Chapter 1

General Introduction
Preface

Diabetes is taking on epidemic proportions not only in western society. According to the World Health Organization more than 180 million cases of diabetes have been reported and as an estimate for the coming years, the number of people with diabetes will be over 375 million by the year 2030. A deeper understanding of diabetes is thus required in order to device better treatment strategies or to even prevent the onset of diabetes. In this general introduction, an overview is provided explaining how normal glucose homeostasis is accomplished through the metabolic action of insulin and how deregulation of insulin action can result in insulin resistance and diabetes. Special attention will be focused on signal transduction of the insulin signalling pathway and protein kinase B.
**Glucose homeostasis**

All living cells use glucose as main or only source of energy. Maintenance of physiological glucose levels is thus essential for proper energy homeostasis. Breakdown of glucose via glycolysis to acetyl groups yields ATP. Subsequently, the remaining acetyl group is degraded by the Krebs cycle to CO₂ and NADH and FADH₂. Oxidation of the latter components by the mitochondrial respiratory chain yields additional ATP. Normal plasma glucose levels are maintained at 5 mM in the fasting state in an average adult human being. Regulation of glucose levels is tightly regulated by glucose itself and through stimulation of insulin secretion. It was in 1921 that Canadian scientists and Nobel price winners Frederick G. Banting and Charles H. Best successfully purified insulin from the pancreas and were able to treat a diabetic dog whose pancreas was removed.

![Diagram of proinsulin precursor molecule](image)

*Figure 1. Overview of the human proinsulin precursor molecule. The A- and B-chain are linked together by disulfide bonds after removal of the C-peptide and signal peptide.*

The peptide hormone insulin (5.8 kDa) is produced in the islet of Langerhans by the β-cells. The name for insulin is derived from the Latin word “insula” which means island. Insulin is synthesized from the proinsulin precursor molecule (Fig. 1). After removal of signal peptide and the C-peptide, the A- and B-chain are linked together by disulfide bonds. In general insulin induces an anabolic effect in cells and tissues. More specifically the effects of insulin include glucose uptake by muscle and adipose tissue, decrease of hepatic glucose production (gluconeogenesis), stimulation of cell growth and differentiation, storage of substrates in adipose tissue, liver and muscle (lipogenesis, glycogen and protein synthesis,
inhibition of lipolysis, glycogenolysis and protein breakdown), increased fatty acid synthesis and esterification of fatty acids, increased amino acid uptake and vasodilatation (relaxation of vessel wall of blood arteries, allowing for more blood flow). Glucagon which is produced in the α-cells of the pancreas counteracts some of the metabolic effects of insulin. Glucagon as catabolic hormone and insulin as anabolic hormone are together able to regulate normal plasma glucose levels.

Insulin release is accomplished when blood glucose levels are increased. As a result, more glucose is taken up by the β-cell via glucose transporter GLUT2 and action of glucokinase. This results in an enhanced flux through the subsequent glycolytic pathway and citric acid cycle resulting in an increased ATP/ADP ratio. As a result the ATP-sensitive potassium (K⁺) channels close, causing the cell membrane to depolarize. Depolarization of the cell membrane results in the opening of the voltage controlled calcium (Ca²⁺) channels, allowing an influx of calcium. The increase of intercellular Ca²⁺ releases insulin from the stored vesicles (Fig. 2). By this mechanism, glucose levels are tightly regulated.

Figure 2. The process of glucose dependent insulin secretion from the pancreatic β-cell. The key players in insulin secretion from the β-cell are glucose transporter GLUT2, glucokinase, ATP-sensitive potassium (K⁺) channel and voltage-dependent Ca²⁺ channel. See text for detailed description.
Disturbance in glucose homeostasis: Diabetes mellitus

Diabetes is a disease state in which glucose concentrations in blood are chronically elevated (hyperglycaemia). Diabetes develops when the amount of secreted insulin by β-cells is insufficient for insulin action. The latter is dependent on the degree of insulin resistance in various insulin sensitive tissues. Different forms of diabetes have been identified. In type 1 diabetes mellitus (T1DM), the β-cells are destroyed causing an absolute insulin deficiency, resulting in hyperglycaemia. T1DM manifests itself around puberty. In case of type 2 diabetes mellitus (T2DM), a patient is still able to produce insulin, however the secreted amounts are not sufficient to lower blood glucose levels to the appropriate physiological level because the major target organs for insulin action (liver, skeletal muscle and adipose tissue) are unable to fully respond to insulin. The latter condition is thus called insulin resistance. Onset of T2DM is at a much later stage in life, however this phenomenon is currently also affecting (obese) youngsters. The acute complications arising from a total absence of insulin, as seen in T1DM, are hyperglycaemia, ketoacidosis and nonketotic hyperosmolar coma. Untreated long term hyperglycaemia induces microvascular damage, leading to blindness and kidney failure. Furthermore, it induces damage to the long sensory nerves resulting in foot problems. In addition, the insulin resistant state as seen in T2DM is a major risk factor for macrovascular complications, increasing death risk due to cardiovascular problems. Treatment of T1DM involves supplementation with insulin by injection, while treatment of T2DM can be controlled by dietary change, medication or injections with insulin depending on the severity. Other types of diabetes have been described. During pregnancy, women can develop diabetes which resembles very much to T2DM and is therefore called gestational diabetes. Many of these women develop T2DM later in life. A mutation in a single gene is can also be sufficient to give rise to diabetes eg. Maturity Onset Diabetes of the Young (MODY) (1-4) and Maternally Inherited Diabetes and Deafness (MIDD) (5). Latent Autoimmune Diabetes in Adults (LADA) is a form of T1DM which progresses very slowly, thus causing confusion with T2DM. The distinction between LADA and T2DM can be made, since in LADA antibodies against glutamatic acid carboxylase are present (6).

Downstream signal transduction from the insulin receptor

In order to better understand how insulin action is accomplished and how insulin action is altered under the insulin resistant state, a deeper understanding of insulin signalling is required. Insulin induces multiple responses (both mitogenic and metabolic) and often the nature of these response is tissue specific. Downstream signalling described below is generally linked to the metabolic effects of insulin. However how different signalling intermediates are able to contribute to the various insulin-stimulated responses is far from complete. The very first step in insulin action is accomplished by binding of insulin to its
corresponding receptor. Binding results in the activation of a downstream signalling cascade which starts at the insulin receptor.

**Insulin receptor** The insulin receptor (IR) is part of a subfamily of receptor tyrosine kinases which include the insulin receptor-related receptor (IRR) and the insulin-like growth factor (IGF)-I receptor. The receptor is made up out of two extracellular α-subunits and two transmembrane β-subunits resulting in a α₂β₂ heterotetrameric complex which are joined by disulfide bonds. The α-subunit is able to inhibit the tyrosine kinase activity of the β-subunit. Thus binding of insulin to the α-subunit activates the tyrosine kinase of the β-subunit. The receptor undergoes a series of intramolecular transphosphorylation reactions in which one β-subunit phosphorylates its adjacent partner on specific tyrosine residues.

The activated IR is now able to phosphorylate tyrosine residues on intracellular substrates that include member of the insulin receptor substrate family (IRS1-6 (7-11)), Grb2-associated binder-1 (Gab-1 (12)), Cas-Br-M (murine) ecotropic retroviral transformin sequence homologue (Cbl (13)), adaptor containing PH and SH2 domains (APS (14)), Src homology 2 containing protein (Shc (15)) and signal regulatory protein (SIRP) family members. IRS and Shc are recruited to the juxtamembrane region via a common NPXY motif, while APS is able to directly bind to the activation loop. The phototyrosine residues are recognized by molecules which contain Src homology 2 (SH2) domains like small adaptor proteins Gbr2 and Nck, the SHP2 protein tyrosine phosphatise and the p85 regulatory subunit of the class IA phosphatidylinositol 3’-kinase (PI3K).

**Phosphatidylinositol 3’-kinase (PI3K)** The PI3K enzyme is composed out of a regulatory and a catalytic unit. The PI3K family is composed of class IA, class IB, class II and class III and the various classes display different substrate specificity. The class IA is the most important with respect to metabolic action after binding of insulin to the IR and is composed of p110α catalytic subunit and its associated regulatory subunit p85. Binding of p85 to p110α stabilizes p110α and thus inactivates its kinase activity. Upon stimulation, p110α is not inhibited by p85 leaving kinase activity of p110α uninhibited (16). At the plasma membrane, PI3K phosphorylates phosphoinositides on the 3’-OH (D3) position of the inositol ring to generate second messengers phosphatidylinositol-3,4-bisphosphate (PI-3,4-P₂) and phosphatidylinositol-3,4,5-triphosphate (PIP₃) from substrates phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PIP₂). Phosphatase and tensin homolog (PTEN) dephosphorylates the 3’-OH position of the inositol ring, acting as a suppressor to PI3K. Proteins with a Pleckstrin homology (PH) domain can bind and localize to the lipid second messenger PIP₃ (review in (17)). The AGC kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) and downstream
phosphorylation substrate protein kinase B (PKB/Akt) are both recruited to the plasma membrane via their PH domain (Fig. 3).

![Figure 3. Activation of protein kinase B (PKB/Akt) after insulin binding to the insulin receptor. For detailed description see text.](image)

**Protein kinase B (PKB/Akt)** The Ser/Thr kinase protein kinase B is composed of a N-terminal PH domain, a C-terminal catalytic domain and a hydrophobic motif and is highly conserved among different species (18). Generated PIP₃ is able to recruit PKB/Akt via its PH domain to the plasma membrane. For full potential of PKB/Akt, phosphorylation occurs at two different residues; phosphorylation of Thr308 in the catalytic domain occurs via PDK1 and phosphorylation of Ser473 in the hydrophobic motif site occurs via PDK2 (mTORC2 (19;20)). Once phosphorylated, PKB/AKT dissociates from the membrane and is able to phosphorylate targets in the cytoplasm and the cell nucleus (Fig. 4). In mammals three genes encode for the PKB/Akt family: *Akt1* (PKBα/Akt1), *Akt2* (PKBβ/Akt2) and *Akt3* (PKBγ/Akt3). The isoforms are products from distinct genes and overall they share a high degree of amino acid identity (>80%). PKBα/Akt1 and PKBβ/Akt2 are expressed ubiquitously, while expression of PKBγ/Akt3 is highest in brain and testes. Studies from knockout animals demonstrate that PKB/Akt is important in development and (glucose) metabolism. Akt1⁻/⁻ mice are smaller compared to their wild type littermates and male Akt1⁻/⁻ mice have increased apoptosis in the testes. Mouse embryonic fibroblasts from Akt1⁻/⁻ mice are more sensitive to apoptotic stimuli (21). Akt2⁻/⁻ mice are both hyperglycaemic and hyperinsulinaemic (22). Akt3⁻/⁻ mice have smaller brains compared to wild type animals (23;24). All single knockout animals are viable indicating that PKB/Akt isoforms can probably compensate for the lack of another. The double knockout for Akt1 and Akt2 has a severe phenotype which includes atrophy of skin and skeletal muscle, dwarfism and early neonatal lethality (25). Double knockout for Akt2 and Akt3 results in animals with glucose and insulin intolerance and reductions in size and weight of brain and
testes (26). Considering the different phenotypes of the knockout mice, PKB/Akt signalling has been linked to malignancies, insulin resistance/diabetes, muscle atrophy, Alzheimer disease and chronic obstruct pulmonary disease (27-33).

**PKB/Akt and PKB/Akt substrates in cellular processes and disease**

PKB/Akt is protein kinase able to regulate a wide range of cellular processes: (glucose) metabolism, apoptosis, proliferation and angiogenesis. In order to achieve these effects different PKB/Akt substrates have been identified and linked to these processes. PKB/Akt specifically phosphorylates substrates that contain the following amino acid motif: Arg-Xaa-Arg-Xaa-Xaa-pSer/pThr (34;35). The number of substrates is ever increasing and a combined search of “protein kinase B”, "phosphorylation” and “substrate” in Pubmed results in over 1500 hits.

The tuberous sclerosis complex (TSC) is a downstream phosphorylation target of PKB/Akt. Phosphorylation of TSC2 by PKB/Akt results in an inhibition of TSC activity (36). As a result, Ras homolog-enriched in the brain (Rheb)-GDP is converted into Rheb-GTP, thus allowing Rheb-GTP to activate mammalian target of rapamycin (mTOR) (37;38). mTOR is a component of two distinct complex called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 complex is sensitive for mTOR inhibitor rapamycin, while mTORC2 is not. mTORC1 is composed out of mTOR, regulatory associated protein of mTOR (Raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and proline-rich PKB/Akt substrate of 40 kDa (PRAS40) (39;40). Direct substrates for mTORC1 include p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein-1 (4EBP1). Via this way mTORC1 is able to regulate protein synthesis.

With respect to metabolism PKB/Akt is able to phosphorylate glycogen synthase kinase (GSK) which results in the downstream phosphorylation and inactivation of glycogen synthase (41). Via phosphorylation of PKB/Akt substrate of 160 kDa (AS160), PKB/Akt is able to regulate GLUT4 mediated glucose uptake (42). The forhead box O (FOXO) transcription factors play an important role as PKB/Akt substrates controlling gene expression. Nuclear exclusion of FOXO inhibits gluconeogenesis while promoting β-cell function (43;44). FOXO proteins exert many different functions and are also able to regulate expression of Bcl2 family members thereby regulating apoptosis (review in (45)).

Involvement of PKB/Akt in the process of programmed cell death or apoptosis can be mediated by phosphorylation and inhibition of pro apoptotic mediators such as Bad, MDM2 (46), FOXO and IκB kinase (IKK (47)). When dephosphorylated, Bcl2 family member Bad is able to complex with BclXL resulting in apoptosis. However phosphorylation of Bad on Ser136 results in 14-3-3 binding and thus inhibits apoptosis (48).
In cell cycle control, PKB/Akt can phosphorylate cyclin-dependent kinase (CDK) inhibitors p21\textsuperscript{CIP/WAF1} (49) and p27\textsuperscript{KIP1} (50) which results in the cytoplasmic retention of these proteins, since they are able to bind 14-3-3. PKB/Akt is thus able through p21\textsuperscript{CIP/WAF1} able to inhibit cell cycle arrest.

The list of identified PKB/Akt phosphorylation substrates is always increasing of which a small sample is included in the following list: ATP-citrate lyase (51), WNK1 (52), NDRG2 (53), PRAS40 (54), Synip (55), filamin C (56), JCF1 (57), VCP (58-60), PIKfyve (61), YB1 (62;63), Par-4 (64). However from the more recent PKB/Akt substrates it not always clear in which cellular processes they play a role.

**Rationale and outline of thesis**

Impairment of glucose metabolism is associated with decreased activity of PI3K and downstream signalling partner PKB/Akt. The PI3K and PKB/Akt signalling pathway is able to exert numerous different effects mediated through specific PKB/Akt substrates. A lot of distinct functions can be described to PKB/Akt substrates, however from a lot PKB/Akt substrates it remains unclear what their specific function is. Therefore in this thesis, the focus is on PKB/Akt substrate PRAS40 and examine how phosphorylation of this substrate is altered under conditions of insulin resistance. Next we examine the function of PRAS40 in respect to insulin action. In **chapter 2**, an overview is provided on different literature concerning PRAS40. Special attention will be paid to PRAS40 and the role of this protein in the regulation of mTORC1. In **chapter 3**, phosphorylation of PRAS40 on Thr246 both *in vitro* and *in vivo* is examined. Using an animal model for insulin resistance we have assessed phosphorylation status of PRAS40 under insulin resistance. **Chapter 4** describes phosphorylation of PRAS40 on Ser183 and the importance of Thr246 in this process. **Chapter 5** describes the subcellular localisation of the PRAS40 protein. In **chapter 6**, we investigate the involvement of PRAS40 in palmitate-induced insulin resistance. All the obtained results are summarised and discussed in **chapter 7**.
Chapter 1

References


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