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Uncover elements of the GLUT4 pathway that are altered by insulin resistance

Bachelor thesis

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Abstract

Type 2 diabetes is becoming a more apparent health problem in the world. This disease is marked by insulin resistance, which causes pathological states due to a too high blood glucose level. Therefore, research is trying to elucidate the details of the insulin resistance mechanism. The GLUT4 pathway is the main focus of this research because insulin, besides exercise, is an element that is able to activate the GLUT4 pathway via PI3K and APS, leading towards glucose uptake. Elucidating the elements of these pathways that are affected and altered by insulin resistance in comparison with normal functioning insulin plus the mechanism it does so, possibly could provide therapeutic interventions to treat or even cure insulin resistance, and so type 2 diabetes. This literature study shows the current knowledge of which elements of the GLUT4 pathway are affected by insulin resistance by going through the GLUT4 pathway. Also therapeutic interventions based on this elements will be discussed.

Introduction

Type 2 diabetes (T2D) is an important health problem. It represents the largest disease in the Netherlands, with approximately 900.000 patients. Every year around 70.000 new cases of T2D are identified in the Netherlands, which comes down to close to 200 per day.[1] It is estimated that approximately 370 million people worldwide have type 2 diabetes, but the prevalence is still rising. This provides high health care costs that go up. This is a reason why T2D research is a hot topic at the moment.

The disease can be caused by a variety of factors, such as the big contributing element called the western world lifestyle. This lifestyle includes high energy diet, little exercise (sitting at work most of the time) and obesity. T2D starts to develop if the risk factors cause deterioration of insulin sensitivity which, in the beginning, can be compensated with stimulated insulin production, a state that is called hyperinsulinemia. After a while, the beta-cells that produce insulin start to deteriorate which compromises the stimulated insulin production, resulting in a variety of health problems like glucose intolerance, insulin resistance, hyperglycemia (high blood glucose level) and possibly fatty liver disease. Since glucose uptake is facilitated by translocation of glucose transporter 4 (GLUT4) to the plasma membrane in response of insulin or exercise, glucose intolerance and insulin resistance are correlated. At the end stage of type 2 diabetes complications like blindness, renal failure and cardiac disease could appear.[2]

The most characterizing factor of T2D is insulin resistance (IR), which is defined as a defect in the ability of insulin sensitive tissues like liver, muscle- and adipose tissue to respond to insulin-stimulated glucose entry.[3,4] This causes disruptions of the glucose homeostasis and will result in hyperglycemia.[5]

The underlying mechanisms of IR are still unclear. Since improper glucose entry into the cell is the first noticeable defect, it is suggested that IR occurs somewhere in the pathway from insulin receptor activation to GLUT4 translocation.[6] The fact that IR really does affect this pathway is demonstrated in adipose- and muscle tissue by subcellular fractionation and photolabeling.[7-11] Lots of research has also been conducted to establish the differences in insulin signaling between healthy and type 2 diabetes tissue. For example, they found that in adipocytes of T2D tissue is a reduced concentration of GLUT4 with 30-70%.[12]

It is obvious that the field of research still tries to elucidate the mechanism of how this insulin resistance (and the insulin pathway at which it exerts its effect) works in detail and hopefully ultimately find a therapy or cure for T2D. But this research proves to be hard. The insulin pathway is complicated with lots of crosstalk which leads to the fact that even nowadays not all aspects of the pathway are known. Also so far is there still no consensus on the importance of the found mechanisms and the way they relate to each other.[13] Therefore in this bachelor thesis there will be focused on the question: at which points, and by what mechanisms does insulin resistance affect the GLUT-4 pathway?

GLUT4 pathways

To be able to say something about how the GLUT4 pathway is affected due to IR, it is necessary to establish the pathway in healthy tissue first. In this way a comparison can be drawn between them. It is a fact that there are two major elements that cause glucose to enter the cell with GLUT4, namely insulin and exercise. Both ways will be discussed below, starting with the insulin-dependent pathway.

Insulin-dependent pathway

Insulin is a hormone produced by the beta-cells in the islets of Langerhans of the pancreas, and after production it is released in the bloodstream. From there it goes mainly to muscle tissue (90%) and adipose tissue (10%). The 10% of insulin that resides in adipose tissue is of big importance since adipose tissue is a key regulator of whole-body energy homeostasis.[14,15]
After reaching the target tissue, insulin binds to the α-subunit of its receptor, the insulin receptor, which causes trans-autophosporylation of its β-subunit. This activates a signaling cascade towards glucose uptake in two different ways, namely by the APS (adaptor protein containing pleckstring homology and Src homology-2 domains) pathway and the PI3K (phosphatidylinositol 3-kinase) pathway which both will be studied in detail.

APS pathway

Upon activation of the insulin receptor, APS binds on the receptor resulting in docking of the c-CBL/CAP complex to APS. This promotes tyrosine-phosphorylation of the proto-oncogene c-CBL.[16-18] This activated c-CBL then interacts with the GEF C3G/CRK complex, which at his turn, activates the small GTPase family member TC10 in lipid rafts.[19-21] TC10 can facilitate GSV exocytosis by forming complexes like CIP4/GAPEX5, that are known to activate the EXO70 subunit of the exocyst complex. The exocyst complex is suggested to facilitate the GSV targeting to the plasma membrane, but the precise mechanism is currently unknown. Also the precise role of the APS pathway besides the PI3K pathway is yet still not understood.[14] Summary of the APS pathway is given in figure 1a.

PI3K pathway

The other insulin dependent pathway that regulates GLUT4 translocation to the plasma membrane is the PI3K pathway. Upon binding of insulin, the β-subunit of the insulin receptor activates its intrinsic tyrosine kinase activity which phosphorylates adaptor proteins like IRS (insulin receptor substrate) 1 and 2. These adaptor proteins recruit PI3K (type 1A phosphatidylinositol-3-kinase) by binding the SH2 domain of its p85 subunit. Also smaller adaptor proteins like Nck and Grb2 dock to the phosphorylated insulin receptor, but their role is still unclear.[22] The reason this pathway is named PI3K pathway is because it is shown with the PI3K inhibitor wortmannin that PI3K is a critical element in this pathway, since wortmannin ensures blocking of downstream effectors of PI3K.[23]
Docking of the SH2 domain of PI3K onto the phosphorylated insulin receptor causes activation of PI3K which results in catalyzed synthesis of PIP3 (phosphatidylinositol-3,4,5-triphosphate) from PIP2 (phosphatidylinositol-4,5-diphosphate). The synthesis of PIP3 happens on the inner layer of the plasma membrane which ensures PIP3 is anchored there after synthesis.
This anchored PIP3 forms a lipid-based platform that attracts PH (pleckstring homology) domain-containing molecules like PDK1 (phosphoinositide-dependent kinase 1) and Akt (also known as protein kinase B (PKB)). Preferentially Akt2 is bound, since its the most insulin-sensitive isoform of Akt. Knock-out mice lacking Akt2 show deficiencies in their glucose metabolism which, besides the evidence that Akt is essential for most of the insulin-regulated metabolic actions, prove Akt2 to be essential in the insulin-regulated pathway.[4,24] Akt2 is also one of the elements in the GLUT4 pathway that exhibits spareness. Only the activation of a small portion of Akt2 is sufficient to induce a maximal response as will be described below.[4] The concept of spareness also will be discussed later on.
Akt2 is activated at the PIP3 platform after a conformational change, which occurs after independent phosphorylation of its threonine^{308} and serine^{473} residue by respectively PDK1 and mTORC2.[25-27] That latest factor was unknown for a long time, but recently the mTORC2 complex was discovered.[28]

Upon activation, Akt2 translocates to different compartments in the cell like endosomes and GSVs (GLUT4 storage vesicles) to activate its most important downstream effectors AS160 (also known as TBC1D4) and the RAL-GAP complex. These two factors control release of GLUT4 onto the plasma membrane, but both in a different way.[29-31]
The PI3K pathway is summarized in figure 1b.

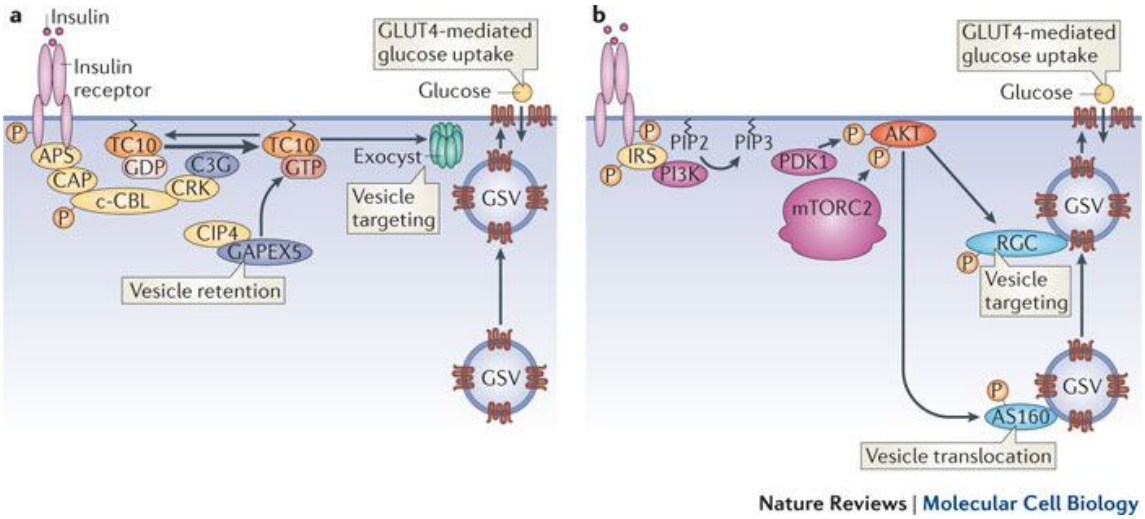

Figure 1. Overview of both insulin-stimulated pathways of GLUT4 exocytosis. (a) APS pathway; (b) PI3K pathway[14]

Exercise-stimulated pathways

As discussed above, there is another way in which skeletal muscle tissue is able to regulate GLUT4 expression. It has been shown that also exercise can increase GLUT4 content, and so glucose uptake.[32,33] And even though insulin and exercise use different pathways, both have almost all of the same effects on glucose metabolism.[34]
Exercise has two ways of doing so, first by CaMKII (Ca^{2+}/calmodulin dependent protein kinase II) and secondly by AMPK (adenosine monophosphate-activated protein kinase). Both pathways increase GLUT4 content by promoting GLUT4 transcription. In various experiments it has been shown that exercise increases GLUT4 transcription ~1.8 fold within three hours of a single bout of exercise in both human and rat muscle tissue.[35-38] This transcriptional increase is followed by an increase 1.5 to 2-fold of GLUT4 content within 16 to 24 hours after the single bout of exercise.[38,39] The way CaMKII and AMPK promote GLUT4 transcription is different, and will be shortly reviewed.

CaMKII pathway

The CaMKII pathway is activated due to rising Ca^{2+} levels in the cytosol of the cell in response to exercise. This cytosolic Ca^{2+} is among others bound by the protein calmodulin (CaM) which can activate CaMKII after undergoing a conformational change.[40] Subsequently CaMKII phosphorylates class II HDACs (histone deacetylase) implemented in HDAC/MEF2 complexes.[41,42]
This complexes occupy the GLUT4 promotor under basal conditions, inhibiting transcription. Upon phosphorylation, the HDAC detaches itself from the complex, allowing MEF2 to bind with HATs (histone acetyltransferases) like p300 on the promotor. This cooperation results in acetylation of both MEF2 and histones in proximity of the MEF2/HAT-complex. This ensures greater accessibility of the promotor region for RNA polymerases and transcription activators, so that the GLUT4 gene can be transcribed which ultimately leads to higher levels of GLUT4 proteins in the cell.[33] This pathway in summary is shown in figure 2.

AMPK pathway

The AMPK pathway is activated due to a rising AMP:ATP ratio in the cell caused by exercise. This activation is followed by phosphorylation of the GLUT4 transcription inhibitor HDAC5 (histone deacetylase-5).[43,44] This phosphorylation weakens the interaction of the transcription inhibitor with the promoter, so the net result is an increase of GLUT4 transcription.[33] Besides this, AMPK also phosphorylates GEF (GLUT-5 enhancer factor) which also controls the GLUT4 promotor, by interacting with MEF2 and HDAC-5. Upon activation, HDAC5 leaves the promotor site, allowing GEF and MEF2 to stimulate GLUT4 gene transcription.[44,45] This again ultimately leads to higher levels of GLUT4 protein content in the cell. In figure 2 is a summary of the AMPK pathway as well.

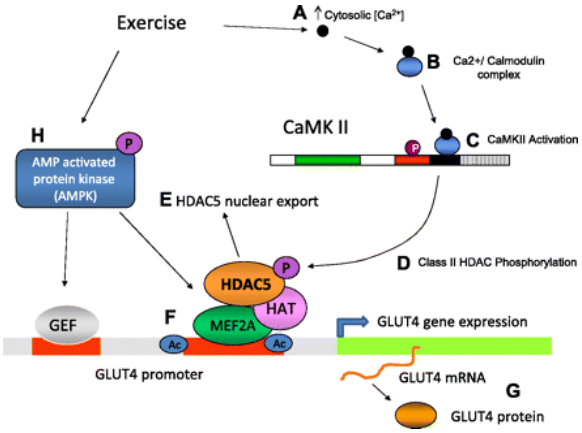

Fig. 2 Overview of the signaling pathways stimulated by exercise in skeletal muscle. On the left the role of AMPK, on the right the role of CaMKII.[33]

But interestingly, AMPK has another way to regulate glucose uptake. Activated AMPK ensures inhibition of the mTORC1 complex(mammalian target of rapamycin complex 1). mTORC1 consists of the elements raptor, MLST8, deptor, pras40 and the tti-tel2 complex and it has important metabolic functions through controlled protein synthesis, membrane-required lipid synthesis, regulation of ATP production and cellular metabolism and at last, also control of the transcription factor SREBP1. Especially this last element is interesting because SREBP1 regulates transcription of the GLUT4 gene, and so indirectly the glucose uptake. Besides this, mTORC1 also interacts with IRS1, but this will be discussed later.

Recapitulating, both the CaMKII and the AMPK pathway ensure higher levels of GLUT4 protein content in the cell by enhanced production of GLUT4. But this doesn't directly have to elevate the GLUT4 levels on the plasma membrane. Interestingly it is shown that muscle contraction (exercise) decreases the rate of GLUT4 endocytosis and so it doesn't elevate GLUT4 exocytosis like the insulin-pathway. It is thought that these effects are mediated through AMPK, which makes AMPK an even more important factor in this pathway.[46] Because no insulin is needed in this pathways at all, are they intrinsically different from the PI3K and the APS pathway which obviously do require insulin.

Exocytosis

As already said before, GLUT4 is the receptor for uptake of glucose from outside the cell. GLUT4 is part of the GLUT family which is defined by 12 transmembrane domain-containing proteins. All GLUTs have the ability to transport glucose in an energy-independent manner into the cell. They do so by utilizing the glucose concentration gradient.[47]
Nowadays the family consists of 14 members, which can be divided in three classes based on structural and transport characteristics.[48,49] GLUT4 is the most studied glucose receptor and

is very well known for being insulin stimulated in insulin sensitive tissues like adipose - and striated muscle tissue, unlike the other GLUT family members.[50,51]
GLUT4 knockout mouse models however do not show diabetes, nor a large decrease in glucose uptake. This suggests that other glucose transporters are present in cells as well, but they will not be considered in this literature study because they make no use of the GLUT4 pathway.[52]

It is proposed that GLUT4 resides in insulin-sensitive storage vesicles under basal conditions as mentioned above. This GSVs are found in different compartments, depending on their fate. Vesicles that will be directly translocated to the membrane upon activation will go there straight from the recycling endosome, which will be explained below. The vesicles also can be transported to the trans-Golgi network to start a futile cycle process, stimulated by the protein RAB31.[14]
Upon stimulation, GSVs can reach the membrane through transport via the cytoskeleton of the cell. The complete mechanism is unknown, but it is proposed that the vesicles travel alongside microtubules and actin filaments with the help of respectively kinesin and Myo1c.[24,53]
Once the vesicle reaches the membrane, aPKC ensures that he will be released from the actin filament, and is handed over to SNARE proteins like SNAP23, VAMP2 and syntaxin 4 to facilitate docking and fusion onto the plasma membrane. This process is controlled by Munc18c.[14,54] Also Synip, a SNARE regulatory protein, is involved in this process by regulating the fusion of the vesicles to the plasma membrane.[55-57]

Endocytosis
Insulin also seems to have an effect on endocytosis of GLUT4. The endocytosis takes place through the clathrin- or cholesterol-dependent pathway. These pathways can be affected by insulin, but that mechanism is unknown. It is already shown that insulin has no effect on the endocytosis rate in muscle cells, but such an effect is not yet seen in adipose tissue.[14, 58,59] As stated above, exercise is shown to decrease the rate of endocytosis in muscle tissue.
It is established that the clathrin-dependent pathway is the main endocytotic mechanism in muscle cells, while both the clathrin- and cholesterol- pathways are used for GLUT4 endocytosis in adipose tissue. Which form is used predominantly in adipose tissue is possibly regulated by a switch, but this is not yet confirmed by research.[60] Both the clathrin- and cholesterol-dependent pathway gather GLUT4 transporters in vesicles, both depending on adaptor proteins respectively AP2 and caveolin. The GLUT4 containing vesicles are internalized and brought to sorting endosomes by transport via RAB5 connected to dynein.[61,62] The sorting endosome, GLUT4 can be transported to the membrane again, or retained intracellular in recycling endosomes, late endosomes or trans-Golgi network in GSVs. This sorting provides precise regulation of membrane-bound GLUT4 and is regulated by the endosome components IRAP (insulin-regulated aminopeptidase), sortilin, VAMP2 (vesicle-associated membrane protein 2) and LRP1 (low-density lipoprotein receptor-related protein 1), but their precise role is unknown at the moment because technically it proves to be difficult to extract GSVs alone since they are part of a complex process.[63-66]

An overview of the whole recycling process of GLUT4 is given in figure 3.

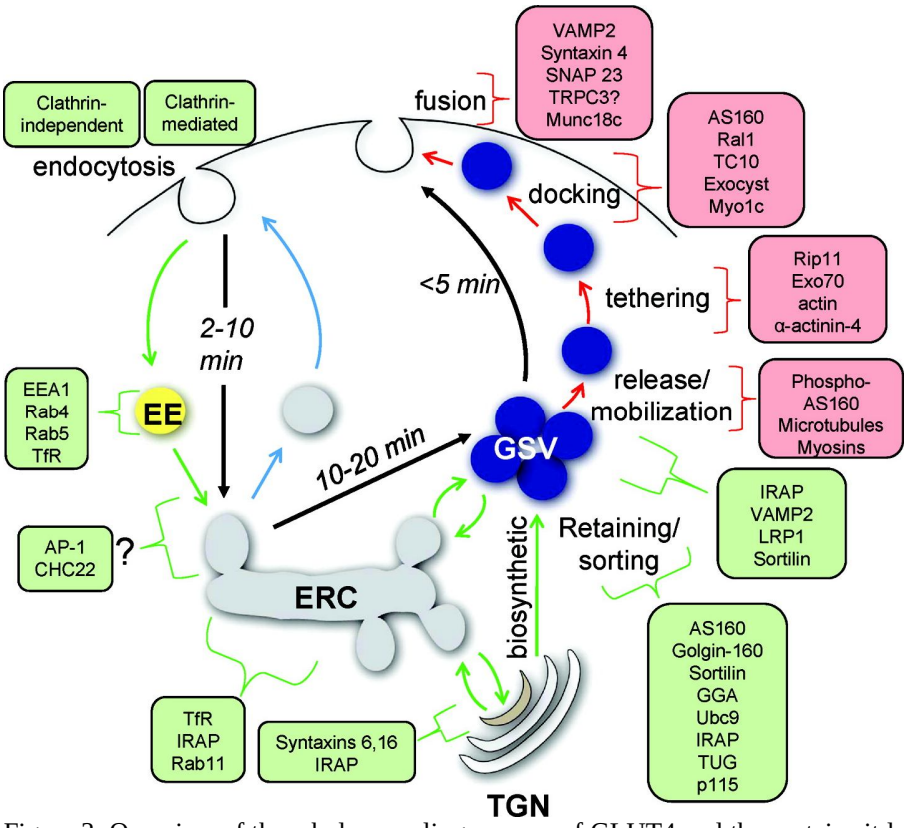

Figure 3. Overview of the whole recycling process of GLUT4 and the proteins it localizes with to establish that step of the process.[67]

IR affecting which pathways?

Because IR itself in mammals can be caused by a variety of causes like inflammation, hyperinsulinemia, poor diet, dyslipidemia and possibly by oxidative stress, it is hard to study the mechanisms in detail. But most of these causes can be traced back to T2D.[4,68] Mutations in the insulin receptor could potentially also cause T2D but this accounts for only 5% of the cases of the disease and so doesn't contribute a lot to the prevalence of T2D.[53]

It would be easy to state that all factors of the GLUT4 pathway that are insulin-dependent, will be affected by IR. However, the glucose uptake pathway is so complex, and has so many crossroads that this cannot be said so easily. This is seen in the fact that insulin resistant muscle cells still exhibit exercise-mediated glucose uptake.[69-72] Due to crossroads even between elements of the insulin stimulated and exercise stimulated glucose uptake, defects in the insulin-mediated pathway don't necessarily have to lead to reduced glucose uptake because they might be taken over by the exercise stimulated pathway. A good example of a crossroad between the pathways is the role of AS160. Besides being an important effector in the PI3K pathway, it also has been shown that AS160 is involved in the regulation of exercise regulated glucose uptake, in combination with AMPK.[61]

Another factor to consider is the that some elements of the insulin signaling cascade exhibit 'spareness', a term introduced by Hoehn et al. in 2008, which means that for example mice with partial knockdown of IRS1 (the effector upon activation of the insulin receptor) show no negative effects on insulin action.[4,73] This means that the system apparently has buffer zones which protect the pathway outcome from minor defects in the upstream elements of the pathway.

Also a phenomenon called selective insulin resistance can occur. This means for example that it is possible that the mechanism of GLUT4 translocation is working, but that insertion in the plasma membrane is blocked. So one part of the system works, another doesn't.[74] It is

possible that spareness and selective insulin resistance are part of the same process, but that is unsure at the moment.
So, as can be concluded, it is hard to study the precise effects of IR to the glucose uptake mechanism. But, some remarks can be made. First of all that the APS pathway seems to be cell type specific which makes its role in IR doubtful, since the pathway cannot account for IR in the whole body.
Also the exercise stimulated pathways can possibly be excluded as the main site of IR action on the GLUT4 pathway. This because these pathways stimulate glucose uptake without the need of insulin. Only pathway that remains to be studied is the PI3K pathway, which seems to be the most obvious site of IR action. How IR affects this pathway will be further discussed in the rest of this bachelor thesis.

Elements of the PI3K pathway affected by IR

Some elements of the pathway have been found to be affected by insulin resistance, and they will be described here according to the role they have in the pathway. The receptor activation elements IRS1, Akt2, mTORC1, AS160, aPKC and Rac1 will be discussed first. After that, the elements of respectively the exocytotic/membrane fusion process (Myo1c, VAMP2, SNAP23, syntaxin 4, Munc18c) and the recycling process (sortilin) will be studied.

It seems obvious that the elements directly activated by insulin will be affected by IR. Good example of this is IRS1, which is required for the signaling cascade after insulin binds to the cell. Until recently, it was widely believed that IRS proteins were the major targets of IR. Gual et al. showed that 3T3-L1 adipocytes exhibit altered phosphorylation of tyrosine on IRS due to IR.[75] But Hoehn et al. have stated in 2008 that the origin and the biggest effects of insulin resistance happen downstream and independent of IRS. They state that the biggest problem with the research that has been investigating IRS is that they only focus on small elements of the signaling cascade instead of looking at the final output like GLUT4 translocation to the membrane. Nowadays more research has focused on elucidating the mechanisms downstream of IRS1, and searching the origin of IR there.[4]
It is suggested that upon the spareness of the signaling system downstream of IRS, defects in IRS can make the situation worse. This is probably what happens in chronic insulin state.[15]
So Hoehn et al. show that defects in the upstream elements of the insulin cascade like IRS1 most likely not lead to IR due to spareness of elements in the cascade. This seems logical, and the research is conducted in a stochastic way, considering only the output of the system as a whole instead of looking at the output of only the next element. But more research has to be done on the exact role of IRS1 and his downstream effectors in the insulin pathway during IR so that possibly conclusive evidence can be obtained.

Downstream of IRS1 is Akt2 which turned out to be necessary in the insulin-stimulated glucose uptake, and Rondinone et al. showed in human adipocytes that IR caused impaired Akt 2 phosphorylation. [76,77] In some researches it has interestingly been shown that Akt2 can also be activated in response of exercise, but the data are not consistent about this fact, and should be further investigated.[32] Ng et al. interestingly state that there probably is a feedforward system of insulin that acts upon Akt 2 which causes downstream inhibition. In cells that are made insulin resistant due to chronic insulin-exposure, readdition of insulin causes an inhibitory signal from PI3K towards Akt 2. Ng et al. suggested that in normal cells this mechanism is silenced and that it is only active during IR.
But these results cannot determine the precise effect of IR on Akt 2, because the results are obtained in different cell lines and with different IR models (chronic insulin vs T2D cells). To be able to deduce reliable results, this differences should be overcome. But altogether, the

results suggest that Akt 2 is affected by IR, possibly by a feedforward inhibitory signal and/or impaired phosphorylation of Akt 2.

Akt2 activation can indirectly lead to activation of a couple of elements,including mTORC1.[78] Brännmark et al. show with a mathematical model that attenuation of the signal from mTORC1 to IRS1 seems to be the most important change in the transition from healthy to T2D tissue, because this change alters IRS1 and almost all intermediaries in the signaling network for GSV exocytosis. Khamzina et al. support this conclusion when they found that obese rodents show high levels of mTORC1, and altered levels of functional IRS1.[79] Interestingly, also many conditions that show IR like inflammation, hypoxia and other internal dysfunctions are known to influence mTORC1 function.[47]
The attenuation of the signal is caused by a weakening of the phosphorylation of ser^{307} of IRS1, and causes reduced signal strength and sensitivity in the signaling network due to higher IRS1 targeting for degradation. The autophosphorylation of the insulin receptor and phosphorylation of Akt on the contrary, show no altered sensitivity to insulin in T2D in the model.[47,78] Some remarks have to be made by this research. First of all, the body of the research is conducted by a mathematical model which doesn't include all aspects of the insulin pathway simply because not all aspects are yet known or understood. Secondly, the model used more than 40 parameters, and even though their values were fitted to the data, it is not unthinkable that the values are not completely how they normally would be in vivo. It is therefore important that the model that they used will be further expanded so that all cross-talks in the insulin pathway are in there as well, so better conclusions can be drawn upon the model. But so far, it seems that mTORC1 is largely affected by IR.

Another element that is activated due to Akt2 and so could possibly be affected by IR is AS160, which is responsible for controlling GSV docking to the plasma membrane.[31,80] But Karlsson et al. found that AS160 protein expression in human skeletal muscle tissue is not affected by IR. However, the AS160 phosphorylation was weakened. Interestingly Bruss et al. found in rat skeletal muscle that exercise increases AS160 phosphorylation. This suggests that impaired AS160 function due to IR can (partly) be overcome with exercise and that this leads to the assumption that even though AS160 is affected by IR, it is not the main site that exhibits problems. Another intriguing conclusion that can be made based on this possible link between PI3K pathway and exercise is that AS160 is capable of combining both pathways for GSV translocation towards the membrane.[81,101] As a matter of fact, one remark has to be made by the results above, and that is that the study of Karlsson made use of just 10 IR subjects and 9 healthy subjects. This groups are too small to obtain reliable and significant results. Besides that, the effect of AS160 is only seen in skeletal muscle, and it is needed to see if AS160 in adipose tissue and other types of tissue also shows attenuation of phosphorylation.

Another downstream effector of Akt2 is aPKC (atypical PKC). Its activation mechanism is still unknown, but knockout mice studies have suggested that aPKC plays a role in glucose transport, but that it is not needed for glucose transport per se since aPKC knockout mice showed only minor impairment in glucose transport.[82] It is even suggested that aPKC plays a role in exercise-stimulated glucose uptake through AMPK, but the results are not yet conclusive.[83] Vollenweider et al. showed that in human muscle tissue there was no differences in expression levels of aPKC in healthy vs IR tissue, although there was a significant decrease in activation in the IR tissue.[84] Together with the observation of Bandyopadhyay et al. that overexpression of aPKC-defective isoforms block GLUT4 translocation, it could be suggested that active aPKC is required for glucose transport.[85] But this system should be further investigated since the abovementioned results are not univocal about the precise role of IR on the functioning of aPKC.

A downstream element of the PI3K pathway is the small GTP-ase Rac1. Its precise role and mechanism are still subject of research but it probably induces actin remodeling

needed for actin-based GSV transport to the plasma membrane.[86] JeBaily et al. showed that ceramide and oxidant-induced IR in L6 myotubes (which are rat muscle cells) prevented activation of Rac1 and the subsequent actin remodeling. The mechanism at which this does so is unknown, but it is been suggested that ceramide and glucose oxidase interfere with GEFs that are possibly responsible for Rac1 activation.[87] This research only started to find a possible link between IR and Rac1, and so it needs to be further investigated to apply this results on other cell lines or even human cells. But the first results seem promising for a link between IR and Rac1.

Next part of the pathway is ensuring GLUT4 translocation from the cytosol to the plasma membrane, as stated above. Motor protein Myo1c is believed to mediate movement of GSVs along actin filaments and it is stated that Myo1c is phosphorylated by an unknown insulin-dependent component.[88] More recently it also has been shown by Toyoda et al. that Myo1c functions in the exercise-stimulated pathway. In the same article the researchers state that IR muscle tissue exhibits lower expression levels of Myo1c in comparison with insulin-responsive muscle tissue. It seems unlikely that the expression level can account for altered GLUT4 expression on the membrane, but it could cause impaired glucose uptake. This can be concluded from the fact that some forms of IR cause even an altered output of the exercise-stimulated pathway.[89] For this research, one remark needs to be made, since it is conducted in muscle tissue from female mice. This means that the results are not necessarily true for human muscle tissue as well, but that should be investigated which has not happened so far. This makes that the results should be treated with some caution in accordance with IR mechanisms in human tissue. But it could be concluded anyway that Myo1c is largely affected by IR by an unknown mechanism, because not only the insulin-stimulated response is altered, but even the exercise-stimulated response.

The next essential step in the pathway is docking of the GSVs onto the plasma membrane facilitated by SNARE proteins like vesicle-associated membrane protein 2 (VAMP2). This is a v-SNARE proteins, anchored in the vesicle to regulate docking to the t-SNAREs anchored in the plasma membrane.[90] Maier et al show that in ZDF (Zucker diabetic fatty) rats, which represents an animal model of diabetes and is used in research of T2D and glucose/insulin (in)tolerance, skeletal muscle has significantly increased levels of VAMP2 in IR tissue.[91] They show the same results in 3T3-L1 adipocytes.[90] This doesn't say however that the function or mechanism of VAMP2 is altered, so these results say nothing of the output of VAMP2 in IR versus healthy tissue.
Also the t-SNARE proteins can be affected by IR. This is suggested for the proteins SNAP-23 (synaptosomal-associated protein of 23 kDa) and syntaxin 4.[92,93] Bostrom et al. demonstrated that IR leads to elevated levels of SNAP-23 in human skeletal muscle even though the mRNA levels of SNAP-23 did not differ between healthy and IR tissue. The protein level of SNAP-23 at the plasma membrane was lower in insulin resistant tissue, but the levels inside the cell were higher, indicating impaired localization.[93] This effect was also seen in the research of Bostrom et al. in HL-1 cardiomyocytes (mice).[92] It has to be said however that the precise mechanism of this effect is unknown, and that it is difficult to distinguish between cause and consequence here. This because it is also implicated that SNAP-23 is involved in the development of IR and it has still not been clearly shown if altered SNAP-23 causes insulin resistance or that insulin resistance causes altered SNAP-23 translocation.
Maier et al. showed that also syntaxin 4 levels (like the VAMP2 levels described above) were increased in ZDF rats in contrast with no difference in GLUT4 expression in healthy muscle tissue. And this applies also to 3T3-L1 adipocytes.[90] Chen et al showed that glucosamine-induced IR in 3T3-L1 cells blocks syntaxin-4 translocation. Also the association between

VAMP2 and syntaxin4, regulated by Munc18c, required for GSV fusion to the membrane, is blocked due to glycosamine.[94] So one research states that expression of syntaxin 4 is elevated, and the other shows decreased levels of syntaxin 4 in the plasma membrane. This could mean three things. First of all, it could be that syntaxin 4 is piling up in the cell, but this doesn't seem logical since neither one of the groups has observed this. It also could be that there is still no consensus on how syntaxin 4 is processed during IR. Last of all it could be that the effects of different IR-inducing factors differ. This third option seems logical, but is also shows a big handicap of IR research. Different models are used with different IR-inducing factors, which could lead to different results that can't be linked to each other.
It can be concluded that both SNAP-23 and syntaxin 4 are definitely affected by IR. For SNAP-23 this ensures impaired translocation to the membrane into compartments in the cytosol, and for syntaxin 4 the mechanism is still unknown.

Another element involved in SNARE complex formation is the regulator Munc18c (also known as STXBP3), which can bind to syntaxin 4 on the plasma membrane if activated. This activation is currently debated since it is thought that Munc18c undergoes insulin-dependent tyrosine-phosphorylation, but it is also been stated that insulin directly regulates Munc18c function.[14,24] Bostrom et al. have shown that IR human skeletal muscle exhibits an elevated level of Munc18c in comparison with healthy skeletal muscle. The observation that high levels of Munc18c are associated with IR, and so with impaired glucose uptake coincides with previous studies showing elevated levels of Munc18c inhibit GLUT4 translocation.[95,96] Bostrom also tested levels of Munc18c in adipose tissue, but they didn't find elevated levels of Munc18c there. This raises the possibility that IR exerts different effects in different tissues.[93] Chen et al. showed that glycosamine-induced IR causes modification of Munc18c.[94] Nelson et al. also used glycosamine-induced insulin resistance in 3T3-L1 cells, and their results suggest that Munc18c trafficking to the membrane is modified in IR.[97] Especially these last two studies are comparable, because they used the same cell line and the same IR-inducing model. They both show that even though the precise mechanism is unknown, Munc18c function and trafficking is altered in IR.

Another really important process in the GLUT4 pathway as described above is the recycling of GLUT4, so it can facilitate the uptake of glucose again. Sortilin is a sorting receptor that regulates GSV formation mainly between the trans-Golgi network and the endosomes.[14,98] Depletion of sortilin leads to GLUT4 degradation which shows that sortilin is of importance.[99] Also palmitate downregulation of sortilin, which is comparable to decreased sortilin expression due to IR, resulted in a decrease in GLUT4 recycling, showing the importance of sortilin.[100] Tsuchiya et al. showed that in C2C12 myoblasts (mice) TNF-α induced IR caused a significant decrease in sortilin expression. The precise mechanism at which sortilin is linked to IR is still under investigation.[100] Again here, the results should be treated with caution because an animal cell line has been used to investigate the effect of IR on elements of the GLUT4 pathway. Next to that, again here another type of IR model has been used, namely TNF- α which means this results don't necessarily have to be true for other IR-inducing models. This means that the results cannot be transferred to human cells that easily. Further research mainly on human muscle- and adipose tissue should reveal if sortilin levels are indeed decreased in response to IR.

A summary of all elements that are possibly affected by IR are listed in table 1.

Protein affected by IR?	What mechanism?	What cells/tissue used?	Which research has seen this?
IRS1	Altered tyrosine phosphorylation No effect of IR on IRS1, but downstream of IRS1	3T3-L1 adipocytes 3T3-L1 adipocytes and L6 myoblasts	Gual (75) Hoehn (5)
Akt2	Inhibitory feedforward signal in IR cells to Akt 2 in response of insulin Impaired phosphorylation of Akt 2	3T3-L1 adipocytes Human adipocytes	Ng (76) Rondinone (77)
mTORC1	Attenuation signal mTORC1 to IRS1, altering all intermediates	Mathematical model based on IR and healthy human adipocytes	Brännmark (47)
AS160	Attenuated phosphorylation of AS160 in IR, no change in protein expression	Human IR vs healthy skeletal muscle tissue	Karlsson (101)
aPKC	Significant decrease of activation of aPKC, no change in expression levels	Human IR vs healthy muscle tissue	Vollenweider (84)
Rac1	IR prevents activation of Rac1 and actin remodeling	L6 myotubes	JeBaily (87)
Myo1c	Decreased expression levels, even in exercise stimulated pathway	Muscle tissue from female mice	Toyoda (89)
VAMP2	Elevated levels of VAMP2	ZDF rats and 3T3-L1 adipocytes	Maier (90)
SNAP-23	Lower levels of SNAP-23 at the plasma membrane, higher levels in cytosol in IR.	Human IR vs healthy skeletal muscle HL-1 cardiomyocytes	Bostrom (92), Bostrom (93),
Syntaxin 4	Elevated levels of syntaxin 4 Blocked VAMP2-syntaxin4 complex	ZDF rats and 3T3-L1 adipocytes 3T3-L1 adipocytes	Maier (90) Chen (94)
Munc18c	Increased Munc18c expression in IR. IR modifies Munc18c and blocks association of Mun18c with the VAMP2-syntaxin4 complex Modified trafficking of Munc18c to the plasma membrane	Human IR vs healthy skeletal muscle 3T3-L1 adipocytes 3T3-L1 adipocytes	Bostrom (93) Chen (94) Nelson (97)
Sortilin	Decreased sortilin expression in IR, decrease of GLUT4 recycling due to downregulation of sortilin	C2C12 myoblasts	Tsuchiya (100)

Table 1. Overview of the elements of the GLUT4 pathway linked to insulin resistance.

Possibly, but not surely affected

Besides the few elements described above that are possibly directly affected by IR, there are more elements insulin responsive, and thus could potentially be affected by insulin resistance as well. Some of them are IRAP, RAB31, LRP1, TUG, RALA, RAB10 and possibly RAB5. These elements are discussed in article 14.

Implications/therapeutic interventions

It is believed that therapeutic interventions directed on insulin resistance will have major beneficial effects on diseases like T2D.[76] Abovementioned are the elements of the GLUT4 pathway that are (most) affected by insulin resistance, so it just seems logical that therapeutic interventions should try to improve these factors, so they work (properly) again.

Like Brännmark et al have shown that attenuation of the positive feedback signal from mTORC1 to IRS1 causes most of the problems seen in insulin resistance due to decreased phosphorylation of IRS1, an apparent target for therapeutic interventions should look to mechanisms that increase IRS phosphorylation due to mTORC1 activation. It also could be by decreasing dephosphorylation of that signal after mTORC1 induced it.[47]

Another possibility is at the AS160 protein. As already stated before, it seems that AS160 can also be stimulated by exercise. It seems that contraction stimulates phosphorylation of this Akt substrate, and so it could serve as a place of crosstalk between the insulin-stimulated and the exercise-stimulated GLUT4 translocation. This possibly could provide a therapeutic starting point.[81, 102]

As seen before, also SNAP-23 is affected by insulin resistance, namely by decreased levels of SNAP-23 at the membrane, and higher levels in the cytosol in skeletal muscle. Because the precise mechanism is still unknown, it is not possible yet to establish therapeutic interventions on it. But it is possible to speculate about how the intervention could be like. For example, if it would be possible to change SNAP-23 localization, so from cytosol to the membrane, it would possibly improve glucose uptake by the cell, thereby treating insulin resistance and so even T2D.[93]

Another possible therapeutic intervention is downregulation of GRK2. GRK2 is a G protein coupled receptor kinase, which is an inhibitor of the PI3K pathway after stimulation of the insulin receptor and thus inhibiting insulin-stimulated glucose uptake. It is shown that GRK2 levels are elevated in insulin-resistant states. Downregulation of GRK2 by 50% improves glucose uptake, so implying an interesting intervention against T2D.

Interestingly, this year Guo et al proposed another mechanism to enhance GLUT4 translocation in insulin resistance, based on guava leaf extract. Experiments on male SHRSP/ZF rats which was orally given guava leaf extract for six weeks, showed that they had improved expressionlevels of IRS1, Akt, PI3Kp85, and improved phosphorylation of IRS1, AMPK and Akt308 in skeletal muscle. Besides this, also significantly higher levels of membrane GLUT4 was seen in contrast with the control animals. These results show promising new ways to treat and maybe even prevent insulin resistance related diseases like T2D.[103]

Conclusions

Since the discovery of insulin, lots has been uncovered about its role in glucose uptake. But even nowadays not every detail of its pathway are known. The precise mechanisms and effects of insulin resistance on this pathway are also unknown, even though some elements of the GLUT4 pathway have been indicated to be largely affected by insulin resistance. They include altered phosphorylation of IRS1 and AS160, inhibition of Akt2 and Rac1, attenuated signal mTORC1, decreased activity of aPKC, decreased expression levels of Myo1c and sortilin and elevated expression levels of SNAP-23, VAMP2, syntaxin 4 and Munc18c.

It is important to not that not all these effects have to occur in every case of insulin resistance. Different causes of insulin resistance lead to different effects. But for all elements it has been shown that they are affected by insulin resistance.[33]

Like it is already been stated, exercise positively affects glucose uptake by promoting GLUT4 translocation to the membrane by its own pathway. This means that exercise can undo a part

of the negative effects of insulin resistance, even though exercise can't restore the PI3K pathway.[33]

To further extent the knowledge of the factors affected by insulin resistance, more research has to be conducted with proteomic analysis for example. First of all, all aspects of the GLUT4 pathway should be established.[24]

To be able to study the effects of insulin resistance on the GLUT4 pathway for possible therapeutic interventions, it is desirable that all research makes use of just one research model. Nowadays a wide variety of models is used, which differ in the mechanism that induces insulin resistance. For example there are models with chronic insulin, dexamethasone, aging, HFD (high-fat diet), TNF-α infusion and use of ceramide and oxidants.[76, 87] This causes that no consensus in results is possible since all models are so different. In accordance with this, is there also need for an animal model that represents the early stages of T2D better as the models nowadays. It is suggested that horses represent this, but this should be further investigated.[14]

But because lots of focus is on elucidating insulin resistance, it should be expected that promising new therapies will become available in the forthcoming years.

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