most used for the analysis of GLUT is the Western Blot. Western Blot is a technique used to separate and visualize the proteins present in a tissue sample. This semi-quantitative technique allows researchers to estimate the levels of a specific protein in a sample. To research GLUT, GLUT proteins need to be separated and identified first. Gel electrophoresis is used to separate the proteins based on molecular weight. The molecular weight is specific for the different types of proteins. The separated proteins are transferred to a membrane, where for each protein present a band will show up if the membrane is stained before the antibody treatment. Labelled antibodies are added specifically for GLUT and a control protein. If a rabbit anti-GLUT4 was used, then the secondary antibody must target that rabbit antibody, it could be for example a swine anti-rabbit IgG. The antibodies bind to the target proteins and then all the antibodies that did not bind are washed off. While using an imager, the stronger the signal the band gives, the more protein is present. The control band is used to compare, normalize and measure the amount of protein present (Mahmood and Yang, 2012).

6.2 Subcellular fractioning methods

Several subcellular fractioning methods exist nowadays. These methods can be used when only a small part of a cell is investigated, like an organelle or a protein, so this small part needs to be separated from the rest of the cell. First the tissue sample is minced and homogenized. The mincing is necessary to break open the cell. Centrifugation is the next step, where the speed and time of centrifugation determine what cell fractions will be separated. These methods are mainly based on homogenizing the tissue sample, using sucrose as a medium and lots of centrifugation. After the separation of the cell fractions, Western blotting is used most of the times to analyze GLUT levels in the muscle tissue samples (Dimauro et al., 2012; Dombrowski et al., 1996).

6.3 Exofacial labeling method

Exofacial labels are used to label glucose transporters at the cell surface. These labels are not able to enter the bilayer, so only the glucose transporters at the cell surface will be labeled (Li and McNeill, 1997). Photolabeling is followed by immunoprecipitation when it comes to investigating GLUTs (Lund et al., 1993). Immunoprecipitation is an extra purification step used to make the difference between GLUT1 and 4 (Li and McNeill, 1997). Before the photolabeling can begin, the muscle tissue samples need to be incubated twice for 30 minutes in Krebs-Henselent bicarbonate buffer, which contains 2 mM pyruvate, 38 mM mannitol and 0,1% BSA with or without insulin. After the incubation, the samples are moved to a dark room and incubated with an ATB-BMPA solution (Lund et al., 1993), which is a photoaffinity reagent used for labeling GLUT4 (Gould, 1997). Irradiation, with intense ultraviolet light, of the tissue samples is the next step for crosslinking, after which they are blotted, trimmed, frozen and homogenized in a sucrose buffer with protease inhibitors. The tissue sample is subjected to centrifugation two times and the supernatant is used for immunoprecipitation. A specific

antiserum for the investigated GLUT is used as an incubation immunoprecipitation medium. After this incubation an electrophoresis buffer is added, and electrophoresis is performed. The radioactivity is measured for each peak as well (Lund et al., 1993).

6.4 Immunocytochemistry, immunohistochemistry and immunofluorescence

Immunocytochemistry focusses on a few cells, where immunohistochemistry focusses on the whole tissue. These techniques are used for the morphological analysis of tissue samples. This technique makes it possible to provide morphological evidence for translocation of GLUT4, which gives a lot of information about the cellular trafficking of GLUT4 (Li and McNeill, 1997). Very thin layers of muscle tissue are prepared out of a muscle biopsy after being fixated with paraformaldehyde. First an anti-GLUT serum, like anti-GLUT4 is used, which will bind to the GLUT protein of interest. A secondary antibody that can bind to the primary antibody is added to the sample. Another substance, like protein A-gold, is added for the staining of the sample, which will bind to the secondary antibody. When the investigated protein is present in the sample, the protein will light up, which can be seen under the light microscope. The staining can also be done with fluorescent molecules, which is then called immunofluorescence. It is necessary to use ultraviolet light to visualize the fluorescent molecules (Kokk et al., 2004; Rodnick et al., 1992).

6.5 Advantages and disadvantages of certain methods

One of the downsides of Western Blot and immunohistochemistry is the fact that differentiation between active and inactive GLUTs in the plasma membrane is not possible. Photolabeling leads to better results, but is also a lot more expensive (Waller et al., 2011). Exofacial labeling technique has a great benefit over subcellular fractioning methods, because of the great increase in sensitivity (Lund et al., 1993). Subcellular fractioning methods have a higher risk of cross- contamination of other membrane fractions (Li and McNeill, 1997).

7. Training: intensity, duration, modality (strength, endurance)

Training of the muscles does not simply consist out of one type of training or one specific exercise. Training can be done at a different intensity, duration and modality. Training can be divided in the main classes of endurance training and strength training. Endurance training uses mainly the aerobic pathway. Strength training consists of an explosive force of contraction and it mainly uses the anaerobic pathway (Vermeulen et al., 2017).

Contraction of muscles is divided in eccentric, isometric and concentric contractions. Eccentric contraction means the muscle elongates while the muscle is active. During an isometric contraction the muscle length stays the same. The muscle creates a certain power output without shortening the muscle fibers. The concentric contraction is best known by most athletes and trainers. The muscle fibers shorten while creating a power output (Radák, 2018). An example of this type of contraction is lifting the head of a horse. The muscles in the topline of the neck shorten, the head gets lifted up (Sjaastad et al., 2018).

Muscles adapt very well when subjected to exercise. After repeated exercise hyperplasia of the muscle tissue will occur, which consists of a thickening of the muscle fibers. The amount of muscle fibers is unchanged (König and Liebich, 2004). After endurance training a rise in

mitochondrial content in the muscle is seen. This will lead to a better oxidative capacity and is a benefit for the aerobic pathway to produce ATP (Stuart et al., 2011).

The intensity and duration of exercise play an important role in glucose uptake in the muscle tissue (Richter and Hargreaves, 2013). Insulin sensitivity increases after exercise training (Funai et al., 2009; Stuart et al., 2011). GLUT 4, hexokinase II and glycogen synthase content in muscle tissue increase when insulin sensitivity is enhanced (Frøsig et al., 2007). The results of different studies on the signaling cascade to enhance insulin sensitivity are inconclusive. One study on humans shows insulin stimulated PI3K activity to increase after short term exercise (Houmard et al., 1999), while another study on humans shows training does not affect insulin stimulated IRS1- associated PI3K activity (Frøsig et al., 2007). A study on rats shows enhanced insulin stimulated PI3K activity after exercise (Zhou and Dohm, 1997). PKB activation and AS160 phosphorylation are both stimulated by insulin and enhanced after training (Frøsig et al., 2007).

Daugaard et al. did a study on GLUT4 content in specific muscle fiber types and the influence of exercise on these GLUT4 proteins. Eight healthy men participated in this study, where they had to do low intensity exercise on a one-leg knee extender apparatus to train the m. vastus lateralis for two weeks. GLUT4 increased with 23% in type I muscle fiber. The content of GLUT4 in type IIa and type IIx did not increase. It must be noted that this kind of training mainly recruits type I muscle fiber. Daugaard et al. hypothesize that increasing the intensity of the training might lead to an increase in GLUT4 content in type II muscle fibers (Daugaard et al., 2000).

A study in humans from Asp, Daugaard and Richter from 1995 shows a decrease in GLUT 4 concentrations after eccentric exercise. On day 0, 1 and 2 after eccentric exercise in humans there was a decrease in muscle glycogen concentration compared to the control contralateral leg. The GLUT 4 concentration also decreased on day 1 and 2 after eccentric exercise compared to the control leg. The GLUT concentrations returned to normal on day 4 after exercise (Asp et al., 1995). According to the study of Kirwan et al from 1992 the insulin sensitivity in the whole-body drops after eccentric exercise. Eccentric exercise leads to muscle damage and enzyme leaking (Kirwan et al., 1992).

Kristiansen et al. were curious about GLUT4 mRNA and transcription rate, so they studied the red and white gastrocnemius muscle of rats during eccentric contraction by electric stimulation. Their findings were a drop of 50% in total GLUT4 content in white gastrocnemius muscle and 32% in red gastrocnemius muscle after two days. The contralateral leg was used as a control study, no stimulus was given to this leg. GLUT4 messenger RNA (mRNA) decreased as well due to the eccentric contractions of the gastrocnemius with 41% in both muscle types. GLUT4 transcription rate was determined in a mix of red and white gastrocnemius muscle and dropped 75%. Even the GLUT4 content in the sarcolemma was decreased with 51% in comparison with the unstimulated leg (Kristiansen et al., 1997). Transcription is forming the complementary strand of DNA, which is called mRNA. With a lower transcription rate, less mRNA is formed. mRNA sequences are translated into proteins during protein syntheses (Nelson and Cox, 2008).

According to the study of Ren et al., exercise leads to an increase in total GLUT4 content, GLUT4 content in the sarcolemma and GLUT4 mRNA in rats. The rats had to swim for 3 hours, 45 minute break, and 3 hours of swimming again. They had to do this two days in a row, with 16 hours between the exercises. These results are from 16 hours after the last bout of exercise.

GLUT4 mRNA rises after 1 day of exercise, but did not continue to increase on the second day of exercise. Total GLUT4 concentration in the epitrochlearis muscle of rats increased 55% after the first day and up to 95% after the second day of exercise. The increase in hexokinase activity was parallel to the increase in total GLUT4 content in the muscle. GLUT4 concentration in the sarcolemma did not change after 2 days of swimming. They stimulated these muscles with an insulin infusion for 30 minutes, which shows a two times higher rise in GLUT4 translocation in the exercised muscle than in the control muscle (Ren et al., 1994).
Kuo et al did an experiment on 5 groups of rats to investigate the effect of carbohydrate supplementation on post exercise GLUT4 expression in muscle tissue. The rats were divided into exercised and fastened, exercised and carbohydrate supplement, sedentary and fastened, sedentary and carbohydrate supplement and sedentary control groups. The rats had to swim for 3 hours, 45-minute break and 3 hours of swimming. They had to swim two days in a row. The carbohydrate supplementation was intubation with a 0,4 ml 50% glucose solution after the first 3 hours and 1 ml after the last 3 hours. Glycogen levels were 50% down after the exercise. Red gastrocnemius muscle restored the glycogen concentration to begin levels after 16 hours of rest, white gastrocnemius muscle could not restore glycogen that fast. Exercise and carbohydrate supplementation lead to a glycogen increase of 76% above the control levels in red gastrocnemius muscle and 42% in white gastrocnemius muscle. Without exercise this was only 40% in red and 15% in white gastrocnemius muscle. Carbohydrate supplementation also has an effect on GLUT4 expression. Without the supplementation of carbohydrates there was a 43% increase above control levels in GLUT4 expression, while GLUT4 expression in red gastrocnemius muscle was 88% higher than the sedentary control group and 68% in white gastrocnemius muscle with carbohydrate supplementation. Without exercise the GLUT4 expression did not significantly rise. The rats that fasted had an 80% rise in GLUT4 mRNA concentration above control levels 16 hours after the exercise. Supplementation of carbohydrates leads to only a 40% increase in GLUT4 mRNA levels in exercised rats. GLUT4 mRNA did not change in the sedentary rats. There seems to be a correlation between GLUT4 expression and glycogen concentration after carbohydrate supplementation (Kuo et al., 1999).

Terada et al conducted a study in 1999 to show the difference between high intensity training (HIT) and low intensity training (LIT) in GLUT4 concentration in epitrochlearis muscles of rats. High intensity training focusses on a very high intensity of training during a short period of time which is repeated multiple times. In this study rats had to swim for 20 seconds while bearing a weight equivalent of 14% of their body weight. This was repeated 14 times with a 10 second interval (Terada et al., 2001). Low intensity training does not require heavy labor of the muscles in a short time but will also be exhausting, because the training takes a long time. The rats in this study had to swim for 6 hours a day separated in two parts of 3 hours with a 45-minute pause in the between. They were allowed to swim without any additional weight during the first 30 minutes. After those 30 minutes they had to carry a weight of 2% of their body weight while swimming (Terada et al., 2001). Both groups, HIT and LIT rats, had to swim 8 days in a row. Both groups were compared to age-matched sedentary rats. GLUT 4 concentration was massively increased in HIT (83%) and LIT (91%) rats compared to the control rats (Terada et al., 2001). There was no significant difference between the HIT and LIT group. This study shows a comparable rise in GLUT4 concentration after HIT and LIT. This also seems to be the maximal GLUT4 expression possible in rat muscles. Kraniou et al. got the same results in humans. High intensity training for a shorter duration and lower intensity training during a longer period lead to the same amount of increase in GLUT4 mRNA and GLUT4 concentration (Kraniou et al., 2006).

Hansen et al. found that a single bout of exercise, 2 hours of swimming, in rats leads to an increase in glucose uptake due to more GLUT4 translocation to the cell membrane. They compared the exercised rats with sedentary rats. Both show the same total amount of GLUT4 protein. This means more of the intracellular GLUT4 translocates to the cell membrane, but the total amount does not change (Hansen et al., 1998).

Pratt et al. discovered that insulin sensitivity in horses does not improve within 24 hours after a single bout of exercise that reduced the glycogen content with 50%. After 24 hours the glycogen content was not replenished yet (Pratt et al., 2007). It takes 48-72 hours for horses to refill the glycogen content after heavy exercise. This is a lot longer than in other mammals, were 24 hours is normal. Also, the kind of food they get after exercise has an effect on glycogen levels. High soluble carbohydrate diets lead to a higher muscle glycogen content 72 hours after the exercise, compared to middle and low soluble diets. The GLUT4 content did not seem to change up to 48 hours after exercise and feeding soluble carbohydrate diets. 72 hours after exercising the GLUT4 concentration is decreased (Lacombe et al., 2004). Stewart-Hunt discovered an increase in GLUT4 concentration after exercise when fed a starch-based diet. This increase did not occur when fed a fat-based diet (Stewart-Hunt et al., 2010). Insulin does not lead to a significant increase in GLUT4 translocation. The glycogen content in resting muscles is high in horses (Waller et al., 2011). Lacombe et al discovered in an earlier study an increase in GLUT4 protein content day 3 after exercise. However, the increase in GLUT4 content seems to be less than in rats (Lacombe et al., 2003). McCutcheon shows in his study an increase in muscle GLUT4 content after 6 weeks of training. There are no significant differences found in horses before or after a single bout of exercise (McCutcheon et al., 2002)

Duehlmeier et al. did experiments with Shetland ponies to see whether inulin affected GLUT4 expression and translocation in the m. semitendinosus. The m. semitendinosus mainly contains glycolytic fibers. The GLUT4 expression did not increase due to insulin, but the concentration of GLUT4 in the sarcolemma did rise. The translocation of GLUT4 was quite low compared to rats and humans (Duehlmeier et al., 2010). Manso Filho et al. discovered that there was no significant difference in GLUT4 expression between different fibers in horses (Manso Filho et al., 2007), This is in contrast with the finding seen in rodents, rabbits and humans (Daugaard and Richter, 2004; Kong et al., 1994; Richter and Hargreaves, 2013).

The study of Vukovich researched the effect of detraining on the GLUT4 concentration. Seven healthy man that were used to exercise at least five to seven times a week had to stop all kinds of exercise for six days, while their diet did not change. Their fastening plasma insulin concentration showed a significant rise after six days without exercise compared to before the experiment. Insulin action also seemed to be less. The GLUT4 protein content was measured in the gastrocnemius muscle of the men and showed a significant decline of 17% in the concentration after the six days without exercise (Vukovich et al., 1996).

8. Human and rodents versus other animals

A lot of studies on GLUT 4 expression and translocation are focused on humans, because of the link with diabetes mellitus type 2. The sequence of the human GLUT4 protein is very similar to the sequences of rat and mouse GLUT4 (Simmons, 2017). Rodents are most common used to investigate the effect of training on mainly GLUT4 expression and translocation. The effects in rodents and humans are similar. Horses react differently to a stimulus of insulin, probably due to a different digestive system and glucose metabolism. The

results obtained in humans and rats cannot be translated to horses and vice-versa (Waller et al., 2011).

Discussion

There is not really one straight answer for the question what the influence of exercise is on GLUT expression. Not all animal species have the same expression of GLUTs in their body and react the same way to exercise. Also, the type of exercise seems to matter for the expression of GLUT4.
There are still a lot of questions what the exact signaling cascade is that leads to enhanced insulin sensitivity. The results of Frøsig et al. in humans showed a decline in IRS1- associated PI3K activity, while studies in rats and other studies in humans show an increase in the PI3K activity (Frøsig et al., 2007; Houmard et al., 1999; Zhou and Dohm, 1997). A possible explanation for these conflicting results is the difference in the way they analyzed the results. The studies that showed an increase in PI3K activity used an immunoprecipitation with antiphosphotyrosine antibody, leading to precipitation of at least 95% of the PI3K pool. The studies that showed decreased PI3K activity used insulin receptor or IRS1 antibodies. This might mean that the increase occurred due to other molecules that the insulin receptor or IRS1 (Houmard et al., 1999). The age, physical condition and possible pathologies like diabetes type 2 of the test subjects may also influence the results. The same goes for the exercise training, the duration of the exercise seems to influence results (Frøsig et al., 2007). More studies need to be done to fully understand the signaling cascade during exercise.

The type of muscle contraction is also important for the change in expression of GLUT4. Eccentric exercise, in both humans and rats, leads to a decrease in GLUT4 content (Asp et al., 1995; Kristiansen et al., 1997). Concentric exercise leads to an increase in GLUT4 content in rats and humans (Daugaard et al., 2000; Ren et al., 1994). GLUT 4 is regulated by insulin, so this might explain why there is less GLUT 4 present after eccentric exercise. Another cause might be an impaired translocation or implantation of GLUT 4 in the cell membrane, because of the muscle damage. It can also lead to a quicker degradation of GLUT 4, which leads to a lower concentration of GLUT 4 on day 1 or 2 after exercise (Asp et al., 1995). Concentric exercise, done within normal ranges, does not lead to muscle damage.

Carbohydrate supplementation in combination with exercise leads to a higher GLUT4 concentration than just the effect of exercise on GLUT4. It can also be said that carbohydrate supplementation combined with exercise leads to a better translation efficiency of GLUT4 mRNA. Exercise and fastening on the contrary leads to a lower translation efficiency. So not only exercise is important, the combination with food is also of great importance. (Kuo et al., 1999). This might be interesting to use in a study of diabetes mellitus type 2 to see the effect on insulin sensitivity through better GLUT4 expression.

There is a maximum level of GLUT4 expression possible in muscle tissue. This maximum expression can be reached with high intensity training, but also with low intensity training. Studies in humans and rats both gave the same results (Kraniou et al., 2006; Terada et al., 2001). It will be interesting to see if these results can help with making training schedules for people (or animals) with insulin resistance. Everyone has a preference in the kind of exercise they do. Some people like powerlifting, which is high intensity training, while other people rather go for a long walk, which is low intensity training. Exercise has already proved its importance in improving insulin sensitivity in insulin resistant people (Stuart et al., 2011) Detraining quickly leads to a decline in GLUT4 content, so also a lower insulin sensitivity. For those people

suffering from insulin resistance it is very important to keep on exercising. Even six days of not exercising is enough to lower the GLUT4 content and therefore also the insulin sensitivity (Vukovich et al., 1996).

Horses are completely different from humans and rodents when it comes to GLUT4 expression (Waller et al., 2011). Even the food of the animals seems to have an effect whether GLUT4 expression rises after exercise or not. A starch-based food combined with exercise led to an increase in GLUT4 protein in horses, while nothing changed in the horses fed a fat-based diet. A possible explanation for this is that the body tries to compensate the loss of insulin sensitivity that accompanies a starch-based diet with more GLUT4 expression. More GLUT4 expression leads to better insulin sensitivity, so less insulin needs to be excreted to become the same results (Stewart-Hunt et al., 2010). The high glycogen content in equine muscles might explain why there is not much GLUT4 translocation. This high glycogen content may trigger a negative feedback system, which leads to less GLUT4 translocation and therefore less glucose entering the muscle cell (Waller et al., 2011). It seems like time is one of the factors that influences GLUT4 expression in horses. Immediately after exercise there is no significant change in GLUT4 content, but after several weeks of training there is an increase in GLUT4 content (Lacombe et al., 2004, 2003; McCutcheon et al., 2002).

According to Waller et al., the results of most of the earlier studies are not as reliable, because they use Western Blot or immunohistochemistry. Exofacial labelling method leads to better results (Waller et al., 2011). It might be that the results of some of the earlier studies are not completely correct due to new insights in analysis methods. It would be interesting to see what the difference is in results when using the newer exofacial labelling method compared to Western Blot and immunohistochemistry. Especially for the studies involving horses this technique might be good, because of the lower rise in GLUT4 content than in rodents and humans (Lacombe et al., 2003).

In conclusion, it can be said that training increases the GLUT4 protein content, except for eccentric exercises. Not all species react the same, but generally it can be said that training for a longer period increases GLUT4 content. Translocation of GLUT4 to the plasma membrane is important for the glucose uptake of the muscle cell. Translocation can be induced by muscle contractions and insulin. Training will be a good way to increase insulin sensitivity in individuals who suffer from insulin resistance.

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