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β-adrenergic regulation of glucose transporters

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“The cloning of humans is on most of the lists of things to worry about from Science, along with behaviour control, genetic engineering, transplanted heads, computer poetry and the unrestrained growth of plastic flowers.”

Lewis Thomas

“All men dream, but not equally. Those who dream by night, in the dusty recesses of their minds, awake in the day to find that it was vanity. But the dreamers of the day are dangerous men, for they may act their dreams with open eyes to make it reality.”

T.E. Lawrence

To my family
ABSTRACT

The transport of glucose across the plasma membrane is a fundamental mechanism to provide cells with its basic requirements for energy yielding processes. It is also vital for clearing glucose from blood into tissues, a process normally stimulated by the hormone insulin in mammals. In disease states such as diabetes mellitus, the insulin mechanism is not functional, resulting in severe complications. This has been a driving force for understanding how insulin regulates glucose transport, and to find insulin-independent glucose transport mechanisms. The sympathetic nervous system, normally activated during stress, also regulates glucose transport. In contrast to insulin, less is known about the sympathetic regulation of glucose uptake. The sympathetic neurotransmitter noradrenaline, act on the G protein-coupled receptor (GPCR) family of adrenergic receptors (ARs). An important subtype of the AR family is the β-AR which is further subdivided into the β₁, β₂, and β₃-AR. The β₃-AR is the predominant subtype in adipocytes, and the β₂-AR is predominant in skeletal muscle.

Glucose is a polar molecule that has to be transported across the hydrophobic plasma membrane by the 13 member family of facilitative glucose transporters (GLUTs). GLUT1 is ubiquitously expressed and provides cells with basal glucose uptake for maintaining energy levels. GLUT4 is the major GLUT isoform that is rapidly regulated by insulin signaling. In this thesis, I investigated the β-AR regulation of GLUT1 and 4, and glucose uptake. I studied these events using skeletal muscle cells and brown adipocytes in culture, model systems which correspond to metabolically active, sympathetically innervated and insulin-sensitive tissues.

Stimulation with β-adrenergic agonists increased glucose uptake in skeletal muscle cells and brown adipocytes. In brown adipocytes this can be divided into two separate mechanisms. Activation of the β₃-ARs and production of cyclic AMP (cAMP) induced the expression of GLUT1, resulting in an increase of GLUT1 protein in the plasma membrane, and a large increase of glucose uptake after 5 hours of stimulation. However, there was a faster mechanism of glucose uptake that surprisingly was not due to an increase of GLUT1 or GLUT4 protein at the plasma membrane, indicating that β₃-adrenergic stimulation may regulate other GLUTs or the intrinsic activity of GLUTs already present at the plasma membrane. In skeletal myotubes, we postulate there is a possible mechanism where β₂-ARs can regulate the intrinsic activity of GLUT1.

Investigating the insulin and β-adrenergic signaling to glucose uptake revealed that they are two separate pathways, in both skeletal muscle and brown adipocytes. We found that insulin signaling, but not β-adrenergic signaling, mediated glucose uptake through class I phosphatidylinositol 3-kinase (PI3K). The β-adrenergic signaling to glucose uptake appeared to involve a PI3K related kinase (PIKK), in skeletal muscle and brown adipocytes. Furthermore, the increase of glucose uptake by β₃-ARs in brown adipocytes is partially mediated by AMP-activated protein kinase (AMPK), which is not activated by insulin.

In an artificially constructed system, consisting of Chinese hamster ovary (CHO) cells stably expressing GLUT4 and transiently expressing β₂-ARs, both insulin and a β-adrenergic activation regulated GLUT4 and increased glucose uptake. These results suggest that β-adrenergic stimulation can translocate GLUT4 in certain systems, perhaps through atypical signaling from the C-terminal of the β₂-AR.

These results show that β-adrenergic signaling increase glucose uptake by regulating glucose transporters. This occurs through distinct signaling pathways, and separately from insulin signaling to glucose uptake.
This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals.

β3-adrenergic receptors stimulate glucose uptake in brown adipocytes by two mechanisms independently of GLUT4 translocation 

β1- and β3-adrenergic receptor stimulated glucose uptake is dependent on a PI3K related kinase in brown adipocytes. 
Manuscript

III. Hutchinson, D., Chernogubova E, **Dallner, O.S.,** Cannon, B. and Bengtsson, T. (2005): 
β-adrenoceptors, but not α-adrenoceptors, stimulate AMP-activated protein kinase in brown adipocytes independently of uncoupling protein-1. 
Diabetologia 48(11): 2386-95

Involvement of a PI3K related kinase in β2-adrenergic receptor stimulated glucose uptake in L6 myotubes. 
Manuscript

Stimulation of β2-adrenergic receptors leads to translocation of GLUT4 and glucose uptake in CHO-GLUT4myc cells. 
Manuscript

β-adrenergic modulation of glucose uptake through GLUT1 
Manuscript
β-ADRENERGIC REGULATION OF GLUCOSE TRANSPORTERS

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ABBREVIATIONS

AC  Adenylyl cyclase
AMPK  AMP-activated protein kinase
ANS  Autonomic nervous system
AR  Adrenergic receptor
ASE  Adipocyte tissue specific element
AS160  AKT substrate 160
BAT  Brown adipose tissue
cAMP  Cyclic adenosine monophosphate
CAP  Cbl associated protein
C/EBP  CCAAT/enhancer binding protein
CRE  cAMP responsive element
CREB  cAMP-response element binding protein
DNA-PK  DNA protein kinase
DAG  Diacylglycerol
Epac  Exchange proteins directly activated by cAMP
GEF  GLUT4 enhancer factor
GLUT  Glucose transporter
GPCR  G-protein coupled receptor
GRK2  G-protein-coupled receptor kinase 2
HFRE  High fat responsive element
HMIT  H+-coupled myo-inositol symporter
IP3  Inositol 1, 4, 5-triphosphate
IR  Insulin receptor
IRAP  Insulin-regulated aminopeptidase
IRE  Insulin response element
IRS-1  Insulin receptor substrate 1
MAPK  Mitogen-activated protein kinase
MEF2  Myocyte enhancer factor 2
MG1E  Muscle specific GLUT1 element
mTOR  Mammalian target of rapamycin
NF1  Nuclear factor 1
O/E  Olf-1/Early B cell factor
PDE  Phosphodiesterase
PDGF  Platelet derived growth factor
PDK1  3'-phosphoinositide-dependant kinase 1
PI3K  Phosphatidylinositol 3-kinase
PIKK  PI3K related kinase
PIP3  Phosphatidylinositol-3,4,5-phosphate
PKA  Protein kinase A
PKB  Protein kinase B (or Akt)
PKC  Protein kinase C
PLA2  Phospholipase A2
PLCβ  Phospholipase Cβ
PLD  Phospholipase D
PPAR  Peroxisome proliferator-activated receptor
PTEN  Phosphatase and tensin homolog deleted on chromosome 10
RICTOR  Rapamycin insensitive companion of mTOR
SHIP  Src homology 2 domain containing inositol 5’-phosphatase 2
SM  Skeletal muscle
SNARES  Soluble NSF attachment protein receptors
SNS  Sympathetic nervous system
SRE  Serum response element
WAT  White adipose tissue
TPA  12-O-tetradecanoylphorbol-13-acetate
TRE  TPA responsive element/Thyroid hormone responsive element
UCP1  Uncoupling protein 1
VAMP  Vesicle-associated membrane protein 2
VMH  Ventromedial hypothalamic nucleus
1. INTRODUCTION

The sympathetic nervous system (SNS) is a part of the autonomic nervous system (ANS) and regulates important processes in mammals, including humans. Activation stimulates the release of primarily adrenaline and to some extent noradrenaline from the adrenal medulla, and focal release of noradrenaline from sympathetic nerve endings. Adrenaline circulating in the blood mediates the acute effects of stress during isolated events to prepare the body for a reaction. However, noradrenaline released from sympathetic nerves regulate processes on a daily basis to uphold homestasis. Target tissues express adrenergic receptors (ARs) responsible for mediating the effects of adrenaline and noradrenaline, accelerating heart rate, releasing triglycerides from fat and glucose from liver, increasing blood flow to skeletal muscle, dilating pupils and airways, and reducing gut movements. Adrenergic activation of white adipose tissue (WAT) promotes β-oxidation and release of triglycerides to mobilize energy for other organs. On the contrary, activation of adrenergic receptors on skeletal muscle cells results in an activation of processes to mobilize energy for the tissue itself, one such process being glucose uptake as discussed in this thesis. Heavily sympathetically innervated brown adipocyte tissue (BAT) is present in rodents, hibernating animals, and human infants and is characterized by its ability to uncouple the electron transport chain in mitochondria and produce heat, stimulated by sympathetic activation in response to cold. Adrenergic activation of BAT not only activates heat production and hypertrophy of the tissue, but also increases glucose uptake.

Increasing or decreasing glucose uptake in target cells is achieved by regulating the glucose transporters (GLUTs) responsible for facilitating the transport of the polar glucose molecule across the hydrophobic plasma membrane. Signals can alter the expression of the transporter, the translocation and levels of the transporter at the plasma membrane, or the intrinsic activity of the transporter.

The insulin signaling pathway to translocation of GLUTs and increase in glucose uptake into target tissues has been in focus in glucose transporter biology and has given us knowledge in the regulation of GLUTs. The increase in the prevalence of diabetes type II encourages the research in the field to further understand the complex mechanisms involved. Furthermore, this incentive has led to the search for alternative ways to stimulate glucose uptake, and the identification of other signaling pathways regulating GLUTs. The sympathetic regulation of blood glucose homeostasis encourages further investigation into signaling and mechanisms involved. This is of particular interest in skeletal muscle and brown adipose tissue. Skeletal muscle is the largest tissue in the human body, and hence accounts for the
most part of insulin induced reduction of blood glucose levels after feeding. Recently, studies indicate the presence of active brown adipose tissue not only in infants, but also in adult humans (Nedergaard, et al. 2007). Furthermore, there is a recent interesting development in the research into BAT/WAT/skeletal muscle cell lineage, suggesting there is a common progenitor of skeletal muscle and brown adipocytes. There might be possibilities in the future to promote the brown adipocyte lineage as an energy wasting therapy for obesity, and perhaps as a blood glucose clearing tissue. There are clearly many reasons that emphasize the importance of understanding skeletal muscle and brown adipocyte physiology.

In this thesis, I focus on defining parts of the β-adrenergic receptor signaling and mechanisms regulating glucose transporters and ultimately glucose transport, in skeletal muscle and brown adipocyte cells.

2. GLUCOSE TRANSPORTERS (GLUTs)

2.1. Introduction to GLUTs

Transport of monosaccharides over the plasma membrane is a fundamental mechanism in providing the eukaryotic cell with its basic requirements for energy yielding processes. In mammals, glucose is the primary monosaccharide, although fructose and other sugars can also be transported into cells. Transport of glucose into cells is vital in reducing glucose levels and maintaining the homeostasis in the blood plasma after feeding. Glucose cannot penetrate the hydrophobic plasma membrane and has to be transported by facilitative glucose transporters (GLUTs) by diffusion. The GLUT family is comprised of 13 known proteins, GLUT1-12 and HMIT (gene symbols SLC2A1-13), and it is now generally considered that all members of the family are identified (Joost and Thorens 2001; Uldry and Thorens 2004) (Table 1).

Since the first isolation of a glucose transporter (GLUT1, (Mueckler, et al. 1985) ) other members has been included, and subsequently a consensus for their terminology has evolved. The GLUTs all share a structure prediction of 12 hydrophobic α-helical membrane-spanning regions with intracellular amino- and carboxyl-termini. The family is divided into three classes based on multiple sequence alignments and common sequence motifs, class I (GLUT1-4), class II (GLUT5, GLUT7, GLUT9, and GLUT11), and class III (GLUT6, GLUT8, GLUT10, GLUT12, and HMIT) (Table 1). They display different substrate specificities and tissue distribution.
A multitude of stimuli can modulate glucose transport in different tissues, in response to different physiological conditions. Three distinct mechanisms have been identified by which this can be achieved (further discussed in this chapter).

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Class</th>
<th>Expression</th>
<th>Substrate specificity</th>
<th>References (first discovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1</td>
<td>I</td>
<td>Erythrocytes, brain, ubiquitous</td>
<td>Glucose</td>
<td>Mueckler et al 1985</td>
</tr>
<tr>
<td>GLUT2</td>
<td>I</td>
<td>Liver, pancreas, intestine, kidney</td>
<td>Glucose (low affinity) fructose</td>
<td>Fukumoto et al 1988</td>
</tr>
<tr>
<td>GLUT3</td>
<td>I</td>
<td>Brain, WAT</td>
<td>Glucose</td>
<td>Kayano et al 1988</td>
</tr>
<tr>
<td>GLUT4</td>
<td>I</td>
<td>Heart, SM, WAT, BAT, brain</td>
<td>Glucose</td>
<td>Fukumoto et al 1989</td>
</tr>
<tr>
<td>GLUT5</td>
<td>II</td>
<td>Intestine, testes kidney</td>
<td>Fructose glucose (low affinity)</td>
<td>Kayano et al 1990</td>
</tr>
<tr>
<td>GLUT6</td>
<td>III</td>
<td>Brain, spleen, leucocytes</td>
<td>Glucose</td>
<td>Doege et al 2000</td>
</tr>
<tr>
<td>GLUT7</td>
<td>II</td>
<td>Intestine, testis</td>
<td>Fructose, glucose</td>
<td>Joost and Thorens, 2001</td>
</tr>
<tr>
<td>GLUT8</td>
<td>III</td>
<td>Testes, brain, SM, WAT</td>
<td>Glucose</td>
<td>Doege et al 2000</td>
</tr>
<tr>
<td>GLUT9</td>
<td>II</td>
<td>Liver, kidney</td>
<td>-</td>
<td>Phay et al 2000</td>
</tr>
<tr>
<td>GLUT10</td>
<td>III</td>
<td>Liver, pancreas, SM</td>
<td>Glucose</td>
<td>Dawson et al 2001</td>
</tr>
<tr>
<td>GLUT11</td>
<td>II</td>
<td>Heart, SM</td>
<td>Fructose glucose (low affinity)</td>
<td>Doege et al 2001</td>
</tr>
<tr>
<td>GLUT12</td>
<td>III</td>
<td>Heart, prostate, SM, small intestine, WAT</td>
<td>Glucose</td>
<td>Rogers et al 2002</td>
</tr>
<tr>
<td>HMIT</td>
<td>III</td>
<td>Brain, WAT</td>
<td>H^+-myo-inositol</td>
<td>Uldry et al 2001</td>
</tr>
</tbody>
</table>

Table 1. The family of facilitative glucose transporters (GLUT). WAT, white adipose tissue; SM, skeletal muscle; BAT, brown adipose tissue (adapted from Joost and Thorens 2001).

First, the gene expression of the GLUT can be altered resulting in an increase/decrease in the number of transporters in the cell and at the plasma membrane.

Secondly, the glucose transporters can be shuttled between intracellular compartments and the plasma membrane in vesicles, a process referred to as translocation.

The third possible mechanism is a change in the intrinsic activity of the glucose transporter located in the plasma membrane.

Specific signals can trigger responses changing glucose uptake into tissues by one or several of these mechanisms.
2.2. Structure of GLUTs

Determining the structure of GLUTs has proven difficult using methods like x-ray crystallography or NMR spectroscopy. However, there are studies on specific members of the GLUT family based on alternative approaches such as homology comparison, mutagenesis studies, and 3D modeling. These studies have contributed with information about their structure that ultimately is important for investigating their physiological context.

Data that confirms the tertiary structure of GLUT1 has been derived from both extensive cysteine-scanning mutagenesis studies (Heinze, et al. 2004; Mueckler and Makepeace 2005), and structure prediction based on similarities with glycerol phosphate transporter and glucose-6-phosphate translocase structure (Salas-Burgos, et al. 2004). These studies predict GLUT1 is a water-filled channel that selectively transports glucose over the plasma membrane. GLUT1 has a N-glycosylation site at residue N-45 in a large extracellular loop, between transmembrane segments 1 and 2, a motif shared by all class I-II GLUTs (Figure 1). Glycosylation of this site has been shown to be important for GLUT1 intracellular targeting, protein stability, and transport efficiency (Asano, et al. 1991; Asano, et al. 1993). Class III GLUTs have a shorter loop between transmembrane segments 1 and 2, and a longer loop between transmembrane segments 9 and 10, with the characteristic N-glycosylation site. Current theory states that glucose binds to specific sites on the extracellular side of GLUT1, inducing a conformal change that promotes transport over the plasma membrane.
A theory on the structure of GLUT3 made by homology modeling further supports the similarities within the GLUT family. Comparison between GLUT3 and a mechanosensitive ion channel (MscL protein) together with general information from aquaporin structure describes GLUT3 as a 12 transmembrane helix protein, with a central hydrophilic pore where glucose can cross the plasma membrane (Dwyer 2001).

GLUT4 has been extensively studied for expression, translocation and transport characteristics, but not much is known about the structure, except for homology comparisons. To date, no direct studies on structure of other GLUTs expressed in skeletal muscle- and adipose-tissue are known to the author.

2.3. Regulation of GLUT gene expression

Regulation of transcriptional activity, post-transcriptional events and translation can alter the number of glucose transporters in cells. This may also correspond to changes in the glucose transport, and have a physiological relevance.

2.3.1. GLUT1 gene expression

GLUT1 was the first glucose transporter to be cloned (Mueckler, et al. 1985), it is ubiquitously expressed, with high expression patterns in erythrocytes and the endothelial cells in the blood-brain barrier (Maher, et al. 1994). GLUT1 is generally expressed at high levels in fetal or proliferative cells, and lower levels in the differentiated cells, of adipose and muscle tissues (Santalucia, et al. 1992; Castello, et al. 1993; Postic, et al. 1994) (Paper II)

The GLUT1 promoter contains a number of recognition sequences for transcription factors and transcription is induced by SP1, cAMP, serum and thyroid hormone in both adipocyte and skeletal muscle cells (Romero, et al. 2000; Sanchez-Feutrie, et al. 2004; Vinals, et al. 1997b)(Vinals, et al. 1997a) (Figure 2). SP1-SP4 is a family of zinc finger (DNA-binding domain) transcription factors, both SP1 and SP3 has been shown to be involved in regulation of GLUT1 mRNA expression in skeletal muscle cells (Sanchez-Feutrie, et al. 2004; Vinals, et al. 1997a; Vinals, et al. 1997a; Fandos, et al. 1999; Fandos, et al. 1999).

Hyperosmotic stimulation has been shown to increase GLUT1 gene expression, by activation and binding of SP1 to the GC rich region located –95/-83 of the GLUT1 proximal promoter, in Clone9 cells (Hwang and Ismail-Beigi 2006). Another site designated MG1E (muscle specific GLUT1 element) has been identified in L6E9 myoblasts, which leads to induction of the GLUT1 gene by SP1, cAMP or serum (Sanchez-Feutrie, et al. 2004).
SP3 is proposed to be a negative regulator of GLUT1 transcription, when associated with the GLUT1 proximal promoter. SP3 is induced during differentiation when GLUT1 gene expression is down-regulated, and binds to a GC box located –91/-86 in the GLUT1 proximal promoter (Fandos, et al. 1999), presumably acting as a repressor of transcription. It is noticeable that these recognition sequences overlap and further studies is required to elucidate the relationship between these two transcription factors.

Serum, platelet derived growth factor (PDGF), and insulin also has been shown to be able to activate GLUT1 transcription in 3T3/NIH adipocytes. Two enhancer elements are identified for this effect, and enhancer 1 seems to be most important (Murakami, et al. 1992; Todaka, et al. 1994). Enhancer 1 contains several homologues sequences that can be regulated by different stimuli. A cAMP responsive element (CRE) is present, and cAMP has been shown to increase GLUT1 transcription in L6E9 skeletal myocytes, 3T3-NIH adipocytes and brown adipocytes (Vinals, et al. 1997b; Cornelius, et al. 1991) (Paper I). A serum response element is also located in enhancer 1, responding to a variety of growth factors. There is also a TPA responsive element (TRE), sensitive to stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA activates conventional and novel protein kinase C (PKC) isoforms, and the TRE site in enhancer 1 can be activated by platelet-derived growth factor. Enhancer 2 is located between exon 1 and 2 of the GLUT1 gene, and also contains putative TRE and CRE sites.

A transcriptional activation and a de novo synthesis of GLUT1 protein is subsequently followed by transport of the protein to the plasma membrane, this is not considered to be a translocation mechanism. The c-terminus of GLUT1 can interact with the GLUT1 transporter binding protein GLUT1CBP (GIPC1), coupling the GLUT1 vesicles to myosin VI movement on actin filaments (Reed, et al. 2005).

Figure 2. The 5’ flanking sequence of the GLUT1 gene. TRE; TPA responsive element, SRE; serum response element, CRE; cAMP responsive element, MG1E; muscle specific GLUT1 element.
2.3.2. GLUT4 gene expression

The GLUT4 gene was cloned and characterized in rat, mouse and human in 1989 by a number of groups (Birnbaum 1989; Charron, et al. 1989; Fukumoto, et al. 1989; James, et al. 1989b; Kaestner, et al. 1989). It is expressed in insulin sensitive tissues such as heart, skeletal muscle and adipose tissue, where insulin stimulation translocates GLUT4 to the plasma membrane to increase glucose transport into the cell. Expression of the transporter is increased in differentiating tissues and coincides with the time when the cells acquire insulin sensitivity (Santalucia, et al. 1992; Castello, et al. 1993; Postic, et al. 1994) (Paper I).

GLUT4 is also regulated on a transcriptional level by a vast number of growth factors and hormones. Many factors are involved in binding to specific regulatory elements in the GLUT4 promoter and altering the transcription rate.

![GLUT4 5' flanking sequence](image)

Figure 3. The 5' flanking sequence of the GLUT4 gene. C/EBP; C/EBP binding site, TRE; thyroid hormone responsive element, MEF2; MEF2 binding site, ASE; adipocyte tissue-specific element, HFRE; high-fat responsive element, NF1; nuclear factor 1 binding site, O/E; Olf-1/Early B Cell Factor binding site, IRE; insulin responsive element.

CCAAT/enhancer binding proteins (C/EBPs) is involved in regulating the differentiation of adipocytes (Christy, et al. 1991), and GLUT4 is a target gene of C/EBPα. C/EBPα bind to a recognition sequence located –258/-250 in the GLUT4 promoter and enhances transcription in 3T3-NIH/L1 adipocytes (Gross, et al. 2004; Kaestner, et al. 1990) (Figure 3).

In C2C12 skeletal muscle cells, it has been shown that myocyte enhancer factor 2 (MEF2) binds specifically to a site located –437/-428, which confers tissue specific expression (Liu, et al. 1994). The same reporter gene study also indicated that the MEF2 site alone, without the two downstream thyroid hormone responsive elements located –419/-414, and –409/-404 (Ezaki 1997) reduced expression to 60 %. Thyroid hormone has been shown to induce GLUT4 expression by increased transcriptional activity in C2C12 myocytes, 3T3-L1

An adipocyte tissue-specific element (ASE), essential for expression of GLUT4 in white adipocytes, has been located at -551/-506 by examining mutated GLUT4 minigene expression in transgenic mice (Miura, et al. 2003). The study also found evidence for a high-fat responsive element (HFRE) at -701/-551. The existence of such an element correlates with in vivo experiments when mice fed with a high fat diet for three months caused decreased GLUT4 mRNA expression in WAT by 50-70% (Ikemoto, et al. 1995). A GLUT4 minigene with the nuclear factor 1 (NF1) site at -700/-688 mutated results in no expression of the GLUT4 minigene mRNA exclusively in WAT, suggesting that both the ASE and the NF1 sites are necessary for white adipocyte tissue GLUT4 expression (Miura, et al. 2004). Interfering with the NF1 binding does however not affect the repression of GLUT4 mRNA expression by high-fat feeding, and needs to be investigated further.

Prolonged exposure to cAMP or insulin down-regulate expression of the GLUT4 gene in 3T3-L1 adipocytes. This has been shown to be partly mediated by the NF1-binding site in the GLUT4 promoter (Cooke and Lane 1999a; Cooke and Lane 1999b), and stimulation with cAMP also repress GLUT4 transcription in skeletal myocytes and brown adipocytes (Vinals, et al. 1997b; Kaestner, et al. 1991) (Paper I). Further studies of the repression of GLUT4 transcription by insulin and cAMP has also implicated the transcription factor Olf-1/Early B Cell Factor (O/E) in 3T3-L1 adipocytes (Dowell and Cooke 2002), but the relationship between O/E and NF1 is not yet understood. A study indicate, that in the human GLUT promoter there is a domain located -724/-712 where a novel transcription factor designated GLUT4 enhancer factor (GEF) binds, and is required for GLUT4 expression in cooperation with MEF2 (Oshel, et al. 2000). However, there are conflicting data as this domain corresponds to the NF1 binding site in the mouse GLUT4 promoter, and disruption of the GEF binding site also disrupts the NF1 site of human GLUT4.

AMP-activated protein kinase (AMPK) is thought to be a key sensor of the energy status in cells (for a recent review see (Kahn, et al. 2005). It is activated by changes in the AMP/ATP ratio and possibly by exercise, and regulates several important cellular processes in skeletal muscle (see further discussion in chapter 4). Physical training has in several studies induced GLUT4 expression, in skeletal muscle (Host, et al. 1998; Kawanaka, et al. 1997; Ren, et al. 1994). It is a possibility that AMPK activation in response to exercise can induce the observed increase in GLUT4, and also GLUT1 protein (Fryer, et al. 2002). AMPK activation can activate GEF and myogenic factor MEF2, and increase their binding to the GLUT4
promoter (Holmes, et al. 2005). However, there are also indications that AMPK is not required for exercise-induced increases in GLUT4 expression (Holmes, et al. 2004).

2.3.3. Expression of other GLUTs in skeletal muscle and/or adipose tissues

Although nearly all research on GLUTs in skeletal muscle and adipose tissues investigates GLUT1 and/or GLUT4, several of the other members of the GLUT family are also expressed in these tissues.

GLUT3 gene expression

In humans, GLUT3 mRNA is abundant in brain where it is specifically expressed in neurons, and at lower levels in adipose tissue (for a recent review see, (Simpson, et al. 2008)). GLUT3 was originally cloned by low stringency hybridization with a GLUT1 probe from a human fetal muscle cDNA library (Kayano, et al. 1988). However, there are conflicting studies concerning the expression in skeletal muscle, with results showing no expression or moderate expression and a possible regulation by insulin growth factor-1 (IGF-1) (Haber, et al. 1993; Shepherd, et al. 1992; Stuart, et al. 2000; Copland, et al. 2007). Even though the expression pattern of GLUT3 suggests it is primarily a “neuronal glucose transporter” results from heterozygous GLUT3+/− mice indicate it has a possible role in metabolism (Ganguly and Devaskar 2008). GLUT3 is vital in fetal development and the heterozygous mice are exposed to limited fetal glucose supply resulting in sexually dimorphic (only male mice are affected) adult onset adiposity and insulin resistance. Further studies into GLUT3 physiology would be of interest not only to elucidate the importance in neurons, but also in metabolism.

GLUT5 gene expression

The GLUT5 gene was first isolated from human small intestine (Kayano, et al. 1990), and then from rat (Rand, et al. 1993), rabbit (Miyamoto, et al. 1994), and mouse (Corpe, et al. 2002), cDNA libraries. GLUT5 has a high specificity for fructose transport and is present in small intestine, kidney, and brain, but also in skeletal muscle and adipose tissues (for a recent review see, (Douard and Ferraris 2008)). Long-term stimulations with insulin increase expression of GLUT5 and fructose transport by transcriptional activation in L6 skeletal myocytes, and the 5’ flanking sequence of the gene has several putative recognition sites for transcription factors such as, IRES, C/EBP, CREB, and PPAR (Hajduch, et al. 2003). There is an ongoing discussion of the relevance of the transition from glucose to fructose in food and
beverage products and the possible relation to the worldwide increase in obesity and type 2 diabetes (Douard and Ferraris 2008; Stanhope and Havel 2008). This may also implicate the regulation and physiology of GLUT5 in the role as a very specific fructose transporter in skeletal muscle and adipose tissues.

GLUT8 gene expression

The GLUT8 gene was cloned from mouse with an approach of database mining (Doege, et al. 2000). It has a high affinity for glucose, and is expressed at high levels in the testis, and at low levels in cerebellum, adrenal gland, liver, spleen, adipose and skeletal muscle tissue. Little evidence exists regarding regulation of expression, but gonadotropins seem to increase GLUT8 expression, correlating with the high expression in testis (Doege, et al. 2000).

GLUT10 gene expression

The GLUT10 gene was cloned and characterized as a GLUT that primarily transports glucose, and is expressed in human heart, lung, brain, liver, skeletal muscle, pancreas, placenta, and kidney (Dawson, et al. 2001). Promoter analysis of the 5’-flanking sequence of the gene reveals putative regulation of several transcription factors as SP1, SP3, AP2, and a possible upstream transcription silencer (Segade, et al. 2005). There are very few publications on GLUT10, but studies on polymorphisms in humans are correlated with changes in angiogenesis and the emergence of arterial tortuosity (Coucke, et al. 2006).

GLUT11 gene expression

The GLUT11 gene is expressed primarily in human heart and skeletal muscle (cloned from human heart), has closest homology to GLUT5 (42 % identical amino acids), and presumably transports both glucose and fructose (Doege, et al. 2001). Interestingly, there is evidence of alternative splicing, resulting in three isoforms expressed in different tissues (Sasaki, et al. 2001; Wu, et al. 2002). Very few studies are made on this novel GLUT, and further investigation is necessary to define its physiological significance.

GLUT12 gene expression

The GLUT12 gene was characterized recently from MCF-7 cells (human breast cancer cell line), and is primarily expressed in human skeletal muscle, heart and prostate, but also adipose
tissues (Rogers, et al. 2002). GLUT12 is together with GLUT4 and GLUT5 the major isoform expressed in human skeletal muscle. Functional data suggests that GLUT12 transports glucose (Rogers, et al. 2003a), and its expression patterns in tissues with high energy demands is interesting for future studies.

HMIT gene expression

The H⁺-coupled myo-inositol symporter (HMIT) was cloned from cDNA libraries of spleen and frontal cortex, and is predominantly expressed in brain, but also kidney and adipose tissues (Uldry, et al. 2001). It selectively transports myo-inositol, which is an important precursor for different types of phosphatidylinositol molecules, important in a vast number of signaling steps, including insulin signaling.

2.3.4. Relevance of GLUT gene expression

The physiological importance of the expression levels of GLUTs are stressed by a number of studies, indicating the relevance of changes in GLUT expression for glucose transport. The expression of GLUTs may change in response to changes in the energy demand of the tissue, or during disease and abnormal physiological states, an effect observed in obesity and cancer.

Decreased levels of GLUT4 expression in adipose tissue is observed in obesity (Olefsky, et al. 1988; Garvey, et al. 1991; Sinha, et al. 1991), and may be related to the insulin resistance caused by this state. On the contrary, high expression levels of GLUT4 in skeletal muscle or adipose tissues of transgenic mice increase these animals capacity for glucose disposal, and renders them highly insulin sensitive (Shepherd, et al. 1993; Tsao, et al. 1996; Tsao, et al. 2001). Exercise increase the expression of GLUT4 in skeletal muscle, presumably to meet the increased energy demand of the tissue (Ren, et al. 1994; Kranjou, et al. 2004). The nerve system is also implicated in regulating expression of GLUT expression in metabolically active tissues. Denervating muscle in rat changes expression levels of both GLUT1 and GLUT4, resulting in a change of glucose transport (Block, et al. 1991; Coderre, et al. 1992).

A known marker for oncogenic transformation is a rapid acceleration of glucose transport to meet the increased energy demand for the acute growth. The major mechanism behind this phenomenon is an increase in GLUT gene expression (for reviews see (Macheda, et al. 2005; Airley and Mobasher 2007), and several oncogenes are involved and are able to induce this response (Birnbaum, et al. 1987; Flier, et al. 1987; Hiraki, et al. 1989). Oncogenes ras and src can both activate GLUT1 expression by interaction with enhancer 1 in the GLUT1 promoter.
There are also examples of GLUTs that are expressed in malignant, but not in the normal cells. GLUT3 has been identified by immunohistochemical analysis in lung, ovarian, and gastric cancers, but are not normally found in these tissues (Younes, et al. 1997). GLUT12 has also been suggested as a GLUT involved in tumor growth, and has been detected in breast cancer cells (Rogers, et al. 2003b). The tumor suppressor p53, known to be mutated in many types of cancers, repress transcriptional activity of the GLUT1 and GLUT4 genes (Schwartzenberg-Bar-Yoseph, et al. 2004). Glucose uptake might be rate-limiting in the development and growth of cancers and could serve as a major target for therapeutic intervention (Airley and Mobasheri 2007).

The changes in expression of glucose transporters and correlating glucose transport observed in rodents could be of importance for human physiology. Therapeutic strategies targeting the expression of GLUTs to counteract diseases are not yet explored, but could provide great possibilities for treatment.

2.4. Regulation of GLUT translocation

Translocation is the mechanism where stimuli shift the equilibrium of glucose transporters, located in vesicles, from intracellular compartments to the plasma membrane. The stimuli often activate a receptor coupling to an intracellular pathway that subsequently interacts with proteins in the GLUT vesicle. When the stimulation is abolished, the glucose transporters are withdrawn in vesicles from the plasma membrane to the intracellular compartment again. This mechanism has been extensively studied for insulin stimulation causing GLUT4 translocation in skeletal muscle and adipose tissues.

2.4.1. Conventional insulin signaling

The involvement of the pancreas in glucose metabolism has been postulated since the 19th century and 1921 Banting and Best discovered insulin, a success that subsequently earned them the Nobel Prize 1923 (Banting, et al. 1991; Banting and Best 1990; Rosenfeld 2002). The first theories describing translocation of an insulin-sensitive glucose transporter were published in 1980 (Cushman and Wardzala 1980; Suzuki and Kono 1980). A vast number of studies on GLUT4 biology is published, mainly as a result of the aim to prevent the emerging problem with diabetes mellitus, and has yielded profound knowledge in insulin signaling and the mechanism of glucose transporter translocation. Diabetes mellitus is a syndrome where defects in the production of or response to insulin results in dysfunctional regulation of blood.
glucose homeostasis. Type I diabetes has a strong genetic association and is often caused by a T-cell mediated autoimmune attack on insulin producing β-cells in the islets of Langerhans in the pancreas. Type II diabetes is connected to lifestyle, and insulin resistance (loss of response from target tissues) is the key abnormality. The insulin signaling pathway and the mechanism of GLUT4 vesicle translocation has been described in several excellent reviews (Bryant, et al. 2002; Watson, et al. 2004), for a recent review see (Zaid, et al. 2008).

The insulin receptor (IR) is a receptor tyrosine kinase, and consists of two extra cellular α- and two transmembrane β-subunits (Figure 4). When insulin binds to the extracelluar α-subunits of the receptor, a conformational change is induced, which cause an autophosphorylation of tyrosine residues in the β-subunits. The activated state of the IR recruits various scaffolding proteins, such as insulin receptor substrate 1 (IRS1) (White 2002). As IRS-1 is associated with the activated IR it is phosphorylated, which then provides a

![Figure 4. The insulin signaling pathway. Activation of the insulin receptor and downstream signaling shifts the equilibrium of GLUT4 vesicles from an intracellular location to the plasma membrane (translocation), causing an increase in glucose uptake.](image)

binding site for the p85 subunit of class 1A phosphatidylinositol 3-kinase (PI3K). PI3K signals and regulates a number of cellular responses, making it a central molecule in many of these processes (Cantley 2002). In turn, the p110 subunit of PI3K catalyzes the formation of
phosphatidylinositol-3,4,5-phosphate (PIP₃) which recruits Akt (also referred to as protein kinase B; PKB) to the plasma membrane. PI3K isoforms are further discussed in chapter III, but recent studies provide strong evidence that PI3K-p110α is the main p110 isoform for insulin signaling (Knight, et al. 2006). PIP₃ also recruits 3’-phosphoinositide-dependant kinase 1(PDK1) which phosphorylates threonine 308 (T308), a key phosphorylation site in the activation loop of Akt (Storz and Toker 2002). Akt has a second phosphorylation site, serine 473 (S473), which is also required for full activation (Welsh, et al. 2005). A study also suggests that mammalian target of rapamycin and rapamycin insensitive companion of mTOR (mTOR/RICTOR) can be the second PDK that phosphorylate S473 of Akt in response to insulin (Hresko and Mueckler 2005). Of the three isoforms of Akt (1, 2 or 3), Akt2 emerges as the most important isoform in insulin signaling. Depletion of Akt2 by RNAi in 3T3L1 adipocytes inhibits insulin signaling, and knockout of Akt2 results in mice with impaired glucose uptake in skeletal muscle (Cho, et al. 2001; Jiang, et al. 2003).

Another target that seems to be downstream of PI3K is protein kinase C (PKC). The family of PKC consists of at least 12 members of serine/threonine kinases that are involved in many signaling events (Way, et al. 2000). The members are divided into three groups, conventional PKC isoforms α, βI, βII, γ (Ca⁺-dependent, activated by phosphatidylserine and diacylglycerol), novel PKC isoforms δ, ε, η, θ (Ca⁺-independent, activated by phosphatidylserine and diacylglycerol), and atypical PKC isoforms ζ and ι/λ (Ca⁺-independent, regulated by phosphatidylserine but not diacylglycerol). PKC signaling has emerged as important steps in insulin signaling and GLUT4 translocation, in particular atypical PKCs (aPKCs)(Farese, et al. 2005), and novel PKC δ (Braiman, et al. 1999). Insulin stimulation induces a rapid remodeling of the actin filaments in L6 skeletal muscle cells, and inhibiting cortical actin almost abolish insulin induced increases of GLUT4 at the plasma membrane (Khayat, et al. 2000; Tong, et al. 2001). PKCζ may be involved in this process in skeletal muscle cells (Liu, et al. 2006), but the effect is not as apparent in adipose cells.

Although the involvement of AKT in insulin-stimulated GLUT4-vesicle translocation is supported by a number of studies in both adipocytes and skeletal myocytes, the precise mechanism is unclear. A link in the interaction between PKB and GLUT4 vesicles are the Rab GTPase-activating proteins AKT substrate 160 (AS160 or TBC1D4) and TBC1D1, both phosphorylated by AKT in response to insulin stimulation, and proposed to be negative regulators of GLUT4 vesicle translocation (Eguez, et al. 2005; Sakamoto and Holman 2008).
Another putative downstream target of AKT, involved in GLUT4 translocation, is the phosphoinositide 5-kinase PIKfyve. Insulin stimulation phosphorylates PIKfyve at serine 318, an effect that is abolished by wortmannin (PI3K antagonist) (Berwick, et al. 2004). Kinase-dead mutants of PIKfyve inhibit GLUT4 translocation, which although its precise involvement is not clear, makes it an interesting target in the insulin signaling.

Two factors that may negatively influence insulin signaling is the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and src homology 2 domain containing inositol 5’-phosphatase 2 (SHIP2). These phosphatases can dephosphorylate PIP3 to PIP2 and suppress insulin signaling, thus inhibition of PTEN and SHIP2 may be a strategy for treating insulin resistance (Sasaoka, et al. 2006).

The insulin-regulated aminopeptidase (IRAP) has also been implicated in insulin signaling and GLUT4 translocation (Keller 2004). It is localized in intracellular compartments under basal conditions, and is rapidly translocated to the plasma membrane in response to insulin. The role of IRAP in GLUT4 translocation is not clear, but it is hypothesized that the aminopeptidase activity is functional at the plasma membrane, where it initiates degradation of non-insulin peptide hormones.

2.4.2. TC10 pathway

There is evidence of a second insulin signaling pathway leading to GLUT4 translocation (primarily in adipocytes), possibly associated with caveolae and lipid raft microdomains. When insulin receptors in these microdomains are activated and undergo autophosphorylation of tyrosine residues, adaptor protein containing a PH and SH2 domain (APS) is recruited and phosphorylated (Liu, et al. 2002) (Figure 5). This in turn recruits Cbl and CAP (Cbl associated protein) to form a complex, and through CAP this complex interacts with flotillins and lipid rafts (Baumann, et al. 2000; Liu, et al. 2005). Cbl associates with another adaptor protein, CrkII, a protein that is constitutively associated to the nucleotide exchange factor C3G (Ribon, et al. 1996). As C3G also localizes to the lipid rafts, it activates the small G-protein TC10 (Chiang, et al. 2001), which in turn can interact with the exocyst complex. The exocyst complex is comprised of several proteins, Exo70, Sec6, Sec8, and SAP97, and is thought to be involved in the tethering of GLUT4 vesicles to the plasma membrane (Inoue, et al. 2003; Inoue, et al. 2006). Furthermore, activation of the TC10 pathway seems to be able to activate and recruit aPKCs to the plasma membrane in a PI3K independent way (Kanzaki, et al. 2004), presumably to participate in GLUT4 translocation events.
Figure 5. The TC10 pathway. Insulin receptor activation recruits APS-CAP-Cbl-CrkII/C3G activating TC10. This recruits the exocyst complex involved in stimulating vesicle tethering, and atypical PKCs (aPKCs) involved in actin remodeling.

However, there are some opposing results. There is evidence that ablation of APS results in inhibition of insulin stimulated glucose uptake (Ahn, et al. 2004), while in another study no effect could be seen when siRNA knockdown of Cbl or CAP was achieved (Zhou, et al. 2004). In spite of this, the TC10 pathway appears to be an important part of insulin signaling.

2.4.3. Tethering of vesicles: SNARES

Fusion of vesicles to the plasma membrane is not a random event, but requires a specific interaction between molecules in the vesicles and the plasma membrane (Figure 6). Two types of SNAREs (Soluble NSF attachment protein receptors), t-SNAREs (target membrane SNAREs) and v-SNAREs (vesicle membrane SNAREs), are the core of the mechanism to bring the vesicles in close proximity to the plasma membrane to allow lipid bilayer mixing. Two potential GLUT4 vesicles v-SNAREs are expressed in adipocytes, synaptobrevin 2 (VAMP2; vesicle-associated membrane protein 2) and cellubrevin (VAMP3), but VAMP2 has been found to be the primary v-SNARE involved in GLUT4 vesicle translocation (Martin, et al. 1998). VAMP2 bind to a complex of t-SNARES Syntaxin 4 and SNAP23 in adipocytes.
A number of SNARE associated proteins, Munc18c, Synip, and Tomosyn, (Thurmond and Pessin 2000; Widberg, et al. 2003; Yamada, et al. 2005) have also been implicated in the process of vesicle/plasma membrane fusion. Furthermore, it is suggested that cortical actin also is involved in the tethering of the vesicles close to the plasma membrane (reviewed in (Zaid, et al. 2008)).

2.4.4. Translocation of other GLUTs

A very interesting finding is that the GLUT4 deficient mice (GLUT4−/−) are growth retarded and has decreased longevity, but does not develop diabetes (Katz, et al. 1995; Stenbit, et al. 1996). Although these mice possibly show insulin resistance, GLUT4−/− mice clear oral glucose tolerance tests as efficiently as wild type controls. This suggests that there are one or several other insulin-responsive GLUTs, able to compensate for the lack of GLUT4. Candidates are GLUT3, 8, 10-12, but there is up to date no definitive theory for the phenotype of the GLUT4 null mice. Expression patterns of GLUT10-12 in insulin-sensitive tissues indicate they may be involved, but there is no evidence of translocation in response to insulin. GLUT8 has been shown to be insulin-responsive, but only in the blastocyst (Carayannopoulos, et al. 2000), and GLUT3 is suggested to translocate and be insulin responsive in neurons (Heather West Greenlee, et al. 2003; Uemura and Greenlee 2006). However, hetezygous GLUT4+/− or conditional depletion of GLUT4 in skeletal muscle or
adipose tissue of mice show the expected insulin resistance and tendency for diabetes (Stenbit, et al. 1997; Li, et al. 2000; Zisman, et al. 2000; Abel, et al. 2001), and overexpression of GLUT4 in GLUT4+/− mice normalizes insulin sensitivity and glucose tolerance (Tsao, et al. 1999). It is evident that GLUT4 has a central role in insulin stimulated glucose uptake during normal physiologic conditions although the observed compensation for ablation of GLUT4 in GLUT4−/− mice is not yet explained.

2.5. Regulation of GLUT intrinsic activity

The least investigated area of glucose transporter biology is the regulation of putative changes in GLUT intrinsic activity, and little direct evidence of such a mechanism exists. However, there are a number of observations that cannot readily be explained by altering expression or translocation of GLUTs.

2.5.1. GLUT1

Changes in the intrinsic activity of GLUT1 have been proposed in a number of studies, and may account for observed increases in glucose transport. In general, this increase in glucose transport cannot be explained by GLUT1 expression or translocation. A majority of the studies has been carried out in erythrocytes, and only little is known about intrinsic activity changes in adipocyte and skeletal muscle tissue. Possible changes in intrinsic activity of GLUT1 protein in 3T3-L1 adipocytes has been observed after treatment with protein synthesis blockers, indicating this mechanism also could be present in this tissue (Harrison, et al. 1992).

2.5.1.1. ATP/nucleotides

GLUT1 has three predicted ATP-binding sites (domain I-III) at residues 111-118, 225-229, and 332-338 (Liu, et al. 2001), which are homologues to Walker ATP binding domains (Walker, et al. 1982). There are evidence indicating that domain I and III are true ATP binding sites, and that ATP binding there is important for GLUT1 mediated glucose transport (Liu, et al. 2001; Levine, et al. 1998). It is unclear if ATP exerts an effect directly on GLUT1 or in cooperation with extrinsic proteins.
2.5.1.2. Structural interaction of GLUT1

It has been observed that GLUT1 protein can form oligomeric structures, tetramers, and that disrupting this association inhibits glucose transport activity in erythrocytes (Zottola, et al. 1995). It is however not concluded whether this mechanism exists in adipocytes and skeletal muscle tissue.

Association of GLUT1 to lipid rafts has been observed, and there is evidence of an increase in localization to these plasma membrane domains during glucose deprivation, in 3T3-L1 adipocytes (Kumar, et al. 2004). This distribution of GLUT1 seems to be regulated by the N-terminal part of the protein, as a chimeric GLUT1 with the GLUT3 N-terminus is not associated to lipid rafts (Sakyo, et al. 2007). A redistribution of GLUT1 transporters from lipid raft domains in plasma membranes correlates with an increase in glucose transport without changing the number of transporters in the plasma membrane, suggesting a change in the intrinsic activity (Rubin and Ismail-Beigi 2003). This theory is also supported by the negative effect on GLUT1 mediated glucose transport by stomatin, a protein that is enriched in lipid raft domains (Zhang, et al. 1999; Zhang, et al. 2001). There is no direct evidence that this causes an alteration in GLUT1 transport activity, but it raises an interesting theory that these domains may be favorable for activation.

2.5.1.3 Other pathways regulating GLUT1 intrinsic activity

In brown adipocytes, there have been studies proposing a mechanism where norepinephrine acting on adrenergic receptors can increase glucose uptake by altering GLUT1 intrinsic activity (Shimizu, et al. 1996; Shimizu, et al. 1998) (Paper II). It is possible β-adrenergic receptors also regulate intrinsic activity of GLUT1 in skeletal muscle (Paper VI).

Furthermore, it has been proposed that activation of the serum- and glucocorticoid-inducible kinase SGK1 may be involved in altering GLUT1 intrinsic activity in insulin-sensitive tissues (Palmada, et al. 2006).

2.5.2. GLUT4

Although GLUT4 is translocated and regulates glucose uptake by an increase in of the number of transporters at the plasma membrane, studies suggest GLUT4 intrinsic activity can be a part of the increase of glucose uptake. Insulin-stimulated docking of GLUT4 vesicles to the plasma membrane precedes the increase in glucose uptake in L6 skeletal myocytes, suggesting there is a signal regulating GLUT4 intrinsic activity (Somwar, et al. 2001b). The mitogen-activated protein kinase (MAPK) p38 has been implicated in this effect, and using
inhibitors on p38 inhibits insulin-stimulated glucose uptake without affecting the translocation of GLUT4 (Hausdorff, et al. 1999; Somwar, et al. 2001a). Another possible event that could alter the intrinsic activity of GLUT4 is the direct nucleotide binding to the transporter (James, et al. 1989a; Lawrence, et al. 1990; Piper, et al. 1993)

3. ADRENERGIC SIGNALING

3.1 Introduction to the sympathetic nervous system

The sympathetic nerves are a part of the autonomic nervous system (ANS) and innervate many organs in the human body. Postganglionic neurons release the primary sympathetic neurotransmitter noradrenaline to accelerate heart beat, relax airways, stimulate glycogenolysis and glucose output from the liver, and stimulates secretion of the hormone adrenaline, but to some extent also noradrenaline, from the medulla of the adrenal gland. In mammals, sympathetic activation in response to cold activates brown adipose tissue (BAT) to produce heat and regulate body temperature. It is generally accepted that blood vessels in skeletal muscle are sympathetically innervated, but it has also been discovered that sympathetic nerves directly innervate skeletal muscle in mammals (Barker and Saito 1981).

3.2. Adrenergic receptors and signaling

3.2.1. Adrenergic receptors

The endogenous natural receptors for the catecholamines adrenaline and noradrenaline are adrenergic receptors that belong to the superfamily of seven-transmembrane G-protein coupled receptors (GPCRs). Adrenergic receptors mediate responses both centrally and peripherally, and are expressed many tissues in the human body. The first subdivision of adrenergic receptors was into α-adrenergic and β-adrenergic receptors based on pharmacological characteristics (Ahlquist 1948). Subsequently, further studies led to the identification of three pharmacologically distinct types (α1, α2, and β) (Figure 7), and currently there is 9 members of the family (Bylund, et al. 1994).

3.2.1.1. The α1-adrenergic receptors

Three subtypes of α1-adrenergic receptors are cloned and genetically defined, α1A, α1B, and α1D (Hieble, et al. 1995). They are expressed in many different tissues in humans, α1A is predominantly expressed in heart, brain, and prostate, α1B in spleen and kidney, and α1D in aorta (Garcia-Sainz, et al. 1999). The three α1-adrenergic receptor subtypes primarily couples
to G_{q/11} proteins to activate phospholipase C\(\beta\) (PLC\(\beta\)) (Wu, et al. 1992; Hubbard and Hepler 2006). PLC\(\beta\) catalyses the formation of inositol 1, 4, 5-triphosphate (IP\(_3\)) and diacylglycerol (DAG) that mediates many responses as second messengers. Increases in the levels of IP\(_3\) trigger release of Ca\(^{2+}\) from the endoplasmatic reticulum, and DAG activates protein kinase C (PKC). However, \(\alpha_1\)-adrenergic receptors can also couple to pertussis toxin sensitive G\(_i\) and G\(_o\) proteins, and G\(_s\) and G\(_{12/13}\) proteins, and activate phospholipase A\(_2\) (PLA\(_2\)) and phospholipase D (PLD) (Hein and Michel 2007). The \(\alpha_1\)-adrenergic receptor subtypes display selective signaling which has been observed as differences in the activation of PLC (Zhong, et al. 2001; Richardson, et al. 2003; Taguchi, et al. 1998).

3.2.1.2. The \(\alpha_2\)-adrenergic receptors

Three subtypes of \(\alpha_2\)-adrenergic receptors have been cloned and defined, \(\alpha_2A\), \(\alpha_2B\), and \(\alpha_2C\). Noradrenaline and adrenaline target these receptors which couples to G\(_i/G_o\) proteins and signaling pathways that inhibit adenylyl cyclase (AC) and cAMP production, activate receptor-operated K\(^+\)-channels, and inhibit voltage-gated Ca\(^{2+}\)-channels (Limbird 1988). The negative coupling of \(\alpha_2\)-adrenergic receptors seems to be important in a short negative feedback to inhibit adrenaline release from the chromaffin cells of the adrenal medulla and noradrenaline from sympathetic nerves (Brede, et al. 2003).

![Figure 7. Conventional adrenergic receptor signaling](image-url)
3.2.1.3. The β-adrenergic receptors

Three subtypes of β-adrenergic receptors are identified, β₁, β₂ and β₃. They are differentially expressed in many tissues, where β₁-adrenergic receptors are highly expressed in heart and brain, β₂-adrenergic receptors are expressed in uterus, skeletal muscle, and lungs, and β₃-adrenergic receptors are expressed at high levels in adipose tissues and gall bladder (Dixon, et al. 1986; Frielle, et al. 1987; Emorine, et al. 1989; Strosberg 1997). It has been known since the 80’s that β-adrenergic receptors primarily couple to stimulatory Ga proteins (Gαs), causing an activation of adenyl cyclase and production of cAMP (Gilman 1987).

In contrast to α₁, and α₂, β-adrenergic receptors display a difference in the pharmacological response to their natural agonists. In several model systems, adrenaline has a greater effect on β₂-adrenergic receptors, and is less potent on β₁ and β₃, while noradrenaline is most effective on β₃-adrenergic receptors, and has less effect on β₁ and β₂ (Bylund, et al. 1994; Strosberg 1997).

The existence of cAMP and its function as a classical intracellular “second messenger” (neurotransmitters and hormones being “first transmitters”) to catecholamines was pioneered by E. W. Sutherland during the late 50’s and 60’s, and for these discoveries he was awarded the Nobel Prize in 1971 {{189 RALL,T.W. 1958;190 Sutherland,E.W. 1966;}}. cAMP triggers a plethora of effects downstream of the GPCR/G-protein/AC pathway, and discussing all of them is beyond the scope of this thesis. Rising levels of cAMP in response to AC activation initiates signaling by ion channels, cAMP-dependant protein kinase A (PKA), and exchange proteins directly activated by cAMP (Epac) (Beavo and Brunton 2002). PKA phosphorylates cAMP-response element binding protein (CREB) that binds to CRE sites in gene promoters, initiating transcription of many target genes (Sands and Palmer 2008). Recently, the complexity of cAMP signaling has become increasingly evident as cAMP concentration is varying throughout the cell, and occurs compartmentalized. This can be correlated to the localization of proteins involved in cAMP signaling and regulation, as AC, phosphodiesterases (hydrolyzes cAMP to AMP), and PKA (Zaccolo, et al. 2006; Lynch, et al. 2007).

3.2.2. Desensitization and atypical signaling

During the 1980s it was made clear that the β₂-adrenergic receptor was phosphorylated following stimulus, and this appeared to be related to the strictly controlled mechanism of receptor desensitization (Lefkowitz 2004)(Figure 8). The proteins that mediate the
phosphorylation of the β2-adrenergic receptor were identified as PKA and β-adrenoceptor kinase (βARK), later renamed G-protein-coupled receptor kinase 2 (GRK2) (Benovic, et al. 1985; Benovic, et al. 1986). β-arrestin1 and β-arrestin2 was also identified as cofactors in the mechanism of desensitization (Lohse, et al. 1992; Attramadal, et al. 1992). Studies on visual arrestins (arrestins that are recruited to the GPCR rhodopsin in rod and cone photoreceptors) show that phosphorylation and the ligand induced conformational change of the receptor recruits β-arrestins, changing the β-arrestin to an active state (Gurevich and Benovic 1992; Hirsch, et al. 1999). Only phosphorylation on the c-terminal tail of the β2-adrenergic receptor by GRK2, and not PKA, recruits β-arrestin (Lohse, et al. 1992), but both GRK2 and PKA mediated phosphorylation cause desensitization (Roth, et al. 1991). Desensitization of the agonist induced response is in part due to β-arrestins ability to block or uncouple the G-protein dependent response from the β-adrenergic receptor (Krupnick and Benovic 1998), but also involve internalization. It was observed before the discovery of GRKs and arrestins that β2-adrenergic receptors were rapidly internalized into the cytosol of frog erythrocytes when exposed to the agonist isoproterenol (Chuang and Costa 1979). Internalized β2-adrenergic receptors are detected after only 2 minutes of agonist exposure and co-localize with the the transferrin receptor (TrR), which is an endosomal marker (von Zastrow and Kobilka 1992). Activated β-arrestins also recruits phosphodiesterases (PDEs), enzymes with an activity for hydrolyzing cAMP (Perry, et al. 2002), and this also seems to cause the β2-adrenergic

Figure 8. The desensitization mechanism of β-adrenergic receptors

33
receptor to switch from coupling to Gs to Gi (Baillie, et al. 2003). The increased degradation of cAMP and G-protein subtype switching also diminishes the agonist response, displaying the several different actions of β-arrestins in β-adrenergic receptor desensitization.

The similarity of β₁ and β₂-adrenergic receptor C-terminal tails correlates with the mechanism of desensitization as GRK2 and PKA phosphorylation, and β-arrestin recruitment, also occurs at the β₁-adrenergic receptor (Freedman, et al. 1995). The β₃-adrenergic receptor on the other hand, has fewer sites for phosphorylation by GRK or PKA (Strosberg 1997; Liggett, et al. 1993).

Recently, β-arrestins was found not only a central protein in receptor desensitization but also gained a new role as signal transducer. The atypical signaling of seven transmembrane g-protein coupled receptors through β-arrestins implicates a growing number of proteins (reviewed in (Lefkowitz and Shenoy 2005)).

### 3.2.3. Phosphoinositide 3-kinases (PI3Ks) and PI3K related kinases (PIKKs)

An important participant in a growing number of signaling pathways, and possibly atypical GPCR signaling, are the family of phosphoinositide 3-kinases (PI3Ks) that phosphorylate inositol phospholipids at the 3’-OH position of the inositol ring. They are tightly bound heterodimers of a regulatory and a catalytic subunit or monomers and are divided into three classes based on homology, where the major focus has been on class I PI3Ks, and less is known about class II-III PI3Ks (Vanhaesebroeck, et al. 1997; Hawkins, et al. 2006)(Table 2).

<table>
<thead>
<tr>
<th>Class</th>
<th>Regulatory subunit</th>
<th>Catalytic subunit</th>
</tr>
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<tbody>
<tr>
<td>Class IA PI3K</td>
<td>p85α</td>
<td>p110α</td>
</tr>
<tr>
<td></td>
<td>p55α</td>
<td>p100β</td>
</tr>
<tr>
<td></td>
<td>p50α</td>
<td>p110δ</td>
</tr>
<tr>
<td>Class IB PI3K</td>
<td>p101</td>
<td>P110γ</td>
</tr>
<tr>
<td></td>
<td>p84</td>
<td></td>
</tr>
<tr>
<td>Class II PI3K</td>
<td>C2α</td>
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<tr>
<td></td>
<td>C2β</td>
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<tr>
<td></td>
<td>C2γ</td>
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</tr>
<tr>
<td>Class III PI3K</td>
<td>p150</td>
<td>hVPS34</td>
</tr>
</tbody>
</table>

Table 2. The regulatory and catalytic subunits of the three classes of PI3Ks
Classification of PI3Ks is generally based on the catalytic subunit present in the heterodimer (e.g. p85α-p110α heterodimer is referred to as PI3Kα), since there is limited data of the specific function of the different regulatory subunits. There are four members of Class I PI3K catalytic subunits and they are divided into class IA (p110α, p110β, p110δ) and class IB (p110γ), with class IA being primarily activated by tyrosine kinases, and class IB primarily activated by GPCRs. Subunits p110α and β are ubiquitously expressed, while subunits p110γ and δ expression are primarily restricted to cells of myeloid origin in the hematopoietic system. This is supported as transgenic mice lacking p110α or p110β are embryonic lethal, while mice lacking p110γ and p110δ subunits survive, but has defects in leukocyte, lymphocyte, and mast cell function (Vanhaesebroeck, et al. 2005). Class IA PI3Ks has an important role in insulin signaling, and inhibitors of the PI3K-p110 as the potent inhibitor wortmannin and the reversible p110 subunit ATP pocket binding inhibitor LY294002 has extensively been used to probe this (Srivastava 1998). However, these inhibitors are pan-specific and inhibit other kinases in the PI3K family, members of the PI3K related kinase (PIKK) family, and some unrelated kinases. Recently, more specific small cell permeable inhibitors were developed from molecular modeling of the interaction of LY294002 with PI3K-p110 isofoms and high throughput screening, and such inhibitors has high potential for drug therapy. Several such inhibitors were used in paper II and paper IV and in vitro IC50 values (inhibitory concentration 50%) from lipid kinase assays are summarized in table 3. Such approaches have yielded evidence for p110α as the primary isoform involved in insulin signaling (Knight, et al. 2006). As a GPCR is activated and the classical Ga signaling occurs, the βγ subunit of the G-protein is also able to mediate signaling (atypical signaling). Observations suggest Gβγ can activate PI3Kβ (Kurosu, et al. 1997). However, the primary Class I PI3K isoform implicated in GPCR signaling through the G-protein βγ subunit is the PI3Kγ, the only class 1b member (Stoyanov, et al. 1995; Stephens, et al. 1997). Recently, it was suggested that PI3Kβ indeed signals downstream of GPCRs, being functionally redundant with the GPCR activated PI3Kγ (Guillermet-Guibert, et al. 2008). The PIKKs are a family of six proteins, mammalian target of rapamycin (mTOR), ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3 related (ATR), suppressor of morphogenesis in genitalia (SMG-1), DNA protein kinase catalytic subunit (DNA-PKcs), and transformation/transcription domain associated protein (TRRAP). All PIKKs but TRRAP are identified as serine/threonine protein kinases, sharing sequence similarities with PI3Ks, and
have established themselves as stress response proteins primarily related to DNA and RNA damage, but also growth and proliferation (Bakkenist and Kastan 2004; Abraham 2004).

<table>
<thead>
<tr>
<th>PI3Ks</th>
<th>LY294002</th>
<th>C15e (0.58)</th>
<th>TGX221 (0.02)</th>
<th>AS605240</th>
<th>PI-103 (~0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p110α</td>
<td>0.63-9.3</td>
<td>0.0020</td>
<td>5</td>
<td>0.06</td>
<td>0.002-0.008</td>
</tr>
<tr>
<td>p110β</td>
<td>0.31-2.9</td>
<td>0.016</td>
<td>0.005</td>
<td>0.27</td>
<td>0.003-0.088</td>
</tr>
<tr>
<td>p110δ</td>
<td>1.33-6.0</td>
<td>0.66</td>
<td>0.1</td>
<td>0.3</td>
<td>0.003-0.048</td>
</tr>
<tr>
<td>P110γ</td>
<td>7.26-38</td>
<td>&gt;10</td>
<td>0.008(0.09)</td>
<td>0.015-0.15</td>
<td></td>
</tr>
<tr>
<td>PI3KC2α</td>
<td>15-100</td>
<td>&gt;10</td>
<td></td>
<td>~1</td>
<td></td>
</tr>
<tr>
<td>PI3KC2β</td>
<td>2.1-30</td>
<td>0.22</td>
<td></td>
<td>0.026-0.043</td>
<td></td>
</tr>
<tr>
<td>PI3KC2γ</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsVPS34</td>
<td></td>
<td></td>
<td></td>
<td>2.3-3.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. IC50 values of inhibitors for PI3K and PIKK family members as determined in in vitro assays. Values are compiled from several studies and show a range when different values were reported (Knight, et al. 2006; Raynaud, et al. 2007; Jackson, et al. 2005; Camps, et al. 2005; Hayakawa, et al. 2006; Knight, et al. 2004). Values in parenthesis are reported IC50 in cellular assays and grey color indicates the primary target of inhibitor.

The family of PI3Ks seems to be a central participant in many pathways, as tyrosine kinase receptor and GPCR signaling, even though the roles of the different isoforms are constantly changing. The evident involvement in GPCR signaling suggests PI3Ks may be a participant in the β-adrenergic pathway to glucose uptake, perhaps through atypical signaling.

4. β-ADRENERGIC REGULATION OF GLUCOSE TRANSPORT

4.1. Introduction

Focally released norepinephrine from sympathetic nerves and epinephrine released from the adrenal medulla affect glucose transport and metabolism in liver, brown adipose tissue, white adipose tissue, and skeletal muscle (Nonogaki 2000; Philipson 2002). Administering β-agonists to humans are likely to cause hyperglycemia due to the acute effect on liver, resulting
in a net output of glucose from the liver to blood (Philipson 2002). However, there are several studies concerning effects of β-agonists or sympathetic activation on fat and muscle that are primary tissues for clearing glucose from the blood, and main target for insulin stimulated glucose uptake. Activation of the ventromedial hypothalamic nucleus (VMH) cause an increased peripheral sympathetic nerve activity and increase glucose uptake in an insulin independent way in brown adipocytes, heart, and skeletal muscle, but not in white adipose tissue or brain (Sudo, et al. 1991).

4.2. In brown adipose tissue

Brown adipose tissue is the organ responsible for non-shivering thermogenesis by uncoupling mitochondrial respiration, through a mechanism mediated by uncoupling protein 1 (UCP1) (Cannon and Nedergaard 2004). It is metabolically active, highly vascularized, rich in mitochondria, and under direct hypothalamic control via sympathetic nerves. In response to cold exposure, norepinephrine from sympathetic nerve endings activates BAT to increase metabolism and produce heat. BAT is primarily an organ in rodents, hibernating animals, and neonatal humans, but there are novel theories of it being present in adult humans (Nedergaard, et al. 2007).

Glucose uptake in BAT is markedly increased by both norepinephrine and insulin (Marette and Bukowiecki 1989; Liu, et al. 1994). Extensive studies of insulin signaling in skeletal muscle and white adipocytes show that insulin translocates GLUT4 vesicles to the plasma membrane to increase the influx of glucose. This mechanism is also displayed by brown adipocytes (Slot, et al. 1991; Omatsu-Kanbe, et al. 1996). Furthermore, crucial components of insulin signaling in skeletal muscle and white adipocytes are important for insulin stimulated uptake in brown adipocytes. Insulin stimulated glucose uptake is impaired in immortalized brown adipocytes from IRS2-KO mice, and retroviral re-expression of IRS2 partially restores the response (Fasshauer, et al. 2000). Similar approaches with a Cre-LoxP system abolishing PDK1 or using brown adipocytes from AKT2-KO mice implies importance of PDK1 and AKT2 (Sakaue, et al. 2003; Bae, et al. 2003). Insulin signaling in brown adipocytes seems to be mediated by the PI3K p110α catalytic subunit causing phosphorylation on threonine 308 and serine 473 of PKB/Akt (Paper II), as seen in skeletal myotubes and white adipocytes (Knight, et al. 2006). In many ways, brown adipose tissue display all the characteristics and signaling steps of an insulin sensitive tissue, such as white adipose and skeletal muscle tissue. Exposing rats to cold stimulates a dramatic increase of glucose uptake into brown adipose
tissue (Vallerand, et al. 1990; Vallerand, et al. 1990; Greco-Perotto, et al. 1987). This tissue has an exceptionally high uptake of glucose per tissue gram in response to cold exposure or norepinephrine in rats, which reinforces significance in regulation of blood glucose homeostasis (Liu, et al. 1994; Shibata, et al. 1989). Brown adipocytes express α1, α2, β1 and β3-adrenergic receptors (Lafontan and Berlan 1993). The effect of norepinephrine on this tissue is mainly mediated by the β3-adrenergic receptor, although α1-adrenergic receptors may be of some importance (Zhao, et al. 1997). Norepinephrine-stimulated glucose uptake is mediated by the β3-adrenergic receptor subtype in brown adipocytes, but α1- and β1-adrenergic receptors can compensate for β3-adrenergic receptor deficiency in β3-KO models (Chernogubova, et al. 2004; Chernogubova, et al. 2005). The response is mimicked by stimulation with 8-Br cAMP (cAMP analog) and diminished by 4-cyano-3-methylisouquinoline (4CM, PKA inhibitor), which indicates the involvement of classical β-adrenergic signaling through Gsα, cAMP and PKA. The observed sensitivity of β-adrenergically and 8-Br-cAMP stimulated glucose uptake to PI3K inhibitor LY294002 suggests the involvement of PI3Ks or related kinases. An interpretation of this observation could be that β3-adrenergic and insulin signaling pathways may converge at the level of PI3K, with GLUT4 translocation possibly being responsible for both increases in glucose uptake. In white adipocytes, β3-adrenergic agonists inhibit insulin stimulated glucose uptake, but this is not observed in brown adipocytes (Carpene, et al. 1993). To further elucidate the mechanism

![Figure 9. Glucose uptake in response to the adrenergic natural ligand norepinephrine and insulin during 0-8 hours of stimulation, in brown adipocyte primary cultures.](image-url)
of norepinephrine stimulated glucose uptake in brown adipocytes and address the question of convergence between insulin and β3-adrenergic signaling, I studied the effects of norepinephrine on GLUT1 and GLUT4 in brown adipocytes (Paper I). Initially we observed a dramatic effect of norepinephrine on GLUT1 and GLUT4 mRNA transcription, stimulating a potent 8-fold increase of GLUT1 and a surprising 2-fold decrease of GLUT4 mRNA levels. The effect of norepinephrine GLUT expression is mediated by the classical β3-adrenergic signaling pathway with the second messenger cAMP. Fractionation and analysis of protein levels of primary brown adipocytes after norepinephrine stimulation for 0, 2 and 5 h show an increase of GLUT1 protein in the plasma membrane fraction and low density microsomes, which is consistent with a de novo synthesis of GLUT1. The dramatic increase of GLUT1 mRNA and protein corresponds to a very high glucose uptake at 5 hours that exceeds insulin stimulated uptake in primary brown adipocytes (Figure 9). Interestingly, when we investigated the transcriptional influence of GLUT1 on norepinephrine stimulated glucose uptake, actinomycin D did not completely block the response. Our results indicate the existence of two separate mechanisms of norepinephrine stimulated glucose uptake, a faster actinomycin D insensitive mechanism and a slower actinomycin sensitive mechanism. The second mechanism is actinomycin sensitive due to the de novo synthesis of GLUT1, but the first mechanism is still elusive. The first mechanism may be due to a translocation of another GLUT or a change in intrinsic activity of a GLUT. We find no evidence that GLUT4 translocation occurs, but changes in GLUT1 intrinsic activity in response to norepinephrine stimulation has been proposed earlier (Shimizu, et al. 1996; Shimizu, et al. 1998). To keep brown adipose tissue primaries viable 4 nM insulin is added to the culture media and this is sufficient to promote some GLUT4 translocation. Norepinephrine stimulation seems to antagonize insulin signaling and cause retention of GLUT4 protein from the plasma membrane to an intracellular location (Figure 10). This observation further supports the

![Figure 10. Subcellular distribution of GLUT4 and GLUT1 with norepinephrine-stimulation. Isolated brown adipocytes stimulated with 0.1 μM norepinephrine for 0, 2, or 5 hours. 10 μg of protein from plasma membrane (PM) or low-density microsome (LDM) fractions was subjected to SDS-PAGE and immunoblotting with GLUT4, GLUT1, β3-AR, or ERK 1/2 antibodies.](image)
apparent differences between insulin and β-adrenergic signaling to glucose uptake.

Insulin and β-adrenergic signaling do not converge to regulate glucose transporters in a similar way. However, enigmatically not only insulin, but also β-adrenergically stimulated glucose uptake is inhibited by PI3K inhibitors LY294002 and wortmannin. The possible involvement of PI3K family members indicated by the ability of these PI3K inhibitors to inhibit β-adrenergic glucose uptake could be attributed to an unspecific effect. This notion seems unlikely as the very potent and specific inhibitor PI-103, completely inhibits both insulin and β-adrenergic signaling in brown adipocyte primary cultures (Paper II). PI-103 selectively targets PI3K-P110α, PI3Kβ, PI3K related kinases (PIKKs) rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 mTOR complexes, and DNA protein kinase (DNA-PK) (Knight, et al. 2006). Its high selectivity is indicated by the absence of activity toward a panel of 70 protein kinases (Raynaud, et al. 2007). Furthermore, the specific mTORC1 inhibitor rapamycin does not affect insulin or β-adrenergically stimulated glucose uptake. Class I PI3K activation seems to universally cause phosphorylation of Akt t308 and s473. Intriguingly, 2 hours stimulation with isoproterenol induced a significant phosphorylation of Akt residue s473, but not t308. Several studies suggest both DNA-PK and mTORC2 specifically phosphorylates Akt on this residue (Hresko and Mueckler 2005; Sarbassov, et al. 2005; Bozulic, et al. 2008; Feng, et al. 2004).

In conclusion, β-adrenergic stimulation causes a potent increase of glucose uptake in brown adipocytes consisting of two mechanisms. Mechanism 1 is not due to transcriptional activation or translocation of GLUT1 or GLUT4, and mechanism 2 is through a de novo synthesis of GLUT1. It also seems unlikely that β-adrenergic and insulin signaling converge, but seem to be completely separate signaling pathways. Our data suggest the involvement of a PI3K related kinase.

4.2.1. AMP-activated protein kinase (AMPK)

AMP-activated protein kinase (AMPK) is considered to be an energy sensor, responding to changes in cellular energy levels. Although ubiquitously found, its high expression levels in metabolically important tissues as skeletal muscle, adipose tissue, liver, heart, pancreas, and brain, indicates its importance in energy homeostasis. Low levels of energy activate AMPK to increase glucose uptake, feeding, fatty acid oxidation, and inhibit lipogenesis, all processes increasing available energy and restoring homeostasis. AMPK consists of a catalytic α subunit, and regulatory β and γ subunits, and is allosterically activated by AMP by
phosphorylation on threonine 172, when energy levels are decreasing in the cell (Kahn, et al. 2005). Recently, it has become appreciated that AMPK can be activated by GPCRs, including β-adrenergic receptors (Hutchinson, et al. 2008). The β-adrenergic agonist isoproterenol stimulates AMPK activity in adipocytes (Moule and Denton 1998; Yin, et al. 2003), and it has been proposed that in brown adipocytes, serial activation of UCP1 and AMPK is necessary for glucose utilization (Inokuma, et al. 2005). However, we show that noradrenaline, acting via β-adrenergic receptors, increases AMPK phosphorylation independently of UCP1 expression and function (Paper III). The activation of AMPK is partially responsible for the β-adrenergically stimulated glucose uptake in brown adipocytes.

4.3. In white adipose tissue

White adipose tissue (WAT) is the main organ for fuel storage when energy intake exceeds energy expenditure in mammals. Fatty acids can be synthesized or taken up, converted to triacylglycerol, and stored in lipid droplets. It is also a target for postprandial increases in glucose uptake in response to insulin stimulation. BAT is more innervated by sympathetic nerves than WAT, though SNS is presumed to have a significant role in WAT as well (Slavin and Ballard 1978; Fliers, et al. 2003; Garofalo, et al. 1996). The SNS acts directly via norepinephrine on β-adrenergic receptors in WAT (for a recent review, see (Collins, et al. 2004). Fasting or sustained physical activity can trigger sympathetic activation in WAT, promoting catabolic processes and fuel mobilization through increased lipolysis and triglyceride release, but lipolysis during fasting is not exclusively regulated through β-adrenergic receptors (Jimenez, et al. 2002). There is a clear role for the SNS as a regulator of lipolysis, and a more elusive role in the regulation of glucose transport in WAT. Infusion of norepinephrine for 10-12 days increase glucose uptake independently of insulin, and chronic administration of the selective β3-agonist CL-316243 seems to increase both basal and insulin-stimulated glucose uptake in WAT (Liu, et al. 1994; de Souza, et al. 1997; Liu, et al. 1998). However, most evidence indicates an antagonistic effect of acute β3-adrenergic receptor activation on insulin signaling. β-adrenergic agonists inhibit insulin stimulated glucose uptake and GLUT4 translocation in rat adipocytes and 3T3-L1 adipocytes via the β3-adrenergic receptor (Carpene, et al. 1993; Ohsaka, et al. 1997; Mulder, et al. 2005), an observation we also have seen in mouse mature white adipocytes (Nevzorova, unpublished observations). The inhibition of insulin signaling has also been shown in human primary cultures of mammary adipocytes, although insulin stimulated glucose uptake was not altered (Jost, et al. 2005). In conclusion, the effects of the sympathetic nervous system on glucose
transport in WAT are primarily mediated by the β3-adrenergic receptor subtype. Longer exposures to norepinephrine or β-adrenergic agonists have positive effects on glucose uptake, and shorter exposures does not increase glucose uptake. In fact, it is possible that acute effects of the sympathetic nervous system negatively influences insulin signaling and glucose uptake.

4.4. In skeletal muscle

It seems evident the sympathetic nervous system can regulate glucose transport in skeletal muscle in an insulin-independent way. Cold acclimation can increase glucose uptake into skeletal muscle without shivering thermogenesis or contractile activity, suggesting involvement of sympathetic nerves (Vallerand, et al. 1990). Central neurochemical stimulation or electrical stimulation of the VMH in rats increase glucose uptake in skeletal muscle without elevating plasma insulin concentrations (Sudo, et al. 1991; Minokoshi, et al. 1994; Lang, et al. 1995). This effect is abolished by the norepinephrine-release inhibitor guanethidine, but not by adrenal demedullation, thus indicating that the response is mediated by norepinephrine and not circulating epinephrine (Minokoshi, et al. 1994). Furthermore, administering the β2-AR agonist clenbuterol improves glucose tolerance due to increased glucose uptake in skeletal muscle (Castle, et al. 2001; Pan, et al. 2001). This may partially be explained as clenbuterol has a hypertrophic effect on skeletal muscle and an increase in the actual mass will correspond to the increased glucose uptake (Yang and McElligott 1989).

Most studies on glucose transport effects with β-agonists in rodents are long term, and do not investigate possible acute short term effects.

Skeletal muscle express high levels of the β2-adrenergic receptor subtype and low levels of the β1-adrenergic receptor subtype, and the effects of endogenous catecholamines as well as adrenergic agonists are primarily mediated by the β2-adrenergic receptor (Liggett, et al. 1988; Roberts and Summers 1998; Nevzorova, et al. 2002). A significant body of evidence now suggests the existence of muscle glucose uptake in response to sympathetic nerve activation and focal noradrenaline release, and this is mediated by the β2-adrenergic receptor. The β2-adrenergic receptors in skeletal muscle mediates the conventional β-adrenergic signaling, activation of adenylyl cyclase, production of cAMP and activation of PKA, resulting in many metabolic effects. Selective β-adrenergic agonists and 8-Br-cAMP increase glucose uptake in L6 myotubes, but only the former is inhibited by wortmannin and LY294002 (Nevzorova, et al. 2002; Nevzorova, et al. 2006). These compounds are inhibitors of the PI3K family, suggesting there is an atypical activation of PI3K in the β-adrenergic response, not mediated by cAMP. Investigating this possibility with more specific PI3K-P110 catalytic subunit
inhibitors, we believe class I PI3Ks are not involved in β-adrenergically stimulated glucose uptake (paper IV). Although there seems to be distinct differences between insulin and β2-adrenergically stimulated glucose uptake in L6 myotubes, we also investigated the regulation of glucose transport by the β2-adrenergic receptor in CHO cells stably expressing GLUT4myc, and transiently transfected with β2-adrenergic receptors (Paper V). In this artificial system, β2-adrenergic receptor activation seems to cause GLUT4 translocation and increase of glucose uptake, possibly by atypical signaling through the desensitization mechanism.

Interestingly, the dual mTOR/PI3K inhibitor PI-103 abolishes not only glucose uptake in response to insulin, but also in response to the β-adrenergic agonist isoproterenol in L6 myotubes. The inhibitory profile of PI-103 suggest the involvement of mTOR1C, mTOR2C, PI3KC2β, or DNA-PK. Pretreatment with the mTOR1C inhibitor rapamycin fails to inhibit glucose uptake in response to isoproterenol indicating mTOR1C is not a factor in the β-adrenergic signaling. Class II PI3KC2β is not a plausible candidate to explain the LY294002 inhibition of β-adrenergically stimulated glucose uptake, as it is relatively insensitive to this inhibitor, with reported IC50 values of up to 30 µM (Falasca and Maffucci 2007). These results suggest that a distinct member of the PIKK family is crucial to β2-adrenergically stimulated glucose uptake.

5. SUMMARY AND CONCLUSIONS

β-adrenergic receptors are among the most ubiquitously expressed receptors, responsible for mediating the effects of the sympathetic nervous system in many tissues, focally as well as to circulating catecholamines. They are important in regulating vital functions in the body, heartbeat and blood flow, dilation of airways, and mobilizing energy from liver and adipose tissues. Although their existence was shown more than 50 years ago, there are constantly new discoveries changing the way we consider β-adrenergic receptor regulation and signaling. It has also been known for quite some time, that β-adrenergic stimulation can regulate glucose uptake in target tissues, but there is not much known about the signaling and regulation in this respect.

In this thesis, I have reviewed and compiled an overlook of the field of glucose transporters and β-adrenergic receptors, and how they are connected. I (and my co-authors) show that β-adrenergic receptor stimulation activates distinct signaling pathways, regulating glucose transporters mechanisms, ultimately altering glucose transport into cells. We are also able to show that this occurs in insulin sensitive tissues, but separately from insulin signaling.
Paper I describes the mechanism behind norepinephrine's potent effect on glucose uptake in brown adipocytes. We show that norepinephrine acting on β3-adrenergic receptors induces a markedly increased glucose uptake in brown adipocyte primary cultures, which can be separated into two distinct mechanisms. First, an acute mechanism that is resistant to actinomycin D (transcriptional blocker), and is not due to GLUT4 or GLUT1 translocation. Secondly, there is a slower actinomycin D sensitive mechanism caused by transcriptional activation and protein synthesis of GLUT1. Furthermore, we also conclude that β-adrenergically and insulin stimulated glucose uptake are produced by completely different mechanisms, and that β-adrenergic stimulation in fact antagonizes insulin dependent GLUT4 translocation.

Paper II concerns the previously observed sensitivity of β-adrenergically stimulated glucose uptake to PI3K inhibitors, and if this is correlated with the different signaling properties of the two β-adrenergic receptor subtypes (β1- and β3-adrenergic receptors) expressed in brown adipocytes. We show that although β3- and β3-adrenergic receptors differ in the production and desensitization of cAMP, glucose uptake stimulated by both receptor subtypes is sensitive to the pan-specific (does not discriminate between isoforms) PI3K family inhibitor LY294002. As the mechanism of desensitization is involved in mediating atypical signaling, and the β1- but probably not the β3-adrenergic receptor recruits this mechanism, we conclude that this type of atypical signaling from β-adrenergic receptors is not involved in mediating glucose uptake. By investigating the sensitivity of β-adrenergically stimulated glucose uptake to LY294002 with several PI3K isoform selective inhibitors, we show that insulin activated class I PI3Ks are not involved in β-adrenergically stimulated glucose uptake, but is most likely dependent on a PIKK.

Paper III shows that noradrenaline acts on β3-adrenergic receptors to activate AMPK, independently of UCP1 expression and function in brown adipocytes. This activation partially contributes to β-adrenergically stimulated glucose uptake, and is likely mediated by cAMP.

Paper IV reveals the differences between insulin and β-adrenergic signaling to glucose uptake in L6 myotubes, and identifies possible candidates in the family of PI3K related kinases (PIKKs) crucial to β-adrenergically stimulated glucose uptake. By using PI3K isoform selective inhibitors, we conclude that class I PI3Ks does not participate in β-adrenergically stimulated glucose uptake, and does not explain the high sensitivity of β-adrenergically stimulated glucose uptake to PI3K inhibitor LY294002. However, we show that a novel inhibitor with a highly selective profile for class I PI3Ks, and members of the PIKK family, inhibits both insulin and β-adrenergically stimulated glucose uptake. We
conclude that the increase of glucose uptake in response to β2-adrenergic receptor stimulation is dependent on a member of the PIKK family, in L6 myotubes.

Paper V deal with the mechanism of β-adrenergically stimulated glucose uptake in an artificially constructed system, and investigate the importance of the C-terminal tail of β-adrenergic receptors in this response C-terminal. In this constructed system, consisting of Chinese hamster ovary (CHO) cells stably expressing GLUT4myc, and transiently transfected with the β2-receptor, not only insulin, but also isoproterenol stimulation is able to induce GLUT4 translocation resulting in an increase in glucose uptake. Transfecting these cells with β2-receptors with a truncated C-terminal tail abolishes the β-adrenergically stimulated glucose uptake, without affecting production of cAMP. This indicates that the C-terminal part of the receptor is important for the glucose uptake observed in this system, possibly due to atypical signaling.

Paper VI investigates the possibility that β-adrenergic receptors can modulate the intrinsic activity of GLUT1 in L6 myotubes. Adopting a new approach of introducing cell penetrating peptides that are homologues to parts of the intracellular sequences of GLUT1 to compete for proteins that interact with these sequences, reveal the possibility that a β-adrenergic signaling pathway may be able not only to increase GLUT1 intrinsic activity as suggested in brown adipocytes, but also decrease it. We show that introducing a cell penetrating peptide homologues to a specific sequence in the large intracellular loop of GLUT1 augments β-adrenergically stimulate glucose uptake, suggesting such a mechanism exists in L6 myotubes.

In conclusion of these studies, it is evident that β-adrenergic receptors are able to regulate glucose transporters in insulin sensitive tissues, such as skeletal muscle and adipose tissues. This is achieved in distinct mechanisms and separate from insulin signaling. Understanding the complex events in this physiological response could further our knowledge of β-adrenergic signaling and the regulation of glucose transporters, which may provide valuable information in trying to understand diseases such as diabetes mellitus.
6. ACKNOWLEDGEMENTS

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May the strange canned fish you always bring, and the vodka be plenty.

Julia Nevzorova, we had a lot of fun! You don’t really hold your liquor well, compared to other Russians. Maybe that’s what you would expect when you have a gr

Daniel Yamamoto, my lab-bench neighbor with only black clothes, and party friend. We had lots of fun (and consumed a lot of drinks!), and I have the scars to show for it. I will miss the pointless practical pranks and dirty jokes. Good luck with your research in Italy!

Robert Csikasz, the true connoisseur that does not really settle for second best. You will shoulder the responsibility of being the most experienced PhD student in our group. I’m sure you will cope with this task well, with some help from extremely rare and expensive beers and “tryffelolja”. When will we ever attempt the “lion-club”(24 beers in 6 hours)?

Anette “Nettan” Östberg, the “new kid on the block”. I trust you will shoulder the important GLUT research with grace, and most importantly, continue the “kiss-labben” saga. I know you will do well, but please, don’t forget to breathe when you are laughing.

Claudia Böhme, our visiting student from the alps! No worries, your glucose uptake experiments will soon be publishing quality (or I might help you).

All the people at physiology

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7. REFERENCES


