Hormonal regulation of phosphodiesterase 3B in adipocytes

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To Andreas

Contents

Abstract

Insulin induced activation of the cAMP degrading enzyme phosphodiesterase (PDE) 3B in adipocytes is important for insulin mediated inhibition of lipolysis. The aim of this thesis was to contribute with knowledge on the acute activation of PDE3B in response to insulin and catecholamines in adipocytes. The activation of PDE3B was investigated from three different perspectives namely; phosphorylation of PDE3B, localization of PDE3B and PDE3B interactions with other proteins. As models to study activation of PDE3B we used primary rat and mouse adipocytes, 3T3-L1 adipocytes and caveolin-1 knock out mouse adipocytes.

The study on PDE3B phosphorylation shows that PDE3B is multisite phosphorylated in response to both insulin and catecholamines. We were able to identify six phosphorylation sites on PDE3B namely; 273, S296, S421, S424/5, S474 and S536. From the studies on PDE3B localization we conclude that PDE3B is localized to caveolae and endoplasmic reticulum (ER) in adipocytes and that caveolae localized PDE3B is specifically activated by catecholamines while PDE3B in ER is specifically activated by insulin. Finally, the studies on PDE3B interactions demonstrate that insulin and catecholamines stimulate the recruitment of PDE3B into large macromolecular complexes in adipocytes. The recruitment is dependent on caveolin-1 especially in response to insulin and the constituents of the PDE3B macromolecular complexes are dependent on the stimuli used. After insulin stimulation insulin signaling molecules such as protein kinase B (PKB) are associated with PDE3B in the large complex while after catecholamine stimulation adrenergic signaling molecules such as protein kinase A (PKA) are associated with PDE3B.

List of papers

I. **Multisite phosphorylation of adipocyte and hepatocyte phosphodiesterase 3B**
Rebecka Lindh, Faiyaz Ahmad, Svante Resjö, Peter James, Jeong S. Yang, Henry M Fales, Vincent Manganiello, Eva Degerman
Biochimica et Biophysica Acta, 2007 Apr; 1773(4):584-92

II. **Plasma membrane cyclic nucleotide phosphodiesterase 3B (PDE3B) is associated with caveolae in primary adipocytes**
Rebecka Nilsson, Faiyaz Ahmad, Karl Swärd, Ulrika Andersson, Vincent Manganiello, Eva Degerman
Cellular Signaling, 2006 Oct; 18(10):1713-2

III. **Insulin-induced formation of macromolecular complexes involved in activation of cyclic nucleotide phosphodiesterase 3B (PDE3B) and its interaction with PKB**
Faiyaz Ahmad, Rebecka Lindh, Marie Weston, Yan Tang, Eva Degerman, Vincent Manganiello
Biochemical Journal, 2007 Jun 1; 404(2):257-68

IV. **Differential regulation of PDE3B in distinct membrane compartments of adipocytes in response to insulin and a β_3-adrenergic receptor agonist**
Faiyaz Ahmad, Rebecka Lindh, Yan Tang, Iida Ruishalme, Anita Öst Peter Strålfors, Eva Degerman and Vincent C. Manganiello
Manuscript

Abbreviations

2D	two dimensional
AC	adenylyl cyclase
AKAP	A-kinase anchoring proteins
AMP	adenosine monophosphate
AS47	47 kDa PKB (Akt) substrate
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
AR	adrenergic receptor
cAMP	cyclic adenosine 3´, 5´-monophosphate
Cbl	Casitas B-linage lymphoma protein
cGMP	cyclic guanosine monophosphate
CL	CL-316243, β_3-adrenergic agonist
C-MAD	C-terminal membrane attachment domain
CNCG	cyclic nucleotide gated ion channel
CRE	cAMP responsive element
eNOS	endothelial nitric oxide synthase
Epac	exchange protein directly activated by cAMP
ER	endoplasmic reticulum
FFA	free fatty acid
GAB	GRB2-associated binder protein
GDP	guanosine diphosphate
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
GMP	guanosine monophosphate
GRB	growth factor receptor bound protein
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HSL	hormone sensitive lipase
HSP	heat shock protein
IGF	insulin like growth factor
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
KO	knock out
LPL	lipoprotein lipase
MAP	mitogen-activated protein
MCP	monocyte chemoattractant protein
MGL	monoglyceride lipase
N-MAD	N-terminal membrane attaching domain
NHR	N-terminal hydrophobic region
PAI	plasminogen activator inhibitor
PDE	cyclic nucleotide phosphodiesterase
PDK	phosphoinositide-depentent kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PM	plasma membrane
PP	protein phosphatase
PPARγ	peroxiome proliferator-activated receptor γ
PtdIns	phosphatidylinositol
RBP	retinol binding protein
S	serine
SH	src-homology
Shc	SH2-domain containing proteins
siRNA	small interference RNA
T1D	type 1 diabetes
T2D	type 2 diabetes
TG	triglyceride
TNF-α	tumor necrosis factor-α
tPP	tryptic phosphopeptide
TZD	thiazolidinedione
Vo	void volume
VLDL	very low density lipoproteins
WT	wild type

INTRODUCTION

General background

In humans the main nutrients responsible for generating energy are carbohydrates, fats and proteins. The nutrients enter the bloodstream from the intestine primarily as monosaccharides such as glucose, triglycerides (TG) in chylomicrons and free amino acids or dipeptides [1]. The uptake of the nutrients from the circulation into the cells of the body and biochemical processing of the nutrients are highly regulated. The regulation is controlled by the action of hormones, neurotransmitters, cytokines and the nutrients own signaling pathways. After food intake the increased glucose level in the circulation stimulates insulin secretion from the pancreatic β-cells. Insulin is the main anabolic hormone acting both on lipid and glucose metabolism in adipocytes of the adipose tissue, myocytes of the skeletal muscle and hepatocytes of the liver. In adipocytes the main effects of insulin are to stimulate glucose uptake, stimulate conversion of fatty acids into TGs and to inhibit breakdown of stored TGs. In myocytes the main effects of insulin are stimulation of glucose uptake and glycogen synthesis. In hepatocytes insulin stimulates glycogen synthesis and inhibits glucose production by inhibiting gluconeogenesis and glycogenolysis. After a meal, the concentration of glucose decreases in the circulation. Thereby insulin secretion is reduced and other hormones take over the metabolic regulation. One such hormone is glucagon, which is secreted from the pancreatic α-cells. Glucagon primarily acts on the hepatocytes by stimulating cyclic adenosine 3´, 5´-monophosphate (cAMP) production. The increased cAMP levels stimulate gluconeogenesis and glycogenolysis which lead to increased glucose output from the liver [1]. Other hormones are the catecholamines; adrenalin and noradrenalin, which are increased in response to stress, physical activity or severe infections. Adrenalin is a hormone secreted from the adrenal medulla and noradrenalin is a neurotransmitter released from the sympathetic nervous system. They both stimulate α- and β-adrenergic receptors (AR) and cAMP production in different types of cells [1]. Increased concentration of cAMP will for example stimulate lipolysis in adipocytes, stimulate glycogenolysis in hepatocytes and inhibit glycogen synthesis in the hepatocytes and myocytes.

In patients with diabetes mellitus the metabolic regulation described above is not working correctly, which leads to elevated levels of circulating glucose and free fatty acids (FFA). The disease is traditionally divided into two types, type 1 diabetes (T1D) which is caused by destruction of the insulin secreting β-cells in pancreas and type 2 diabetes (T2D) which is caused by insulin resistance and

insulin deficiency. T1D develops during childhood or adolescence when β-cells are destroyed, often by an auto-immune reaction, which reduces or abolishes insulin secretion. T2D usually develops later in life and is initiated by increased insulin resistance which is very common in obese patients. The insulin resistance causes increased glucose and lipid load and a higher insulin demand. Individuals who can not uphold high rates of insulin production from the β-cells will eventually develop clinically evident diabetes. Two other classes of diabetes are gestational diabetes occurring in 3-5 % of all pregnancies and other specific types of diabetes such as maturity onset diabetes of the young (MODY) or latent autoimmune diabetes in adults (LADA) are a very small heterogeneous subclass comprising 1-2 % of the diabetic patients [2]. The division of diabetes into T1D and T2D is very categorical and in the future it is likely that we will see a more dimensional classification especially since the genetic and metabolic heterogeneity of diabetes have become clear. In a dimensional classification, diabetes would more or less be a spectrum of diseases where T1D and T2D can be viewed as endpoints in the spectrum instead of categories [3].

Adipose tissue

Looking back 60 years adipose tissue was purely regarded as the body insulator and protector of the body organs from injury. These functions are still valid, but the understanding of adipose tissue functions has greatly expanded since then. There are two types of adipose tissue; white adipose tissue and brown adipose tissue [4]. This thesis focuses on white adipose tissue, which in the following will be referred to as adipose tissue. Primarily adipose tissue is an organ used to store and release energy and today it is also considered to be an endocrine organ since many factors are secreted from the tissue to control a variety of physiologic functions. Human depots of adipose tissue located throughout the body have different functions, for example fat pads of the toes, heels and fingers are purely for mechanical support while the subcutaneous and visceral depots contribute to whole body energy homeostasis [5]. The importance of adipose tissue to control whole body energy homeostasis becomes obvious when looking at lipoatrophic patients that lack adipose tissue and their contrasts, the severely obese patients. In both groups the patients have insulin resistance and a number of other defects connected to deranged whole body energy homeostasis [6, 7].

Adipose tissue in lipid metabolism

In general terms, energy is taken up and stored by adipose tissue during food intake and released in the form of FFAs during fasting. Energy in the form of

TGs are transported in the blood from the intestine in chylomicrons after food intake and from the liver in very low density lipoproteins (VLDL), after both food intake and fasting, to be taken up by the adipose tissue and muscle[1]. TGs from the chylomicrons and VLDL are hydrolyzed to FFAs by lipoprotein lipase (LPL) located on the surface of the capillary endothelium. The FFAs enter the adipocyte and are there re-esterified with glycerol-3-phosphate to form TGs stored as lipid droplets in the adipocyte. Another way for the adipocyte to accumulate FFAs is through de novo synthesis from carbohydrates, in a process called lipogenesis, which occurs in humans during high carbohydrate intake [8]. Most carbohydrates in this process come from glucose taken up into the adipocytes by glucose transporter (GLUT) 4. During fasting the TGs are hydrolyzed to form glycerol and FFAs in a process called lipolysis. FFAs are secreted from the adipocyte and taken up by the liver to form lipoproteins or by the muscle to be used for energy production. Lipolysis is stimulated mainly by catecholamines which are elevated during situations of increased energy demand, for example during physical exercise or stress. Insulin on the other hand, which is elevated during energy intake, inhibits catecholamine stimulated lipolysis. For many years two lipases have been known to be involved in the lipolytic process; hormone sensitive lipase (HSL) [9] and monoglyceride lipase (MGL) [10]. Recently another lipase has been identified and shown to be important for the lipolytic process namely, adipose triglyceride lipase (ATGL) [11]. From several different studies it seems like ATGL is involved in the first step of TG hydrolysis to form FFA and diacylglycerol (DG), DG is further hydrolysed by HSL to form FFA and monoacylglycerol (MG) which can finally be hydrolysed by MGL to form FFA and glycerol [12, 13]. In response to catecholamines, HSL is phosphorylated on several different sites by protein kinase A (PKA) which generates an activation [14] and translocation of the enzyme to the lipid droplet [15, 16]. At the lipid droplet perilipins cover the surface and thereby limit the HSL access. After catecholamine stimulation HSL gains access to the lipid droplet when PKA phosphorylates perilipin which makes the perilipins translocate away from the droplet [17]. ATGL has been shown to be constantly associated with lipid droplets independent of stimulation and has not yet been shown to be activated by any stimuli [11], although the ATGL mRNA levels can be affected by for example fasting/feeding and glucocorticoids [18]. Insulin inhibits activation of PKA and lipolysis in a cyclic nucleotide phosphodiesterase (PDE) 3B dependant manner, causing inhibition of catecholamine stimulated lipolysis [19]. The signaling cascades responsible for the regulation of lipolysis are illustrated in figure 1 and further discussed in the sections: *insulin signaling and cAMP signaling*.

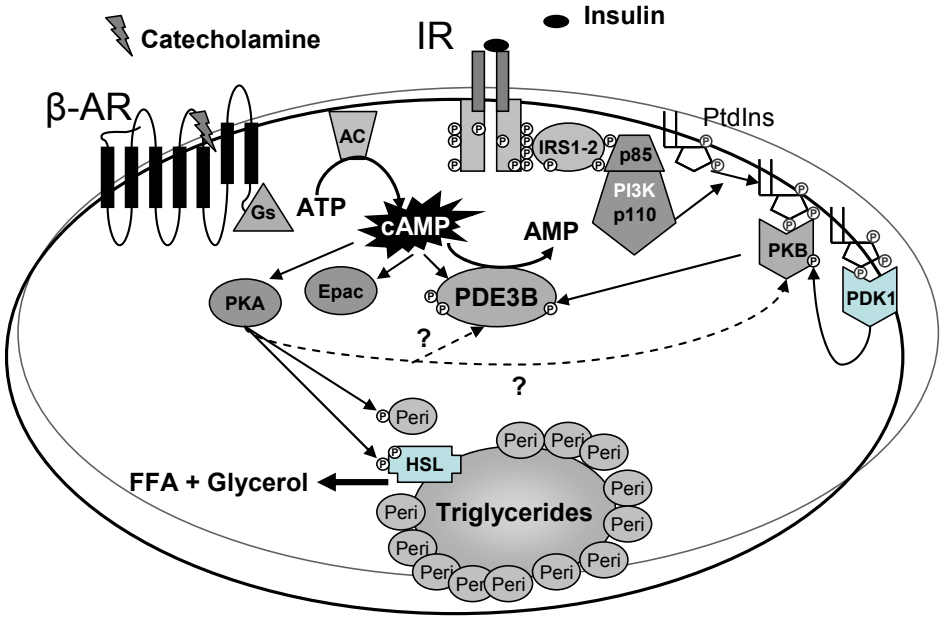

Figure 1: Hormonal regulation of lipolysis in adipocytes
Catecholamines stimulate lipolysis through binding to the β-adrenergic receptor (β-AR) which induces a rise in cAMP levels and a subsequent stimulation of protein kinase A (PKA) and hormone sensitive lipase (HSL). Stimulated lipolysis is inhibited by insulin which activates cyclic nucleotide phosphodiesterase (PDE) 3B and the hydrolysis of cAMP. Gs; stimulatory G-protein, AC; adenylyl cyclase, peri; perilipin, HSL; hormone sensitive lipase, FFA; free fatty acids, IR; insulin receptor, IRS; insulin receptor substrate, PI3K; phosphatidylinositol 3-kinase, PKB; protein kinase B, PDK; phosphoinositide-depentent kinase, Epac; exchange protein directly activated by cAMP, PtdIns; phosphatidylinositol 3,4,5-triphosphate.

Adipose tissue as an endocrine organ

The adipose tissue has been established as an endocrine organ during the last decade. According to Trayhurn et al [20] there are today over 50 proteins/peptides known to be secreted from the adipocyte and thereby called adipokines. A few of these adipokines, like leptin and adiponectin are primarily produced by the adipocytes compared to other cells [21]. The adipokines are structurally diverse and have different physiological effects, for example plasminogen activator inhibitor (PAI)-1 which maintains vascular haemostasis and angiotensinogen which is involved in the regulation of blood pressure [22, 23]. Most adipokines are however involved in one or two of the following categories; (1) the inflammatory response such as tumor necrosis factor-α

(TNF-α), leptin, interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP) -1 and resistin [20] and/or (2) energy homeostasis such as adiponectin, leptin, TNF-α, omentin, visfatin, resistin and retinol-binding protein (RBP) 4 [5]. When it comes to the regulation of energy homeostasis the adipokines can be divided into two categories the pro-hyperglycemic such as resistin, TNF-α and RBP4 and the anti-hyperglycemic such as leptin, adiponectin, visfatin and omentin [5]. Leptin, which is the most studied adipokine has a large variety of effects. It binds to receptors in hypothalamus to reduce appetite and promote energy expenditure [24]. Leptin also increases insulin sensitivity in muscle and hepatocytes, reduces insulin secretion in β-cells, modulates steroid production from the ovaries and affects immune development [25]. As more and more proteins and peptides are added to the list of adipokines the picture of the adipose tissue as an endocrine organ gets more and more complex.

Adipose tissue and type 2 diabetes

Obesity is associated with a number of health problems such as T2D, cardiovascular disease, insulin resistance and fatty liver disease. Although only 10 % of obese patients have diabetes most of them are insulin resistant [26] and most of the patients with diabetes are obese. The exact mechanisms for the development of diabetes and insulin resistance in response to increased fat mass are still not perfectly clear, but the information on the subject has increased during the last decade. The main mechanisms are based on the fact that obese patients have; (1) high levels of FFA and thereby increased ectopic fat accumulation in liver and muscle and (2) high amounts of inflammatory markers/adipokines and infiltration of macrophages in the adipose tissue.

In both obese and T2D patients levels of FFAs are higher than in control subjects [27]. The higher FFA levels can be a caused by expanded adipose tissue per se or deranged lipid metabolism. Deranged lipid metabolism can be seen especially in patients with high amounts of visceral adipose tissue. Studies have shown strong association between omental fat mass (especially visceral adiposity), dyslipidaemia and T2D, whereas such association was less consistent with respect to subcutaneous fat mass [28]. Adipocytes from visceral and subcutaneous fat depots seem to have different metabolic properties, especially regarding regulation of lipolysis [29]. Visceral adipocytes have higher lipolytic rates in response to catecholamines and weaker antilipolytic effects in response to insulin as compared to subcutaneous adipocytes. Large deposits of visceral adipose tissue will thereby generate higher plasma FFA levels than large deposits of subcutaneous adipose tissue [30, 31]. High amounts of FFAs will, especially in a long term perspective, generate fat accumulation in different non-adipose cells. There are however other factors

that effect the ectopic fat accumulation such as leptin levels [32] and the balance between energy delivery/uptake and oxidation [33]. Accumulation of TGs especially in skeletal muscle cells, hepatocytes and β-cells seem to correlate with increased insulin resistance and diabetes [34, 35]. In skeletal muscle cells one hypothesis for the correlation between TG accumulation and development of insulin resistance could be the inhibition of hexokinase which causes decreased glucose disposal and uptake [36]. This hypothesis has during the last decade been challenged by evidence indicating that it is an impaired insulin stimulated glucose uptake per se which is responsible for the decresed glucose disposal in T2D patients and not hexokinase [37]. FFA affect glucose transport by activating protein kinase C (PKC) which in turn serine phosphorylates insulin receptor substrate (IRS)-1 and inhibits the insulin signal [38]. In hepatocytes the effect of increased TG accumulation is not entirely clear, but studies indicate that the activity of PKC is increased in hepatocytes of fatty livers and in livers of T2D patients. The activated PKC leads to decreased insulin signaling and decreased glycogen synthesis and increased gluconeogenesis [33]. In β-cells, exposure of FFAs during a short time period causes increased insulin secretion [39]. This stimulatory effect is however reversed during chronic exposure of FFAs which causes accumulation of TGs in the β-cells, dysfunction of insulin release and eventually apoptosis of the β-cell [32].

In addition to a correlation between obesity and high levels of FFAs, there is a strong correlation between obesity and high concentrations of inflammatory markers such as C-reactive protein and IL-6 indicating the presence of a chronic, low-grade inflammation in obese patients [40, 41]. It is now apparent that the inflammation is caused by increased secretion of different pro-inflammatory adipokines such as TNF-α, IL-6, MCP-1, leptin and reduced secretion of the anti-inflammatory adiponectin from adipose tissue [41]. In addition to increased inflammatory markers, obese patients also have an increased number of infiltrated macrophages in adipose tissue [42, 43]. The macrophages are immune cells that secrete cytokines and protect mammals from invading danger. Macrophages, preadipocytes and adipocytes share several common features and there have been reports suggesting that the macrophages infiltrating the adipose tissue come from trans-differentiated preadipocytes [44]. More likely though, is that the majority of the adipose tissue macrophages are derived from circulating monocytes infiltrating the adipose tissue [43], recruited by MCP-1 (also called C-C motif chemokine ligand 2 (CCL-2)) secreted from the adipose tissue [45, 46]. The chronic activation of pro-inflammatory pathways can be a mechanism for development of insulin resistance [41]. The mechanism for the development of insulin resistance has especially been studied for TNF-α, which causes inhibitory

serine phosphorylation of IRS-1. The serine phosphorylation reduces both insulin induced tyrosine phosphorylation of IRS-1 and the ability of IRS-1 to interact with the insulin receptor (IR) [47].

During development of obesity adipose tissue undergoes extensive remodeling, which is dependent on the coordinated interplay between increased adipocyte size, increase in number of adipocytes (adipogenesis) and angiogenesis [48]. The differentiation of preadipocytes to adipocytes during adipogenesis involves a very complex process, but one "master switch" has been identified to be the transcription factors peroxiome proliferators-activated receptor γ (PPARγ) specifically expressed in adipocytes [49]. Interestingly a class of PPARγ agonists called thiazolidinedione (TZD), reduces insulin resistance in patients with T2D, which highlights a central role for adipocytes in the development of insulin resistance and as a target for drug design. The increased adipogenesis in response to TZD lowers the lipid load in other tissues and thereby reduces the lipotoxic effects. Additional studies have shown that TZDs increase the secretion of high molecular weight adiponectin, the adipokine which is anti-inflammatory and improves insulin sensitivity [50]. Whether improvement of insulin sensitivity in response to TZD is mainly an effect of decreased lipid load or reduced inflammatory status of the adipose tissue is not known, but most likely both effects are important. In summary it seems likely that the adipokine/inflammatory status together with the lipid load are important for the development of T2D and insulin resistance in obese patients.

Caveolae

Caveolae which is Latin for "little caves" was first described in 1955 by Yamada when he studied epithelial cells from gallbladder with transmission electron microscopy [51]. He observed 50-100 nm invaginations of the plasma membranes (PM) which he called *caveolae intracellulare*. These structures were clearly different form the larger and more electron-dense, clathrin-coated pits. It then took another 40 years of research in the field of membrane biology and the discovery of the membrane protein family of caveolins to discover the molecular nature of the caveolae, seen in figure 2B. During the last decade it has become evident that membranes can co-exist in two different forms namely the liquid-ordered and the liquid-disordered state. The liquid-disordered state consists of phospholipids in a fairly loose structure with quite low levels of cholesterol. Among this loose lipid composition are regions of the liquid-ordered state which are more rigid structures composed of cholesterol and shingolipids, also called lipid-rafts. The biochemical property of caveolae resembles that of the liquid-ordered state and lipid-raft with high concentration

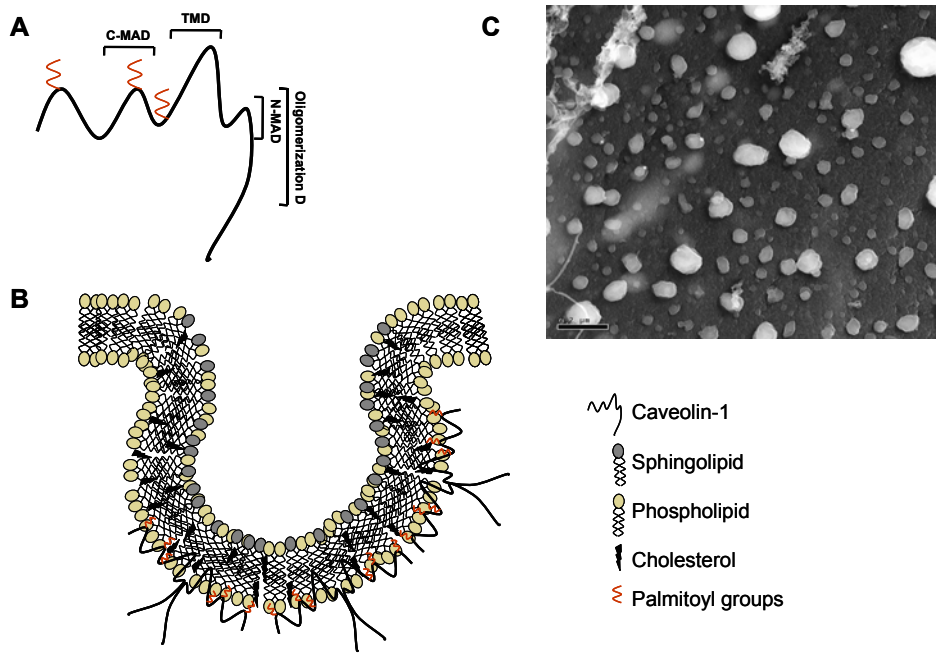

Figure 2: Caveolae and caveolin-1
A, structure of caveolin-1 with palmitoyl groups, C-terminal membrane attachment domain (C-MAD), double-pass transmembrane domain (TMD), N-terminal membrane attaching domain (N-MAD) and oligomerization domain (D). **B**, organization of caveolae membrane, with oligomers of caveolin-1 (illustrated as dimers for simplicity), cholesterol and high concentration of sphingolipids. **C**, caveolae of the intracellular face of the plasma membrane from primary human adipocytes. Transmission electron micrograph prepared by Anita Öst and Iida Ruishalme.

of cholesterol and sphingolipids (glycosphingolipids and sphingomyelin) [52]. What then distinguishes caveolae from lipid-rafts is its morphology at the PM and the presence of the stabilizing protein caveolin [53]. Caveolin was identified to be a component of the caveolae membrane coat in 1992 by Rothberg et al [54] and since then the knowledge about the caveolae has exploded into various fields such as cellular trafficking and signaling. The knowledge has been applied in many different systems since caveolae are found in many cell types, although they are most abundant in adipocytes, fibroblasts, smooth and striated muscle cells, epithelial cells and type 1 pneumocytes [53].

Caveolin

Caveolin exists in three isoforms, caveolin-1, -2 and -3, which are 21-24 kDa proteins that are located on the cytoplasmic surface of the caveolae [53, 55-57]. The tissue expression of the isoforms is diverse, caveolin -1 and -2 are mostly expressed in adipocytes, endothelial cells, pneumocytes, smooth muscle cells and fibroblast where the expression of caveolin-2 is dependent on the caveolin-1 expression [58]. Caveolin-3 is expressed mostly in skeletal and cardiac myocytes [56]. The expression of these isoforms fully correlates with the presence of caveolae in these tissues. The structure of caveolin-1, which is the most studied isoform can be viewed in figure 2A. The C-terminal domain has three palmitolated cysteins [59] and one C-terminal membrane attachment domain (C-MAD) [60]. The middle part consists of one double-pass transmembrane region, one N-terminal membrane attaching domain (N-MAD) and an important oligomerization domain, which can form oligomers containing 14-16 monomers of caveolin-1 or caveolin-1 together with caveolin-2 [53, 60]. Caveolin-1 is important for the formation of caveolae, recruitment of numerous signaling molecules to the PM and the regulation of the activity of interacting proteins [61].

Functions of caveolae

Caveolae and caveolins have been suggested to be involved in several cellular functions such as endocytosis, transcytosis, mechanosensing, cholesterol homoestasis, lipid syhthesis and cell signaling control. The exact involvement of caveolae and caveolin in many of these functions is however unknown. The involvement of caveolae in endocytosis is under debate. It has been shown that caveolae are remarkably stable and are not involved in constitutive endocytosis [62, 63]. There are however some agents that can induce endocytosis of caveolae, these are cholesterol, glycosphingolipids [64] and also certain viruses and toxins like SV40 virus, cholera toxin and tetanus toxin. It has been shown that these agents, viruses and toxins stimulate endocytosis of caveolae to Golgi apparatus or endoplasmic reticulum (ER) via the caveosome but not to the endosome, which is the normal route for clatrin-coated pits [53]. Regarding mechanosensing, caveolae might be important for the ability to sense membrane tensions or other changes of the physical properties of the plasma membrane, which could be important in for example remodeling of blood vessels [65]. The involvement of caveolae in cholesterol homoestasis is without doubt. There is a unique relationship between caveolins/caveolae and cholesterol, where caveolin-1 can bind 1-2 molecules of cholesterol and acts as a “delivery device” for cholesterol [66]. The relationship between caveolin and cholesterol is also supported by the fact that the caveolin-1 gene has a sterol

regulatory element and by the fact that adding free cholesterol causes the level of caveolin-1 mRNA to increase [53].

Caveolae are often described as communication platforms where signals can be effectively transduced and where cross-talk between different signaling pathways can occur. This idea was first described by Lisanti et al [67] as they found that a majority of the proteins associated with caveolae compared to the rest of the plasma membrane were signal transduction molecules. Since then the list of caveolae associated proteins has dramatically expanded with signaling proteins including receptors, downstream targets, channels, enzymes etc., but the relevance of the caveolae localization and to what extent the identified proteins are specifically located in caveolae can be questioned for some of the studies [65]. What is known for sure though, is that caveolae are important in G-protein coupled signaling [68], endothelial nitric oxide synthase (eNOS) signaling [53, 65] and insulin signaling [69, 70]. Interestingly accumulating evidence proves that the caveolin molecule can act as a scaffolding protein by directly interacting with different signaling molecules and modulate the activity of the interacting protein. In most cases interactions have been shown to inactivate signals, although for the insulin receptor the interaction seems to be generating the opposite effect [71].

The various functions of caveolae imply an important role of them in different disease conditions [72]. For example it has been shown that caveolin-1 acts as a tumor suppressor and mutations in the caveolin-1 gene have been found in a variety of human cancers [73]. Caveolin-3 has instead been shown to be important in the development of muscular dystrophy. A mutation in the caveolin-3 gene in humans has been identified to cause hypertrophic cardiomyopathy [74] and caveolin-3 knock out (KO) mice have mild myopathic changes [75]. The important role of caveolae and caveolin in lipid metabolism and insulin signaling in adipocytes and how defects in caveolae and caveolin can cause the development of diabetes are discussed in the next section [69].

Caveolae in adipocytes

Adipocytes have a very high quantity of caveolae as seen on electron micrographs of the adipocyte PM, figure 2C. Based on ultrastructural comparisons, the adipocyte seems to have higher concentrations of caveolae that any other cell and caveolae seem to account for around 30 % of the surface area of the PM. To study the functions of caveolae in different tissues the caveolin-1 KO mice were developed by two different research groups [76, 77]. The caveolin-1 KO mice totally lacked caveolin and caveolae in tissues where

caveolin-1 normaly is expressed. In the adipocyte the main caveolin isoforms expressed are caveolin-1 and -2, but since the expression of caveolin-2 is dependent on the caveolin-1 expression, the adipocytes from the caveolin-1 KO mice totally lacked caveolae [78]. The caveolin-1 KO mice were lean and resistant to diet induced obesity compared to the caveolin-1 wild type (WT) mice, suggesting that the cavolin-1 deficient mice have problems with lipid metabolism or adipocyte function [78]. The adipose tissue from the caveolin-1 KO mice was further analyzed morphologically. The adipocytes in the tissue were poorly differentiated, smaller and had a smaller lipid droplet than adipocytes from the caveolin-1 WT animals. In the same study it was also shown that the caveolin-1 KO mice had higher levels of TGs, higher amount of post-prandial FFA and lower leptin and adiponectin levels in the circulation [78]. Together the results clearly show that caveolae are important for systemic lipid homeostasis and caveolae deficient adipocytes do not have a normal TG storage capacity. Adipocytes from the caveolin-1 KO mice have also been isolated and further characterized regarding lipid metabolism. Results from a study by Cohen et al [79] showed that adipocytes from caveolin-1 KO mice have reduced lipolytic activity in response to a β_3-adrenergic receptor (AR) agonist compared to caveolin-1 WT mouse adipocytes. The reduced lipolytic response was shown to be an effect of depleted perilipin phosphorylation which reduces the HSL access to the lipid droplet. Interestingly PKA, which phosphorylates perilipin was highly present and active in the KO mice, indicating that caveolin-1 facilitates the PKA-mediated phosphorylation of perilipin. In the same study it was also shown that caveolin-1, perilipin and PKA form a tight complex in response to a β_3-AR agonist in 3T3-L1 adipocytes, which is also supported by a study showing that PKA can interact with caveolin-1 [80]. The reduced lipolytic response can however not explain the small lipid droplets in adipocytes of the caveolin-1 KO mice. The small droplets can however be explained by studies suggesting a role for caveolin-1 also in lipid droplet formation [79, 81]. A very interesting study by Öst et al [82] showed that TGs can be synthesized in a specific subclass of caveolae. Caveolae and caveolin-1 thereby seem to have an important role in the regulation of TG breakdown, formation and storage, but the exact regulatory mechanisms for caveolae and caveolin-1 in adipocyte lipid metabolism remain to be unraveled.

The idea that caveolae provide microdomains, which generate closeness of proteins in different signaling cascades, is interesting. The important role of caveolae and caveolin-1 as scaffolding device to generate specific signal transduction in a compartmentalized manner has been shown for β-adrenergic receptor signaling [83]. This concept can perhaps be expanded to other signaling pathways like the insulin signaling pathway in adipocytes. It has been

shown that the IR is located in caveolae in adipocytes by different microscopic and caveolae isolation methods [70, 84]. The IR has also been shown to interact with caveolin-1 and -3 in a stimulatory fashion [71]. In addition GLUT4 has been found to translocate to caveolae in adipocytes [69, 85]. The important role for caveolae and caveolin in insulin signaling is supported by results from the caveolin-1 KO [86] and caveolin-3 KO [87] mice. The caveolin-1 KO mice were less responsive to insulin in vivo and developed hyperinsulinemia after high fat feeding as compared to caveolin-1 WT mice. These results were probably due to the reduction of IR levels by 90%, in the adipocytes. The caveolin-3 KO mice were insulin resistant and glucose intolerant. The effects were mainly attributed to the skeletal muscle, although defects could surprisingly also be seen in both adipocytes and hepatocytes where no caveolin-3 is expressed indicating indirect effects on these tissues. Without doubt a lot of evidence support an important role for caveolae in adipocyte insulin signaling, but there are contradictory data especially regarding the localization of the IR to caveolae and the translocation of GLUT4 to caveolae in response to insulin [88, 89]. The differences in the results regarding caveolae localization of the IR can however be explain by a recent study which showed that the IR is associated with the neck of caveolae in 3T3-L1 adipocytes and that the caveolae neck and bulb have different solubility [90]. Small differences in experimental protocols of caveolae enrichment techniques will thereby generate totally different results regarding the localization of the IR.

Insulin signaling

After insulin binds its receptor different signaling cascades are initiated, which leads to cell growth, differentiation and, most important for this thesis, different metabolic effects as seen in figure 3. The main metabolic effects of insulin include increased glucose uptake in muscle cells and adipocytes, inhibition of glucose production in hepatocytes and promoted storage of energy substrates in hepatocytes, adipocytes and muscle cells. The increased storage of energy is an effect of stimulated lipogenesis, glycogen synthesis and protein synthesis and inhibited lipolysis, glycogenolysis and protein breakdown [1]. Exactly which effects that are initiated when insulin binds its receptor are dependent on several different features of the recipient cell; cell-specific expression of signaling molecules such as IRS substrates, kinases and lipases, intrinsic activities of signaling molecules, cell-specific localization of signaling molecules and multiprotein complexes.

The IR is a receptor tyrosine kinase which belongs to the insulin receptor family together with the structurally related insulin like growth factor (IGF) receptor and the insulin related receptor for which no ligand has been found [91]. The IR is tetrameric with two α-subunits connected to each other by one disulfide bond and two β–subunits, each connected to one α-subunit by one disulfide bond. The α-subunits are located entirely on the outside of the PM and contain the insulin binding sites. Without insulin the α-subunits inhibit the tyrosine kinase activity of the β-subunits located inside the cell. Upon insulin binding the inhibition disappears and the kinase activity of the β-subunits leads to trans-phosphorylation of the IR, which generates conformational changes of the IR that further increase the tyrosine kinase activity of the receptor [92]. Several substrates for the activated IR have been identified, which is illustrated in figure 3. Among these are the IRS 1-6 [93, 94], various SH2-domain containing proteins (Shc) [95], GRB2-associated binder protein (GAB)-1 [96] and Casitas B-linage lymphoma protein (Cbl) [97]. Some of the substrates can dock to the phosphotyrosines of the IR with phosphotyrosine binding (PTB) or Src-homology (SH)-2 domains. In turn the phosphorylated tyrosines of the IR substrates can act as docking sites for proteins such as the SH2 containing proteins phosphatidylinositol 3-kinase (PI3K) and growth factor receptor bound protein (GRB) 2. These proteins thereby become activated and/or associate with other downstream signaling molecules to set off cascades of signaling events.

Some of the most important mitogenic effects of insulin are mediated through GRB-2 signaling. After insulin binds its receptor the IR substrates; IRS, GAB-1 and Shc are phosphorylated and bind to GRB-2 which activates the mitogen-activated protein (MAP) kinase pathway. The activated MAP kinase cascade leads to different proliferative effects [98]. The majority of the metabolic effects are mediated by the IRS-PI3K signaling pathway which is discussed below, but there is also the Cbl pathway which mediates glucose uptake. The Cbl protein is recruited to the trans-phosphorylated IR by APS (adaptor protein containing pleckstrin homology and SH3) where it is phosphorylated. Following Cbls recruitment and phosphorylation it relocalizes to lipid rafts via binding to Cbl associated protein (CAP), which interacts with Cbl via a SH3 domain and to lipid rafts via interactions with flotillin [97]. The recruitment to lipid rafts generates activation of additional proteins which cause membrane translocation of the intracellular glucose transporter, GLUT4 [99].

The IRS proteins are particularly important among the IR substrates, as they mediate most of insulin's metabolic effects and also some mitogenic effects. The N-terminal part of the IRS isoforms has one pleckstrin homology (PH)

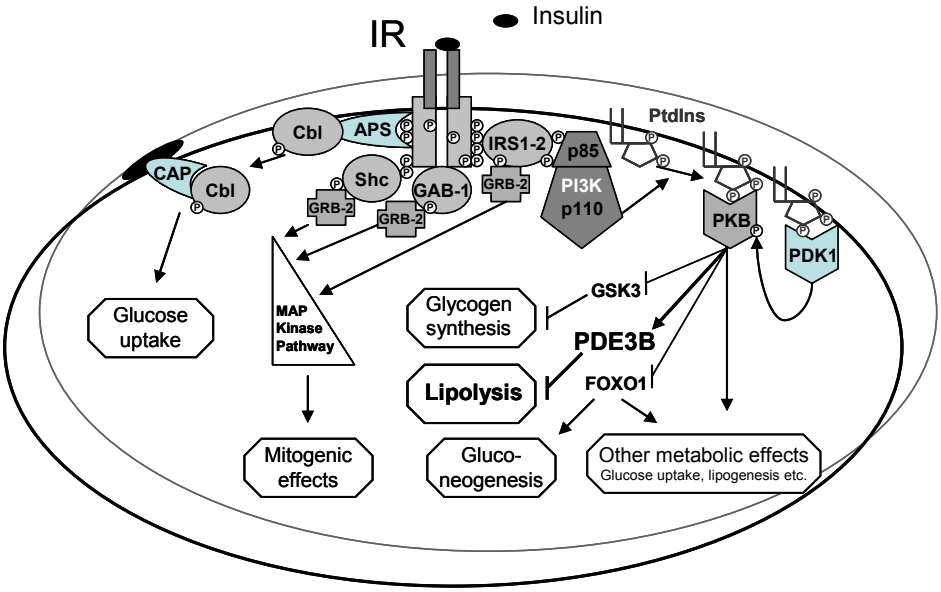

Figure 3: Overview of insulin signaling pathways
Insulin binds its receptor and initiates different signaling cascades which leads to different cellular effects. IR; insulin receptor, APS; adaptor protein, CAP; Cbl associated protein, Cbl; casitas B-linage lymphoma protein, Shc; SH2-domain containing proteins, GAB; GRB2-associated binder protein, IRS; insulin receptor substrate, PI3K; phosphatidylinositol 3-kinase, PKB; protein kinase B, PDK; phosphoinositide-depentent kinase, GSK; glycogen synthase kinase, PDE; cyclic nucleotide phosphodiesterase and FOXO which is a transcription factor.

domain and one PTB domain which are both important for binding to the IR. The C-terminal part of the proteins is less conserved and contains multiple tyrosine phosphorylation sites. For example IRS-1 contains 21 potential tyrosine phosphorylation sites of which several have been shown to be phosphorylated in response to insulin [94]. The different IRS isoforms have both distinct and overlapping signaling properties, tissue distribution and subcellular localization [100]. The IRS-1 and -2 are for example both associated with intracellular membranes, whereas IRS-3 and -4 are located at the PM [101]. The most widely distributed isoforms are IRS-1 and -2. Studies from IRS-1 and IRS-2 KO mice indicate that IRS-2 plays a major role in hepatocyte insulin signaling and pancreatic beta cell growth/differentiation while the IRS-1 isoform plays a major role in the adipose tissue and skeletal muscle [102, 103]. The exact role for the other isoforms are more difficult to dissect as the IRS-3 and -4 KO mice only show mild defects [104] [105] and the IRS-5 and -6 isoforms have very low tissue expression.

PI3K, which interacts with IRS proteins, is one key player in the insulin signaling network. Class IA PI3K is the major type of PI3K, which is activated by receptor tyrosine kinases like the IR [106]. Class IA PI3Ks are tightly bound heterodimeres with a 110 kDa catalytic subunit and a smaller regulatory subunit. The catalytic subunit is one out of three different isoforms, p110α, p110β or p110δ and the regulatory subunit is one out of the five isoforms, p85α, p85β, p85γ, p55α and p50α [107]. In resting cells the regulatory subunit stabilizes the catalytic subunit and conformationally inhibits the lipid kinase activity of the catalytic subunit [108]. In insulin stimulated cells the PI3K heterodimer is recruited to specific phosphotyrosine residues on IRS proteins by the two SH2 domains in the regulatory domain. The recruitment brings the catalytic subunit in proximity to its lipid substrate, allowing it to generate phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) [109]. There has been some debate regarding the true physiological substrate of the Class I PI3K, but it is now generally considered that PtdIns(4,5)P_2 is the substrate to form PtdIns(3,4,5)P_3. The other "activating" PtdIns, PtdIns(3,4)P_2 is indirectly generated from PI3K since it is a breakdown product of PtdIns(3,4,5)P_3 created by the SH2-containing inositol phosphatase, SHIP [107]. The PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are the major signals generated from Class I PI3K although it is likely that PI3K also is involved in additional protein-protein interactions or scaffolding functions in signaling complexes. For example activated PI3Kγ (with p110γ) is thought to bind PDE3B in the heart, independent of its lipid kinase activity [110]. The generated PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 can interact with several proteins containing PH domains such as phosphoinositide dependent kinase (PDK)-1, protein kinase B (PKB) and atypical isoforms of protein kinase C (aPKC). The interaction between PtdIns(3,4,5)P_3/ PtdIns(3,4)P_2 and the PH domain translocates the PH domain containing protein from the cytosol to the membrane which brings it into close proximity with substrates or binding partners. The interaction itself can in certain cases also regulate the protein activity since binding of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 to a PH domain can depress an intermolecular inhibition mediated by the PH domain. PDK-1 is constitutively active independent of PH domain binding and has a major role in the PI3K dependent activation of PKB and aPKC [111]. The binding of PKB and PDK-1 to PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 makes PDK-1 come in close proximity to Thr308/9 of PKBα/β which becomes phosphorylated [107]. In addition to Thr308/9, PKB is also phosphorylated at Ser473/4, in response to insulin, by TORC2, a complex of mammalian target of rapamycin (mTOR), rictor and G-protein β-subunit like protein (GβL) [112, 113].

PKB is a serine/threonine kinase that exists in three isoforms PKBα, PKBβ and PKBγ (also known as AKT1-3). Both PKBα and PKBβ are widely expressed, while PKBγ is present mainly in the nervous system. The PKBβ is particularly abundant in insulin sensitive tissues such as the adipose tissue and interestingly mice lacking PKBβ develop diabetes [114]. PKB has numerous intracellular targets, which mediate a large number of metabolic effects. One example of a PKB target is PDE3B which is phosphorylated and activated by PKB and inhibits catecholamine stimulated lipolysis by hydrolysing cAMP (figure 1). Another target of PKB is glycogen synthase kinase (GSK) 3, which is deactivated by the phosphorylation of PKB causing activation of glycogen synthase and production of glycogen [115, 116]. The transcription factor FOXO1 is also phosphorylated by PKB. The phosphorylated FOXO1 interacts with 14-3-3 and is thereby kept in the cytosol and cannot activate the transcription of gluconeogeneic enzymes or suppress genes involved in for example lipogenesis [117]. Insulin's signaling pathways and effects are summarized in figure 3.

cAMP signaling

An important mediator of intracellular signaling is cAMP. This second messenger controls a variety of metabolic effects such as gluconeogenesis, lipolysis and glycogenolysis as well as differentiation, proliferation, secretory processes, muscle contraction and apoptosis. As illustrated in figure 4, cAMP is generated from ATP by adenylyl cyclase (AC), which occurs at the PM in

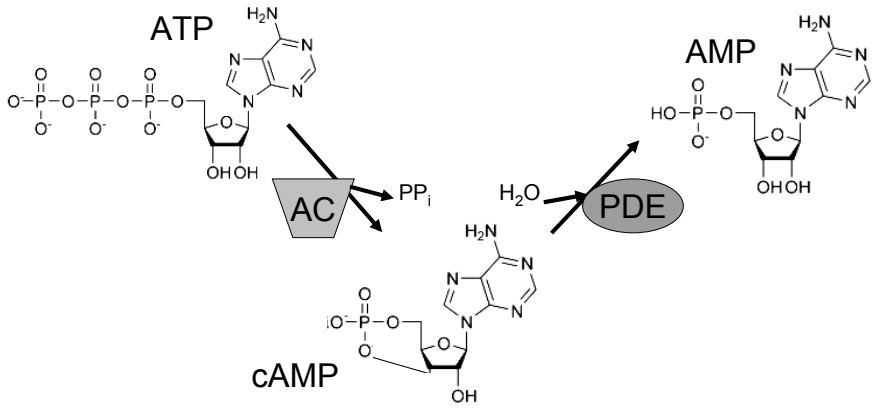

Figure 4: Synthesis and degradation of cyclic adenosine 3´, 5´-monophosphate (cAMP)
AC; adenylyl cyclase, PDE; cyclic nucleotide phosphodiesterase.

response to various hormones and neurotransmitters. AC is activated in response to specific hormones and neurotransmitters which bind to their G_s protein coupled receptors. The activated receptor leads to an exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) at the α-subunit of the heterotrimeric G-protein, G_s. The GTP bound α-subunit dissociates from the whole G_s and can then bind and activate AC [118]. The most common type of GPCRs coupled to G_s are the β-AR (β_1-AR, β_2-AR, β_3-AR), which bind adrenalin and noradrenalin. The β_1-AR and β_2-AR are broadly distributed throughout the tissues of the body while β_3-AR is found mainly in adipocytes [119]. The generated cAMP diffuses throughout the cell to its effector proteins as seen in figure 5. PKA is one important effector which is activated upon cAMP binding and can phosphorylate a myriad of substrates
for example perilipin and HSL in adipocytes. Other ways through which cAMP exerts its effects are via the activation of cyclic nucleotide gated ion channels (CNGC) which is involved in transduction of olfactory and visual signals [120]. cAMP can also bind and activate Epac (Exchange protein directly

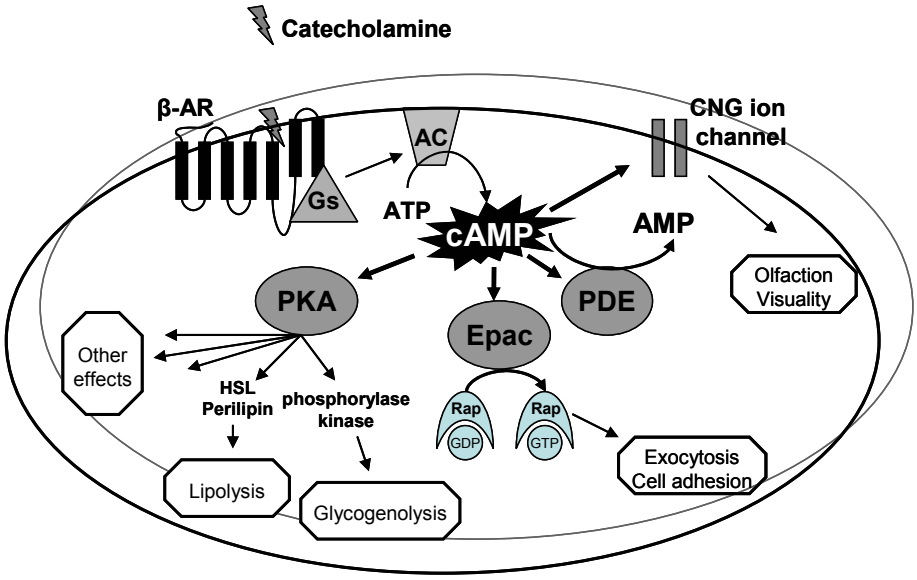

Figure 5: Overview of cAMP signaling pathways
Catecholamines bind the β-adrenergic receptor (β-AR) which gives rise to production of cAMP, which stimulates different signaling cascades. G_s; stimulatory G-protein, AC; adenylyl cyclase, PKA; protein kinase A, Epac; exchange protein directly activated by cAMP, PDE; cyclic nucleotide phosphodiesterase, CNG; cyclic nucleotide gated, HSL; hormone sensitive lipase, Rap; small GTPases.

activated by cAMP) proteins which are guanine-nucleotide-exchange factors (GEF) for the small GTPases Rap1 and Rap2 [121]. Epac proteins havemultiple biological functions; they mediate cell adhesion, cell-cell junction formation or exocytosis for example during insulin secretion. Finally cAMP can also bind to the catalytic domain of PDEs. At this location cAMP is hydrolysed to adenosine monophosphate (AMP). The intracellular concentrations of cAMP are tightly regulated and seem to be temporally, spatially and functionally compartmentalized. For example it has been shown that β-cells respond to glucagon and GLP-1 with marked cAMP oscillations [122]. The cAMP oscillations seemed to be important for local rapid events such as exocytosis while prolonged cAMP elevation instead mediated cell survival and proliferative signals. The tight regulation is facilitated primarily by AC and PDEs, which together thereby modulate the specificity, duration and intensity of the cAMP signals. The specificity of the cAMP signal is also generated by the intracellular organization of signaling proteins within the cell [123, 124]. The organization is facilitated by different anchoring proteins, for example A-kinase anchoring proteins (AKAP) keep PKA to distinct subcellular locations, which makes PKA ready to respond to local cAMP concentrations. Recently AKAPs have also been suggested to be important for the organization of PDEs [125].

Phosphodiesterase 3B

Cyclic nucleotide phosphodiesterases (PDE)

PDEs were discovered 40 years ago [126, 127] approximately the same time as the discovery of cyclic nucleotides. Since then the understanding of the PDEs has increased markedly, although the more we understand, the more complex the emerging picture of the PDEs becomes. A phosphodiesterase is an enzyme that catalyzes the hydrolysis of phosphodiester bonds and in this work the focus has been on the class I cyclic nucleotide phosphodiesterases. This group is highly specific for catalyzing the hydrolysis of the 3´cyclic phosphate bond of cAMP and/or cyclic guanosine monophosphate (cGMP) generating AMP guanosine monophosphate (GMP) respectively. The cGMP system will not be discussed further since the focus of this thesis is on cAMP. The production of cAMP from adenosine triphosphate (ATP) achieved by AC and the hydrolysis by PDE is illustrated in figure 4. The PDEs are highly regulated and keep the cAMP under tight control, which is important considering the many functions of cAMP in different tissues. Another fact that points to the important role of PDEs is the identification of 21 PDE genes in mouse, rats and humans which is estimated to give rise to >100 different mRNA products [128]. Although not all protein products from the different mRNAs have been identified, most products

do have different physiological effects. These effects range from cell division and metabolism to immunological aspects and light perception.

To keep track of the different PDEs, a common nomenclature has been decided upon. The PDEs have been categorized into 11 different families (PDE1-11) based on enzymatic properties such as specificity for its substrates (cAMP/cGMP), kinetic properties and structural similarities. Structural domains present in the members of some families include small molecular binding (GAF) domains, Ca2+/calmodulin (CaM) binding domains, N-terminal hydrophobic regions (NHR) domains, upstream conserved regions (UCR), response regulator receiver (REC) and per-arnt-sim (PAS) domain[129]. The activity and localization of PDEs are very well regulated by different mechanisms such as binding of cGMP (in the GAF-domains), binding of Ca^{2+}/calmodulin, phosphorylations, interactions with regulatory proteins and alteration in gene expression. To add to this complexity the cells have organized the PDEs in a brilliant way to generate well regulated cAMP/cGMP levels. For example cardiac myocytes express several different PDEs and these are localized to different compartments and signaling complexes, which control local concentrations of cAMP/cGMP [130].

To unravel the role of the different families and subfamilies in the complex group of PDEs it has been important with family specific PDE inhibitors and KO animals lacking specific subfamilies of PDEs. KO animals lacking the PDE subfamilies PDE1B, 3A, 3B, 4A, 4B, 4C or 4D and family selective inhibitors have been developed by research groups and pharmaceutical companies as the involvement of PDEs in many pathological conditions has emerged[128]. Unfortunately none of the inhibitors are specific to subfamilies and most of them are only partly family specific like Sildenafil also known as Viagra© from Pfizer. This is a PDE5 specific inhibitor, although it inhibits PDE6 as well with a higher IC_{50} value. Currently the catalytic domain of seven PDE families [128] have been crystalized and hopefully this will generate more subfamily specific inhibitors to use both as pharmaceuticals with less side effects and as tools to gain understanding of the complex functions and regulation of PDEs.

The PDE3 family

Among the first to study phosphodiesterases in adipocytes were Loten et al [131] and Manganiello et al [126] in the 1970s. It was found that a “low Km cAMP phosphodiesterase” was present in adipocytes and could be stimulated by insulin [131]. A particulate “low Km cAMP phosphodiesterase” was later found to be the main target of insulin stimulation [132, 133]. These studies led to the identification of the adipocyte insulin sensitive PDE now called PDE3B

[134, 135]. The PDE3 family is distinguished from other PDEs mainly by three features; (1) The PDE3 family has the ability to hydrolyze both cAMP and cGMP with high affinity (K_m around 0.1-0.8 μM) in a competitive manner; (2) The PDE3 family has a much lower maximal velocity (V_{max}) for cGMP than cAMP and has therefore been called the cGMP inhibited low K_m phosphodiesterase; (3) The PDE3 family is sensitive to specific inhibitors like milrinone, cilostamide, cilostazol and enoxione.

The PDE3 proteins are the products of two different, but highly related genes, which give rise to the subfamilies PDE3A and PDE3B [135, 136]. The PDE3A gene encodes three different isoforms PDE3A1, PDE3A2 and PDE3A3 [137, 138] while the PDE3B gene generates one isoform. The overall predicted structural organization of PDE3A1 and PDE3B is identical, while PDE3A2, and PDE3A3 are shorter versions of PDE3A1. The N-terminal part, which is shorter in the PDE3A2 and PDE3A3 isoforms, contains a regulatory domain with hydrophobic regions. PDE3B is a 135 kDa protein with around 1100 amino acids depending on the species. As shown in figure 6 the most N-terminal part of PDE3B contains a large hydrophobic region with 5-6 predicted transmembrane helical segments, NHR1 followed by a smaller hydrophobic region, NHR2 [139].The C-terminal part of PDE3 has a conserved catalytic domain followed by a hydrophilic region. The catalytic domain is similar among all PDEs, but the PDE3 family contains a 44aa insert that does not align with sequences in the corresponding regions of other PDEs and differs between

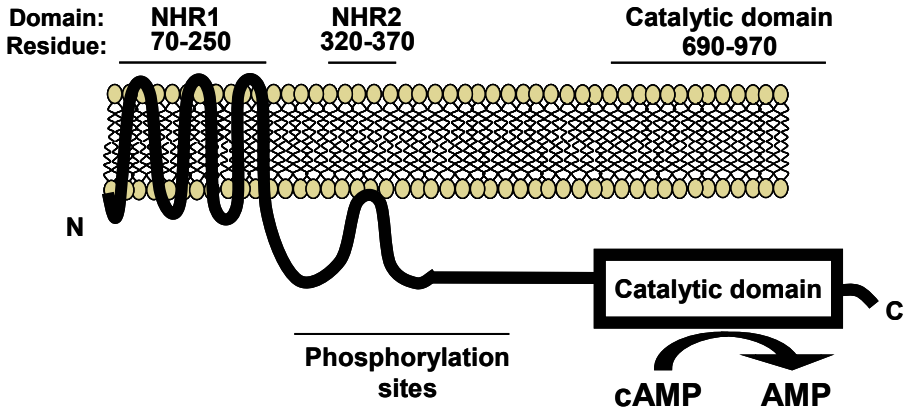

Figure 6: Structure of PDE3B
Predicted structure of cyclic nucleotide phosphodiesterase (PDE) 3B. NHR; N-terminal hydrophobic regions.

PDE3A and PDE3B [135, 136]. PDE3B and PDE3A appear to have distinct distributions although some overlap occurs. After in situ hybridization, Northern blot or immunohistochemical analysis PDE3B is found in white and brown adipocytes, hepatocytes, renal collecting epithelium, spermatocytes, β-cells and developing brain while PDE3A is found in platelets, heart, vascular smooth muscle cells, developing brain and oocytes [19, 140, 141]. In some types of cells the functional role of the enzyme has been studied using PDE3 inhibitors, which suggests a role for PDE3A in cAMP-mediated processes like platelet aggregation, vascular and airway smooth muscle relaxation, oocyte maturation, inflammation and cell proliferation and a role for PDE3B in insulin secretion and lipolysis [19, 142]. Studies on the PDE3A and PDE3B KO mice have made it possible to further evaluate the role of each subfamily. In a study comparing PDE3B and PDE3A KO mice it was shown that PDE3A is the dominant PDE3 isoform that regulates platelet aggregation, peripheral vascular relaxation and cardiac contractility. It has also been shown that the PDE3A KO female mice are infertile since the meiotic maturation of oocytes is blocked, which is not the case for the PDE3B KO mice [143]. Roles of the different PDE3 isoforms have also been studied to some extent. For example in the human myocardium different roles for the PDE3A isoforms in the regulation of sarcoplasmic reticulum and cytosolic cAMP levels have been suggested [144].

PDE3B in energy metabolism

PDE3B is highly abundant in cells important for regulation of normal energy metabolism like adipocytes, hepatocytes and pancreatic β-cells. The presence of PDE3B in these cell types implies an important role for this enzyme in regulating different metabolic processes. In adipocytes the role of PDE3B has been studied extensively and it is now well established that PDE3B is rapidly activated in response to insulin and that this activation is important in antagonizing catecholamine stimulated lipolysis. By using selective PDE3 inhibitors PDE3B has been shown to be essential for the antilipolytic actions of insulin in isolated rat adipocytes [145], 3T3-L1 adipocytes [146] and human adipose tissue in vivo [147]. Furthermore, insulin-induced PDE3B activation in adipocytes has been shown to be important for both insulin stimulated glucose uptake and lipogenesis [148-150]. A recent study from 2007 shows that insulin in a PDE3B dependant manner restores cAMP-suppressed expression and secretion of leptin and adiponectin in adipocytes [151]. In addition to insulin catecholamine and forskolin can also rapidly stimulate PDE3B in adipocytes [152-154]. The catecholamine induced activation of PDE3B is probably a negative feedback mechanism of increased cAMP and hinders an excessive increase of cAMP. In hepatocytes PDE3B has not been studied as extensively as in adipocytes, but it has been shown that PDE3B is activated in response to

leptin, glucagon, insulin and growth hormone [155-157]. The exact role for the activation is not yet established although studies using different cAMP analogs indicate a role for PDE3 in reducing glucagon stimulated glycogenolysis and gluconeogenesis [158]. Furthermore it has been shown that isolated hepatocytes incubated with PDE3 inhibitors have increased glycogenolysis, gluconeogenesis [159] and glucose output [160] compared to control cells. As seen on electron micrographs of mice livers in figure 9, PDE3B seems to be localized primarily to smooth ER of the hepatocytes, which indicates that PDE3B is involved in metabolic processes since smooth ER is the main compartment of synthetic metabolic processes such as lipid syntehsis [161].

When it comes to pancreatic β-cells it has been shown that PDE3B has an important role in the regulation of glucose and glucagon-like peptide-1 (GLP-1) stimulated secretion of insulin in isolated β-cells and INS-1 cells [162, 163]. PDE3B seems to mediate the IGF-1 and leptin induced inhibition of GLP-1/cAMP stimulated insulin secretion [141, 164]. A study by Walz et al shows that PDE3B is localized at the insulin granule and can inhibit both first and second phase of insulin release [165]. PDE3B might also be important in hypothalamus where leptin stimulates satiety signals. A study by Zhou et al has shown that injection of PDE3 inhibitors into the brain completely blocks the satiety and weight-reducing effects of leptin in hypothalamus [166]. Although Zhao et al state that this is the effect of PDE3B inhibition since PDE3B is expressed in hypothalamus, the effect of PDE3A can not be ruled out as PDE3A might also be expressed in adult hypothalamus.

The important role for PDE3B in adipocytes, hepatocytes and β-cells has been confirmed in studies with whole animals injected with PDE3 inhibitors and in studies with PDE3B KO mice. Rats injected with different PDE3 inhibitors clearly showed elevated plasma insulin, elevated plasma FFA and slightly elevated glucose levels as compared to control animals [167-169]. These results support a role for PDE3B in the inhibition of GLP-1 and/or glucose stimulated insulin secretion from β-cells and in the antilipolytic actions of insulin in adipocytes. The slightly elevated glucose levels were shown to be the result of increased glucose production, which supports a role for PDE3B in the inhibition of glucose production from the hepatocytes. The in vivo studies with PDE3 inhibitors have been complemented with studies of subfamily specific PDE3B KO mice. Results from the PDE3B KO mice support an important role for PDE3B in different metabolic functions of the adipocytes, β-cells and hepatocytes [170]. The PDE3B KO animals had increased fasting FFA and glycerol levels together with blocked suppression of lipolysis by insulin compared to WT mice, showing an important role of PDE3B in the regulation of lipid metabolism. Insulin secretion was increased in the PDE3B KO mice

which could be measured both in the whole animal and in isolated β-cells, which shows the role for PDE3B in the regulation of insulin secretion from the β-cell. The PDE3B KO mice were insulin resistent with a slightly lower whole-body glucose uptake compared to WT mice. The main cause of the insulin resistence was a defect in the liver since the insulin-induced suppression of endogenous glucose production was markedly lower in the PDE3B KO mice compared to WT mice. The livers of the PDE3B KO mice had increased levels of phosphoenol-pyruvat carboxykinase (PEPCK), glucose-6-phosphatase and PPARγ coactivator (PGC)-1α mRNA, which is consistent with enhanced glucose production. Interestingly the liver of the PDE3B KO mice also had increased TG levels and mRNAs of pro-inflammatory cytokines like IL-1, IL-6 and TNF-α compared to WT mice. Unpublished results from the adipose tissue of the PDE3B KO show that an ablation of PDE3B causes increased energy dissipation and fatty acid oxidation. The lack of PDE3B appears to change the phenotype of the white adipose tissue so that it displays features of brown adipose tissue. In summary results from the PDE3B KO mice show that the expression of PDE3B is important for the adipose tissue phenotype and whole body metabolism.

As described in the adipose tissue section, dysregulated lipid metabolism can cause development of T2D. As PDE3B is important in the regulation of lipid metabolism, a possible involvement of the enzyme in diabetes has been explored. Patients with untreated T2D and T1D have been shown to have decreased PDE3B activity in the subcutaneous adipose tissue [171]. Interestingly if the diabetes was treated the reduction of PDE3 activity was normalized. Studies of PDE3B have also been performed in the KKAy mice and db/db mice which are models of obesity, insulin resistence and diabetes. The KKAy and db/db mice had decreased levels of PDE3B mRNA, protein and activity in the adipose tissue, but after treatment of diabetes with a PPARγ ligand the PDE3B levels were normalized in the animals [172-174]. The animal and human studies indicate a possible role for PDE3B in the development of diabetes.

Regulation of PDE3B in adipocytes

Changes in adipose tissue PDE3B expression of diabetic patients and mice is interesting and the regulatory mechanism of PDE3B expression has been studied to some extent. The expression of PDE3B in 3T3-L1 adipocytes seems to be affected by long-term incubation of many different agents. For example PDE3B expression is down regulated in response to TNF-α, ceramides and isoproterenol and up regulated in response to insulin [175-177]. The possibility that TNF-α and/or catecholamines mediate down regulation of PDE3B during

diabetes development remains to be further elucidated. The PDE3B gene promotor region has one proximal and one distal cAMP responsive element (CRE) [178]. During 3T3-L1 adipocyte differentiation when PDE3B is markedly up regulated, the CRE regions interact with phosphorylated CRE-binding protein (CREB) which indicates a critical role for the CRE regions in the regulation of PDE3B expression [179].

Compared to long-term regulation of PDE3B expression acute hormonal activation of PDE3B has been more extensively studied and the knowledge goes back over 40 years at the time of the phosphodiesterase discovery. The activation of PDE3B has been shown to be the result of serine phosphorylation of the enzyme both in response to insulin and catecholamines [180]. When it comes to which sites are phosphorylated in response to insulin and isoproterenol and responsible for the activation, some discrepancy exists. In 1994 Rascon et al [181] was the first to identify a phosphorylation site on rat adipocyte PDE3B, namely serine (S) 427 (S421 in mouse) which was phosphorylated in vitro after addition of active PKA to the enzyme. In primary rat adipocytes it was then shown that S302 (S296 in mouse) was the major site phosphorylated in response to both insulin and isoproterenol [182]. In another study using site-directed mutagenesis the results showed that S273 was the only site important for PDE3B activation in response to insulin in 3T3-L1 adipocytes [183]. In addition, phosphorylation of S296 was shown to be the main site responsible for isoproterenol induced activation of PDE3B. As expected, it has also been shown that protein phosphatases (PP) and especially PP2A, are important for the regulation of PDE3B [184, 185]. In my studies one major focus has been to resolve the discrepancies regarding PDE3B phosphorylation sites. My results show that PDE3B is phosphorylated on multiple sites both in adipocytes and hepatocytes which partly can explain the discrepancies described here (see present investigations).

The regulatory process whereby phosphorylation of PDE3B occurs in response to insulin is PI3K dependent [186, 187]. Accumulating evidence suggests that PKB is the kinase responsible for insulin induced phosphorylation of PDE3B. Partially purified PKB from insulin stimulated primary rat adipocytes co-eluted with insulin stimulated PDE3B kinase activity during ion-exchange chromatography [187]. Two other studies supported the co-elution study by showing that recombinant PKB could phosphorylate PDE3B in vitro in 3T3-L1 adipocytes and FDCP2 cells [183, 188]. When it comes to the kinase responsible for catecholamine induced PDE3B phosphorylation one potential candidate is PKA, as it is activated by cAMP. A study by Zmuda-Trzebiatowska et al recently showed that the PKA inhibitor, H89, could block β_3-AR agonist induced activation of PDE3B in adipocytes, indicating a role for

PKA in catecholamine stimulated PDE3B activation [149]. Interestingly, the same study shows that PKB is phosphorylated on Ser473/4 in response to both a β_3-AR agonist and insulin in a PKA dependent manner. Together the results suggest that PKB is involved in catecholamine stimulated activation of PDE3B in adipocytes and that the activity of PKA is important for the activation of PKB in response to both β_3-AR agonist and insulin, illustrated with a dotted line in figure 1.

Other aspects important for proper understanding of hormone regulation of PDE3B are PDE3B's localization in the cell and possible interaction partners. The localization is important especially when compartmentalization aspects come into the picture, as discussed before. The first studies of PDE3B showed that the enzyme mainly was associated with membrane structures of adipocytes [133, 134]. Several years later this knowledge was expanded as it was shown that the N-terminal hydrophobic parts, NHR1 and NHR2, are essential for this association to the membranes and for PDE3B to migrate into high molecular weight structures during gelfiltration [134, 189]. In 2000 it was shown by Shakur et al that PDE3B is localized to the ER in 3T3-L1 adipocytes and that this localization is dependent on the N-terminal hydrophobic parts, NHR1 and NHR2 [139]. My studies have further gained insight into the localization of PDE3B and the regulation of PDE3B at different locations. Gelfiltration results showing that PDE3B appears in a high molecular weight complex indicate that PDE3B interacts with other proteins. Recent studies have identified several PDE3B interaction partners e.g. the insulin receptor in human adipocytes [190], 14-3-3 in rat adipocytes [191], a 50 kDa protein in rat adipocytes [192], a 47 kDa PKB (Akt) substrate (AS47) in 3T3-L1 adipocytes [193] and PI3Kγ in mouse heart [194]. Interestingly the 14-3-3 interaction is dependent on PI3K activation and phosphorylation of PDE3B on S273 or S296. The 50 kDa protein and AS47 are probably the same protein and the studies show that the protein is phosphorylated in response to insulin and that the amount of AS47 protein regulates the quantity of PDE3B expressed. The studies I have performed have led to new insights into protein interactions of PDE3B in adipocytes and the connection of PDE3B interactions to its localization.

To summarize, a lot is known and has been studied about the regulation of PDE3B in adipocytes but still there are things we cannot grasp. The more we understand of the PDE3B regulation the more complex is the evolving picture, with the addition of one dimension after another.

PRESENT INVESTIGATION

Aim

The overall aim of this thesis was to contribute knowledge regarding regulation of lipid metabolism. Important regulatory pathways of lipid metabolism in adipocytes are insulin and catecholamine mediated stimulation of phosphodiesterase 3B (PDE3B). Thus, the focus of this thesis has been on the acute activation of PDE3B in response to insulin and catecholamines in adipocytes. The activation of an enzyme like PDE3B can be studied with different perspectives, but based on the knowledge at the start of this work, the specific aims of this thesis were the following.

- Identify sites phosphorylated in response to insulin and catecholamines in PDE3B of primary adipocytes. (Paper I)

- Clarify PDE3B's localization in adipocytes and characterize the role of PDE3B's localization for its activation by insulin and catecholamines. (Paper II and IV)

- Identify PDE3B interacting proteins in insulin and catecholamine stimulated adipocytes. (Paper III and IV)

Experimental outline

The methodologies used to reach the specific aims are described thoroughly in the papers. Here is a brief overview of the experimental outline also summarized in figure 7. The cells used in the experimental set-up were either 3T3-L1 adipocytes or primary mouse or rat adipocytes. 3T3-L1 adipocytes were differentiated from cultured 3T3-L1 fibroblasts (American Type Culture collection, Manassas, USA) by adding IBMX (a non-specific phosphodiesterase inhibitor), dexamethesone (synthetic steroid hormone) and insulin for 8-16 days. Primary adipocytes were isolated from epididymal adipose tissue of male Sprague-Dawley rats, human adipose tissue or from caveolin-1 KO mice by collagenase digestion. The isolated primary adipocytes were used immediately after preparation and the differentiated 3T3-L1 adipocytes were used immediately after differentiation. In certain experimental set-ups the adipocytes were pre-treated with different agents before hormone stimulation (figure 7A). The adipocytes were pre-treated with atorvastatin or methyl-β-cyclodextrin to lower the cholesterol content in the cell and plasma membrane or caveolin-1 siRNA to knock down the caveolin-1 expression. In some studies the adipocytes were infected with adenoviruses to express WT and mutated forms of PDE3B and PKB. In other experiments the adipocytes were labelled with ^{32}P or incubated with different inhibitors before hormone stimulation. After pre-treatment, the adipocytes were stimulated with insulin and/or catecholamines like isoproterenol (β-AR agonist) or CL-316243 (β_3-AR agonist) for 10-20 min (figure 7B). The stimulated adipocytes were in most cases homogenized and the homogenates were subjected to different separation strategies like subcellular fractionation to study the subcellular locations of PDE3B, gelfiltration to study PDE3B complex formation, caveolae enrichment to investigate PDE3B's localization, two dimensional (2D) phosphopeptide mapping to separate PDE3B phosphopeptides and immunoprecipitation to purify PDE3B and PDE3B interacting proteins (figure 7C). The different fractions or whole cells were finally analyzed using a wide variety of techniques and approaches (figure 7D). The fractions were analyzed by PDE3 activity measurements, immuno-blotting to detect different proteins, cholesterol/protein measurements and phospho-imaging to look at phosphorylated PDE3B and PDE3B peptides. Purified phosphopeptides were analyzed by mass-spectrometry to identify the phosphorylated PDE3B peptides. Whole cells or plasma membranes of whole cells were analyzed with confocal microscopy and electron microscopy to look at the subcellular localization of PDE3B and co-localization of PDE3B with PKB or caveolin-1. Whole cells were in certain set-ups also analyzed for glycerol release to draw conclusions on the rates of lipolysis. The results were summarized and the significance of each experiment was evaluated.

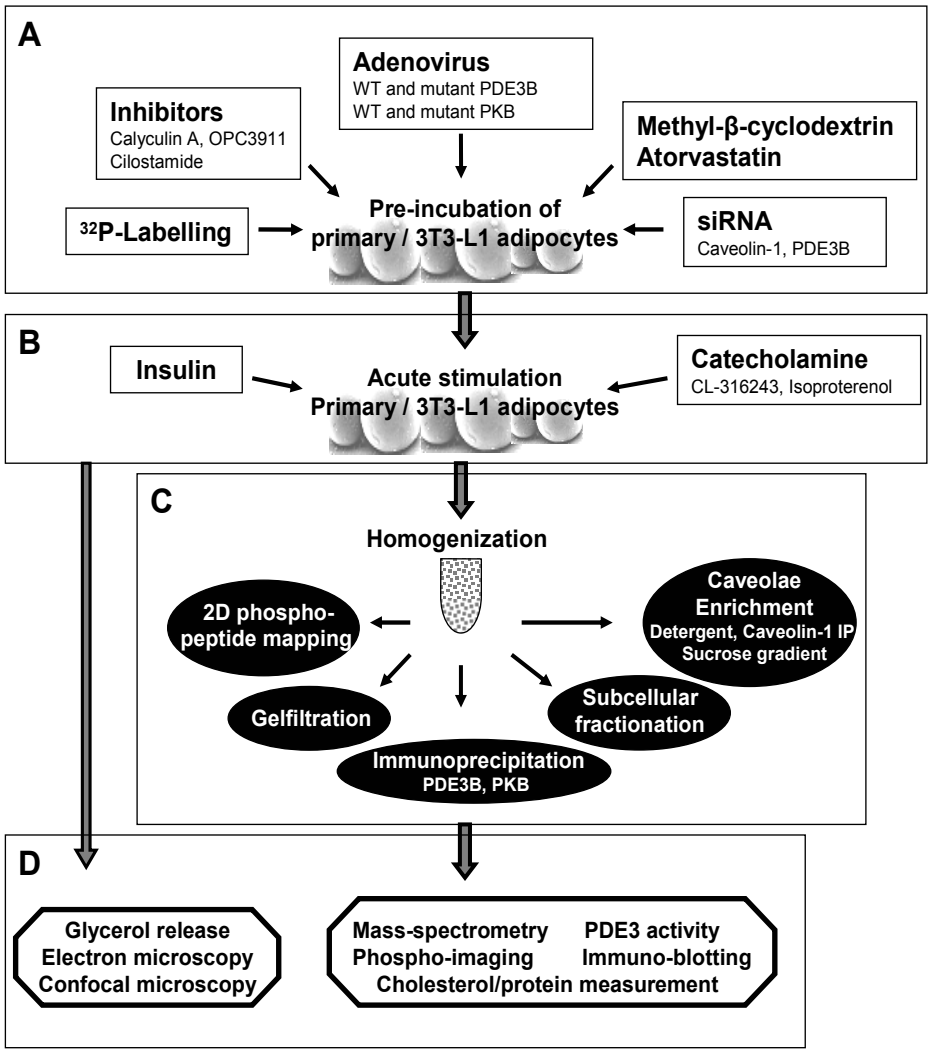

Figure 7: Experimental overview
Primary rat/mouse adipocytes or 3T3-L1 adipocytes were pre-treated with different substances (**A**) and then stimulated with insulin or catecholamines (**B**). The stimulated cells were in most experiments homogenized and the homogenates were subjected to different fractionation techniques (**C**). The fractions or whole cells were finally analyzed using a wide variety of approaches (**D**). siRNA; small interference RNA, IP; immunoprecipitation, 2D; two dimensional.

PDE3B phosphorylation in adipocytes (Paper I)

Background

Previous studies had shown that the activation of PDE3B in response to insulin and catecholamines were important for the regulation of adipocyte lipolysis [131, 132, 145, 153, 154] (figure 1). Insulin activated PDE3B antagonized catecholamine induced lipolysis, while catecholamine activated PDE3B was thought to primarily be a negative feedback mechanism of increased cAMP levels. It had also been shown that activation of PDE3B was the result of serine phosphorylation of the enzyme in adipocytes [180]. Regarding the identity of the serines phosphorylated in adipocyte PDE3B in response to insulin and catecholamines few studies had been published. A study by Rahn et al had identified S302 (corresponding to S296 in mouse) as the major site phosphorylated in adipocytes in response to both insulin and isoproterenol by 2D tryptic phosphopeptide (tPP) mapping of endogenous PDE3B from primary rat adipocytes [182]. Another study by Kitamura et al had used site-directed mutagenesis of PDE3B in 3T3-L1 mouse adipocytes. In this study S273 was shown to be important for insulin-mediated phosphorylation and activation of PDE3B whereas S296 was demonstrated to be important for isoproterenol but not insulin-mediated phosphorylation and activation of the enzyme [183]. The knowledge regarding sites phosphorylated in PDE3B in response to insulin and isoproterenol were therefore partly contradictory at the time. In addition to the tPP containing S302, other tPPs had been weakly detected on the 2D phosphopeptide maps of endogenous adipocyte PDE3B in the study by Rahn et al [182]. Several endogenous PDE3B tPP had also been seen on 2D phosphopeptides maps after PP1 and PP2A inhibition in adipocytes by okadaic acid and calyculin A [185]. Both of these studies indicated that PDE3B in adipocyte was phosphorylated at several different sites, although most of the tPPs were not possible to identify due to the limited amount of phosphorylated PDE3B. Based on these partly contradictory results and incomplete information we wanted to clarify which sites are phosphorylated in response to insulin and isoproterenol, and elucidate if PDE3B is multisite phosphorylated in adipocytes. Therefore the specific aim of this study was to identify the sites phosphorylated in response to insulin and catecholamines in PDE3B of primary adipocytes.

Results and discussion

To identify the sites phosphorylated in PDE3B it was important to generate sufficient amount of material for PDE3B site analysis, as this was one of the

previous limitations of identification. Thus, we used an adenoviral system to overexpress PDE3B in primary rat adipocytes. The adenovirus used expressed flag-tagged mouse PDE3B (flagPDE3B) and generated a 10-25 fold PDE3B overexpression after 6-14 h of adenoviral infection of primary rat adipocytes (paper I, figure 1). The overexpressed flagPDE3B was carefully characterized with regard to localization, regulation and function. As seen in figure 1C of paper I, the overexpressed flagPDE3B was localized to the same subcellular compartment as endogenous PDE3B i.e. in the plasma membrane and ER/Golgi fraction. The overexpressed PDE3B was also activated in response to various stimuli in the same manner as the endogenous PDE3B and mediated regulation of lipolysis (paper I, figure 2A and 4). From the described results it was concluded that the adenoviral generated overexpression of flagPDE3B in primary rat adipocytes is a useful and relevant model for studying PDE3B. We then started our search for PDE3B phosphorylation sites by generating 2D tryptic phosphopeptide maps of flagPDE3B phosphorylated in response to different stimuli. Primary rat adipocytes expressing flagPDE3B were labelled with ^{32}P and treated with insulin, isoproterenol or calyculin A. FlagPDE3B was immunoisolated and trypsin digested. The PDE3B tPPs were then separated on 2D tryptic phosphopeptide maps. Several tPPs denoted tPP1-13 were generated from the adipocyte PDE3B (Paper I, figure 5A-E) in response to the different agents. The results clearly showed that PDE3B is phosphorylated at multiple sites. Interestingly the PDE3B phosphopeptide maps generated from insulin and isoproterenol stimulated adipocytes were very similar. Of the 9 PDE3B tPP seen on the 2D phosphopeptide maps after insulin and isoproterenol stimulation, 8 were seen after both stimuli while one tPP was seen only after isoproterenol stimulation. The large similarities indicate that the signaling pathways of insulin and isoproterenol might converge upstream of PDE3B, maybe at the level of PKB. The separated and enriched PDE3B tPPs were also identified using mass-spectrometry (HPLC-MS/MS). We were able to identify six out of the 13 tPPs which were phosphorylated on serine(S) 273, S296, S421, S424/5 (peptide phosphorylated on S421 and either S424 or S425), S474 and S536 (Paper I, table 1). As shown in figure 5A-E of paper I, S273, S296 and S421 were phosphorylated in response to stimulation of rat adipocytes with insulin as well as isoproterenol whereas S424/5, S474 and S536 were phosphorylated mainly in response to calyculin A. Although the 2D phosphopeptide maps of PDE3B were similar after insulin and isoproterenol stimulation, there was one exception, namely tPP7 (figure 5B-C). Unfortunately we were not able to identify tPP7, but the difference indicates that there are also distinct regulatory mechanisms for insulin and isoproterenol at the level of PDE3B.

The results clearly show that PDE3B is multisite phosphorylated and S273, S296 and S421 are phosphorylated in response to insulin as well as isoproterenol in primary adipocytes. These results are in agreement with the study by Rahn et al [182], which shows phosphorylation of S302 in rat PDE3B (corresponding to S296 in mouse) in response to both insulin and isoproterenol in primary adipocytes. The results are not in perfect agreement with the study by Kitamura et al [183], but the differences can perhaps be explained by the different cell types and techniques used in the studies. Paper I is the first report on phosphorylation of the 135 kDa PDE3B in hepatocytes and evidence for the relevance of the identified PDE3B phosphorylation sites in primary adipocytes are the very similar results obtained from experiments in hepatocytes. In paper I figure 5G-J we show that the phosphorylation pattern of PDE3B generated from insulin and forskolin (cAMP increasing agent) stimulated hepatocytes were very similar, supporting that the same sites are phosphorylated in response to both insulin and forskolin. We also identified S273, S296 and S421 to be phosphorylated in hepatocytes stimulated with insulin and several other sites in response to calyculin A as seen in paper I, table 2. The results from the hepatocytes support that PDE3B is multisite phosphorylated, that insulin and cAMP increasing agents generate similar PDE3B phosphorylation patterns and that insulin stimulates the phosphorylation of S273, S296 and S421 on PDE3B.

The identification of a number of PDE3B phosphorylation sites S273, S296, S421, S424/5, S474, S536 and the demonstration of additional non-identified PDE3B tPPs show that the enzyme for sure is subjected to multisite phosphorylation in adipocytes. Multisite phosphorylation of PDE3B suggests that PDE3B is regulated by complex mechanisms. The different phosphorylation sites can have a wide variety of functions for example control of catalytic activity, docking with other proteins, subcellular localization, and protein degradation [195]. Regarding catalytic activity it is known that PDE3B is activated in response to phosphorylation and Kitamura et al [183] have shown that S273 and S296 are important for the activation of PDE3B, but if there are additional activating sites or any phosphorylation site that inhibits the activation is not known. When it comes to docking with other proteins it has been shown that PDE3B phosphorylation on S296 and S273 induce interaction with 14-3-3 [191]. Interestingly we have also shown in paper III that phosphorylated PDE3B interacts with other proteins in a multiprotein complex, these results are further discussed in the section *PDE3B interacting proteins in adipocytes*. Regarding subcellular localization, phosphorylation of PDE3B does not seem to change the subcellular localization of the enzyme as seen in paper IV, figure 2. The exact role for each of the different phosphorylations is not known, but it seems like phosphorylation of S273 and S296 in PDE3B can have direct effects on enzymatic activity by inducing conformational changes.

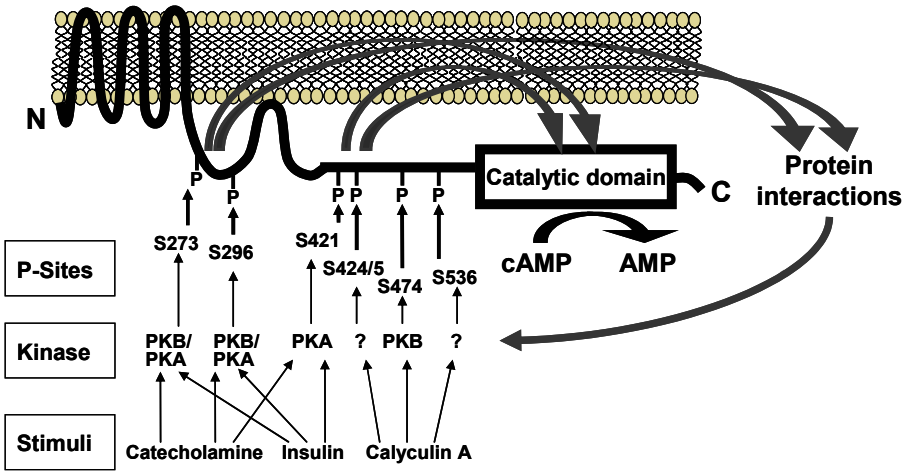

Figure 8: PDE3B phosphorylation in adipocytes
Identified PDE3B phosphorylation sites in response to different stimuli, possible kinases involved in the phosphorylation of the different sites in PDE3B based on search in Scansite 2.0 and possible effects of the phosphorylations. PKA; protein kinase A, PKB; protein kinase B, S; Serine, P-sites; phosphorylation sites, P; phosphor.

The phosphorylations can likely also have indirect effects through the induction of interactions. The interactions with other proteins could lead to additional phosphorylations or closeness to its substrate cAMP (figure 8).

The kinase/es responsible for the phosphorylation of the different PDE3B phosphorylation sites is/are not known. It could be that one kinase is responsible for the phosphorylation of several amino acids in PDE3B or that each phosphorylation site has one responsible kinase. In a search for possible PKA and PKB phosphorylation sites within PDE3B using Scansite 2.0 [196] several sites occurred with medium stringency. The search found four PKB phosphorylation sites with similar scores; S273, S296, S378 and S474, and four PKA sites; S273, S274, S296 and S421. Interestingly three of the predicted PKB sites (S273, S296 and S474) and three of the predicted PKA sites (S273, S296 and S421) were identified in this study, which indicates a possibility for these kinases to be involved in several of the phosphorylations. Another interesting aspect is that S273 and S296 are both potential targets for PKB as well as PKA catalyzed phosphorylations and both sites are phosphorylated in response to insulin and isoproterenol, which indicate an intricate regulatory system. One hypothesis on the matter is that insulin and β-adrenergic signaling pathways converge upstream of PDE3B at the level of PKB. It has been shown that PKB is a PDE3B kinase [183, 187, 188] and it is known to be

phosphorylated/activated both by insulin, β-AR agonists and phosphatase inhibitors in adipocytes [149, 197]. Another hypothesis is that isoproterenol mediates its effect on PDE3B via PKA and insulin mediates its effects via PKB and both kinases will phosphorylate PDE3B on S273 and S296. A role for PKA is supported by a previous study showing that PKA phosphorylates rat adipocyte PDE3B on S427 (corresponding to S421 in mouse) in vitro [181] and S421 is in this work identified to be phosphorylated by all stimuli. The sites identified in paper I, table 1 and possible kinases responsible for the phosphorylation of PDE3B and effects of the phosphorylations are summarized in figure 8.

PDE3B localization in adipocytes (Paper II and IV)

Background

The first studies on the localization of PDE3B in adipocytes had shown that the enzyme mainly was associated with membrane structures of adipocytes [133]. Studies from primary adipocytes had specified this localization to be primarily in the ER membrane fraction, but also to some extent in the PM fraction [198]. In another study with 3T3-L1 adipocytes the membrane association of PDE3B had been specified to the ER without any PM localization and the N-terminal hydrophobic parts, NHR1 and NHR2 of PDE3B were shown to be essential for the ER localization [19]. Based on these partly contradictory results we wanted to further clarify the localization of PDE3B in adipocytes by using primary adipocytes and several different subcellular fractionation methods. In addition we wanted to characterize the role of PDE3B's localization for its activation by insulin and catecholamines since not much was known in this regard.

Results and discussion

Primarily the localization of PDE3B was studied using two different species of primary adipocytes and two different methods for subcellular fractionation. Mouse primary adipocyte membranes were subjected to continuous sucrose gradient (10-45 %) centrifugation to separate different subcellular fractions, while rat adipocytes were subjected to discontinuous sucrose gradient in combination with sequential centrifugations. As seen in paper II, figure 1, PDE3B was found primarily in the PM and ER. The PM and ER PDE3B were isolated and further investigated with gelfiltration. The results showed that PM PDE3B and not ER PDE3B eluted as large molecular assemblies (>4 000kDa) as seen in paper II, figure 2a and 3b. The PM gelfiltration fraction containing

large molecular assemblies also contained high amounts of the lipid raft markers cholesterol and flotillin-1 and the caveolae marker caveolin-1 (paper II, figure 3). Based on the gelfiltration results, PM PDE3B was further investigated regarding its possible association to caveolae. Caveolae are highly abundant structures in the adipocyte PM and are enriched with the protein caveolin-1. Caveolae have also been shown to be a platform for G-protein coupled [68] and insulin signaling [69] and were therefore highly relevant for the localization of PDE3B. Primary adipocytes were therefore subjected to different caveolae enrichment techniques which were based on the fact that caveolae are resistant to certain detergents, have high buoyant density after Na_2CO_3 extraction and contain caveolin-1. In adipocyte PM, PDE3B together with caveolin-1 were resistant to solubilization with 1% Triton X-100 and 2% CHAPS (paper II, figure 2b). During sucrose gradient fractionation of

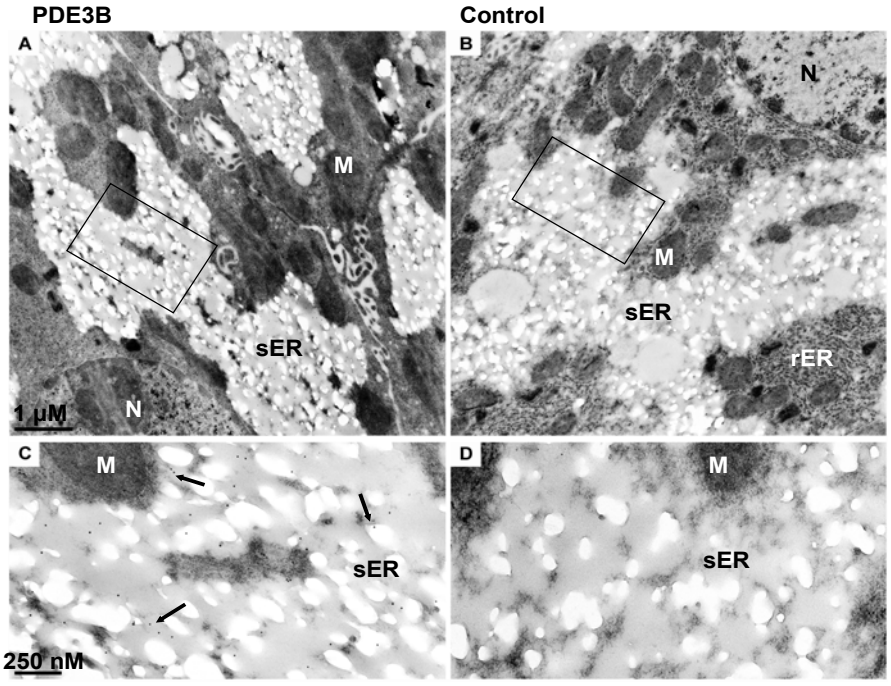

Figure 9: Electron micrograph of PDE3B in hepatocytes
Mouse livers were flushed *in situ* with PBS followed by paraformaldehyde solution. Small pieces were cut out and embedded in Lowicryl. Sections were prepared and incubated with PDE3B antibodies and secondary rabbit antibody attached to gold particles (**A, C**) or only the secondary with gold (**B, D**). The lower picture is an enhancement of the marked area. Different organelles of the cells are marked and arrows show examples of gold particles (n=4). M; Mitochondria, N; Nucleus and sER; smooth ER.

adipocyte Na_2CO_3 extracted PM, PDE3B co-migrated with caveolin-1, cholesterol and flotillin-1 (paper II, figure 4) and in detergent resistant PM PDE3B co-immunoprecipitated with caveolin-1 (paper II, figure 5). The results from the different caveolae enrichment techniques showed that PDE3B was present in fractions enriched in caveolae, which indicate that PDE3B is associated with caveolae. To directly demonstrate the localization of PDE3B to caveolae, immuno-gold labelling and electron microscopy of plasma membrane sheets from primary human adipocytes were performed. Around 90 % of the immuno-gold labelled PDE3B was associated with caveolae, which showed that most PM PDE3B was localized to caveolae (paper IV, figure 3). Together the results clearly demonstrate that adipocyte PDE3B is localized to ER and caveolae. The exact proportion of adipocyte PDE3B in caveolae and ER varies depending on the adipocyte origin and technique used for subcellular fractionation, but in our studies around 35-50 % of total PDE3B was in the PM/caveolae fraction and the rest in the ER compartment. Regarding the ER localized PDE3B, we performed electron microscopy of mouse livers and found the main part of the immuno-gold labelled PDE3B in the smooth ER, as seen in figure 9. The localization to smooth ER is interesting as smooth ER is an important compartment for metabolic regulation [161]. The role of caveolae for PDE3B activity and expression in the PM was studied using primary rat adipocytes treated with methyl-β-cyclodextrin and adipocytes from the caveolin-1 KO mice, which lack caveolae [78]. The lack of caveolae resulted in reduced expression and activity of PDE3B (paper II, figure 6) which proves an important role for caveolae/caveolin-1 in the expression of PDE3B. The localization of PDE3B to caveolae is interesting since caveolae have become more and more established as an important platform to initiate, organize and generate specificity to intracellular signaling. For example, signaling components mediating effects of insulin and β-AR agonists have been localized to these structures [68]. As PDE3B is phosphorylated and activated by insulin as well as β-AR agonists we wanted to further investigate if PDE3B was activated by both stimuli in the caveolae and ER compartment or if there was any difference between the activation of PDE3B in the different compartments.

To characterize the role of PDE3B's localization for its activation by insulin and a β_3-AR agonist in adipocytes, we performed experiments on 3T3-L1 adipocytes. The 3T3-L1 adipocytes were labelled with ^{32}P and stimulated with insulin or CL-316243. The labelled and stimulated adipocytes were subjected to subcellular fractionation and analyzed regarding PDE3 activity, PDE3B phosphorylation and PDE3B expression. Interestingly as seen in paper IV, figure 2 PDE3B localized in ER was phosphorylated and activated only in

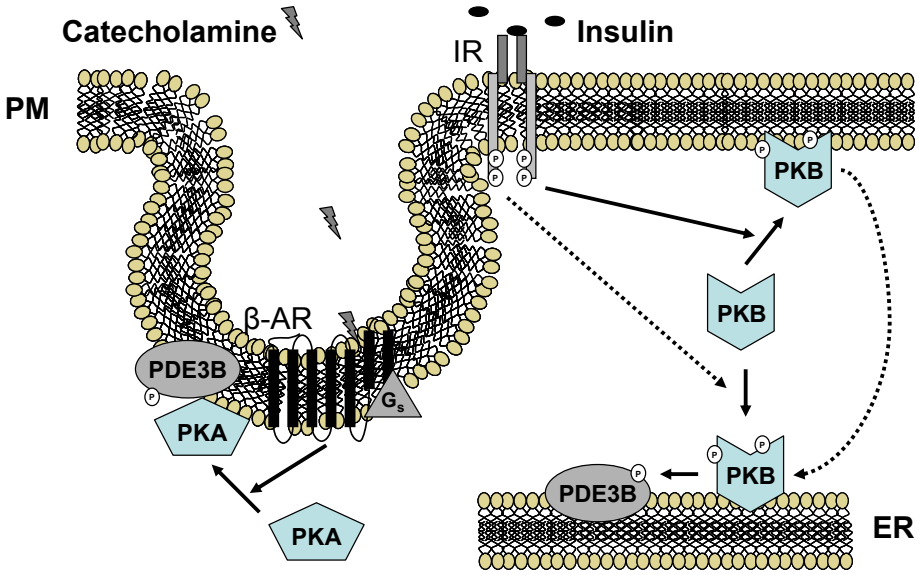

Figure 10: Hypothesis for activation of PDE3B at different locations
PDE3B is located in the caveolae and ER in adipocytes. Insulin activates PDE3B localized in the ER via translocation of PKB to ER and CL activates the caveolae pool of PDE3B via translocation of PKA to caveolae. PM; plasma membrane, ER; endoplasmic reticulum, PDE; cyclic nucleotide phosphodiesterase, PKA; protein kinase A, PKB; protein kinase B, AR; adrenergic receptor, G_s; stimulatory G-protein, IR; insulin receptor, P; phosphor.

response to insulin while PDE3B in caveolae was phosphorylated and activated only in response to CL-316243. As seen in the same figure there was no acute translocation of PDE3B between the different locations. From these results it seems likely that caveolae localized PDE3B is specifically activated by catecholamines and that PDE3B in the ER is specifically activated by insulin. To further investigate if PDE3B was differentially regulated in ER and caveolae we used adipocytes from the caveolin-1 KO mice and developed small interference RNA (siRNA) to knock down the expression of caveolin-1 in 3T3-L1 adipocytes. The adipocytes were stimulated with insulin or CL-316243, and analysed with regard to lipolysis, PDE3 activity and phosphorylation of different proteins. In the caveolin-1 KO mouse adipocytes and the caveolin-1 siRNA treated 3T3-L1 adipocytes, activation of PDE3B in response to CL-316243 was lost, while the response to insulin was partly intact (paper IV, figure 9 and 10). These results further demonstrate that caveolae PDE3B is specifically activated by CL-316243. The lack of caveolin-1 also reduced the CL-316243 induced lipolysis and phosphorylation of CRE binding

transcription factors, HSL and perilipin while the phosphorylation of PKB in response to insulin was intact (paper IV, figure 8). These results show that not only PDE3B is dependant on caveolae localization for CL-316243 activation, but the whole machinery for regulating lipolysis is dependant on caveolae and caveolin-1. The fact that caveolae PDE3B is specifically activated by a β_3-AR agonist and ER PDE3B specifically activated by insulin, is very interesting. The effects downstream of PDE3B are especially interesting since the intracellular concentrations of cAMP are tightly regulated. The activation of PDE3B in different compartments like caveolae and ER by different stimuli can perhaps help to create spatially compartmentalized cAMP and thereby modulate the specificity of the cAMP signals.

To further characterize the activation of PDE3B at the different locations, the localization of two possible PDE3B kinases, PKA and PKB, were studied in 3T3-L1 adipocytes after stimulation. In paper IV, the results in figure 1 show that CL-316243 stimulates translocation of the regulatory domain (R) of PKA to the PM, and insulin stimulates translocation of phosphorylated PKB to both the ER and PM. Insulin did not stimulate any PKA-R translocation and CL-316243 did not stimulate any PKB translocation. The localization of PKB and PDE3B was also studied after insulin stimulation by confocal microscopy as seen in paper III, figure 7A-G. The images show that PKB and phospho-PKB is translocated to the ER and PM after insulin stimulation, while the co-localization of PKB and phospho-PKB with PDE3B was estimated to be greater in the ER region than in the PM region. From the described results it is possible to put forth the following hypothesis; insulin activates PDE3B localized in the ER via translocation of PKB to ER and CL-316243 activates the caveolae pool of PDE3B via translocation of PKA to caveolae. If PKB translocate directly to the ER or if PKB is activated at the PM and then translocated to the ER is not known. The hypothesis is illustrated in figure 10.

PDE3B interacting proteins in adipocytes (Paper III and IV)

Background

It had been shown that solubilized endogenous PDE3B from primary rat adipocytes [134, 199, 200] and recombinant PDE3B [189, 199] elute at or close to the void volume (V_o) during gelfiltration on sephacryl S300 and TSG-3000SW columns. These gelfiltration experiments indicated that PDE3B was present as multimers or macromolecular complexes with other proteins. The study by Kenan et al had also shown that NHR1 and NHR2 regions of PDE3B

are essential for the appearance of PDE3B in the macromolecular complexes [189]. It had also, in analogy with the described results, been shown that PDE3B interacts with several proteins. PDE3B had been shown to interact with the IR [190] and 14-3-3 proteins [191] in a hormone dependent manner in adipocytes and in a hormone insensitive manner with PI3Kγ in mouse heart [110] and a 47 kDa protein of unknown function in 3T3-L1 adipocytes [192, 193]. The interactions of PDE3B with proteins both in a hormone sensitive and hormone insensitive manner are probably very important for the regulation of PDE3B and for effects downstream of PDE3B. Thus, we wanted to investigate the constituents of the PDE3B complexes and identify PDE3B interacting proteins in insulin and catecholamine stimulated adipocytes.

Results and discussion

Primarily we wanted to characterize possible PDE3B containing macromolecular complexes in the internal membranes (i.e not in the PM) of insulin-treated 3T3-L1 adipocytes. 3T3-L adipocytes were therefore stimulated with insulin and internal membranes were isolated, solubilized and fractionated by gelfiltration. As seen in figure paper III, figure 2, PDE3B exhibited a molecular mass of around 1 000 kDa without stimulation, which is larger than its own size (135 kDa) and indicates interactions with other molecules. After incubation of adipocytes with insulin the apparent molecular weight of around 50% of PDE3B protein and activity increased and eluted at the V_o, >3 000kDa (paper III, figure 2). Furthermore, all of the phosphorylated PDE3B from insulin treated adipocytes eluted at >3 000 kD, indicating that activated PDE3B is present in a macromolecular complex after insulin stimulation (paper III, figure 2). Insulin-treatment similarly altered the elution patterns of several other signaling molecules, including IRS-1, PI3K p85, PKB, 14-3-3, heat shock protein (HSP) 90 and PP2A, indicated the presence also of these molecules in macromolecular complex/es after insulin stimulation (paper III, figure 3). The presence of PDE3B, IRS-1, PI3K p85, PKB, 14-3-3 and HSP-90 in macromolecular complex/es was prevented by prior treatment of adipocytes with wortmannin, a PI3K inhibitor (paper III, figure 2B and 3). There results show that the insulin-induced formation of macromolecular complex/es with PDE3B, IRS-1, PI3K p85, PKB, 14-3-3 and HSP90 are PI3K dependent. To study if PDE3B actually interacted with the molecules in the complex a co-immunoprecipitation strategy was used. Samples of gelfiltration fractions from control or insulin-treated 3T3-L1 adipocytes were immunoprecipitated with antibodies against PDE3B. The results showed that IRS-1, PI3K p85, PKB, 14-3-3, HSP90 and PP2A co-immunoprecipitated with PDE3B from the high molecular weight (>3 000 kD) fractions of the insulin-treated adipocytes (paper III, figure 4). These results suggest that, in insulin-treated adipocytes, PDE3B

in the internal membranes is part of a macromolecular complexes comprising IRS-1, PI3K, PKB, HSP90, 14-3-3, PP2A, and perhaps additional unidentified proteins. PDE3B in the macromolecular complex was further studied and we could show that N-terminal truncated PDE3B without NHR1 and NHR2 (the first 604 amino acids deleted) were not recruited to the complex in response to insulin, which shows that the N-terminal part is essential for PDE3B's recruitment to a macromolecular complex (paper III, figure 5B). The truncated form of PDE3B, siRNA down regulation of PDE3B (paper III, figure 5) or inhibition of PDE3B activity by cilostamide (paper IV, figure 6) did not prevent insulin-induced recruitment of signaling molecules into the >3 000 kDa macromolecular complex, which shows that PDE3B is not necessary for insulin-induced complex formation and is not the central scaffold molecule. Taken together, these results suggest that insulin stimulates the recruitment of PDE3B into large macromolecular complexes together with PKB, IRS-1, PI3K, HSP90, 14-3-3, PP2A and perhaps other signaling molecules, in 3T3-L1 adipocyte membranes.

After identification of insulin-induced formation of macromolecular complexes involved in the activation of PDE3B we wanted to further investigate if a β_3-AR agonist such as CL-316243 induces a similar large macromolecular complex in 3T3-L1 adipocytes. To characterize possible macromolecular complexes induced by CL-316243 and compare it to complexes formed by insulin, total membranes with both PM and internal membranes form insulin and CL-316243 stimulated 3T3-L1 adipocytes were solubilized and fractionated by gelfiltration (paper IV, figure 4). After incubation of adipocytes with insulin or CL-316243, PDE3 activity increased, and 50 % of total PDE3B eluted at >3 000 kDa instead of at 1 000 kDa as is the case in control condition. Furthermore, all of the phosphorylated PDE3B from CL-316243 treated adipocytes eluted at >3 000 kDa (paper IV, figure 4B). The results show that CL-316243 like insulin induces the formation of macromolecular complexes containing PDE3B. As seen in paper IV, figure 4A, CL-316243-treatment specifically altered the migration pattern of β_3-AR, HSL and PKA-R into macromolecular complexes of >3 000 kDa. The migration of other proteins, including 14-3-3, PP2A, perilipin, AC and caveolin-1, were altered in the same way in response to both CL-316243 and insulin. The migration pattern of IRS1, PI3K p85, PKB and HSP90 into macromolecular complexes were specifically stimulated by insulin. To study if PDE3B actually interacted with the molecules in the CL-316243 induced complex and compare the CL-316243-induced complex to to the insulin-induced complex, a co-immunoprecipitation strategy was used. Samples of gelfiltration fractions from both insulin and CL-316243 stimulated adipocytes were immunoprecipitated with an anti-PDE3B antibody and then analysed. Several signaling proteins co-immunoprecipitated

with PDE3B in the high molecular weight (>3 000 kD) fractions from insulin and CL-316243 stimulated 3T3-L1 adipocytes (paper IV, figure 5). The results suggested that PDE3B in the CL-316243 stimulated adipocytes interacts with PKA-R, β_3-AR, 14-3-3, PP2A, AKAP and caveolin-1 in a macromolecular complex. PDE3B in insulin stimulated adipocytes interacted with a slightly different set of proteins in the macromolecular complex as compred to CL-316243 such as IRS-1, PI3K P85, PKB, HSP90 14-3-3, AKAP, PP2A, and caveolin-1. The presence of caveolin-1 and 14-3-3 both in the insulin and CL-316243 stimulated macromolecular complexes indicate that these molecules are the main scaffolding proteins in the formed complexes with PDE3B. As the molecular weight of the identified components in the macromolecular complexes does not add up anywhere close to 3 000 kDa it could be that 14-3-3 and caveolin-1 proteins act as scaffolds to attract several protein copies of the identified proteins to the same complex. The 14-3-3 proteins have been shown to directly interact with phosphorylated PDE3B in adipocytes, it is thereby likely that 14-3-3 interacts closely with the phosphorylated PDE3B in the complexes after both insulin and CL-316243 stimulation [191]. Caveolin-1 is probably also an important scaffold protein since knock down of caveolin-1 more or less blocks the formation of complexes in 3T3-L1 adipocytes (paper IV, figure 10). In support of this hypothesis, caveolin-1 has been shown to directly interact with PKA [80] and this interaction seems to be important for the β_3-AR agonist stimulated complex formation between perilipin, caveolin-1 and the catalytic subunit of PKA in adipocytes [79]. Another explanation to the large size of the formed complex is that the complex contains several other so far not identified components. A third explanation to the large size is that it is located in a raft structure which could be part of the complex and possibly also keep the complex together. An illustration of the described results can be seen in figure 11.

As seen in paper IV, figure 4A, upper panel, the macromolecular complexes induced by CL-316243 and insulin coincided with elution of increased amounts of cholesterol with the large complexes and activated PDE3B. This result indicates that cholesterol is a part of the formed complex maybe as part of a lipid raft structure. To further study the role of cholesterol 3T3-L1 adipocytes were treated with atorvastatin prior to stimulation and gelfiltration. Atorvastatin is a HMG-CoA reductase inhibitor that reduces the formation of mevalonic acid, a precursor in the biosynthesis of cholesterol and isoprenoids, which are necessary for prenylation and subsequent membrane anchoring and activity of signaling effectors. The treatment was associated with reduced recruitment of PDE3B into macromolecular complexes in response to both insulin and CL-316243 and destabilization of these complexes (paper IV, figure 13). The described results suggest that cholesterol and / or membrane anchoring of

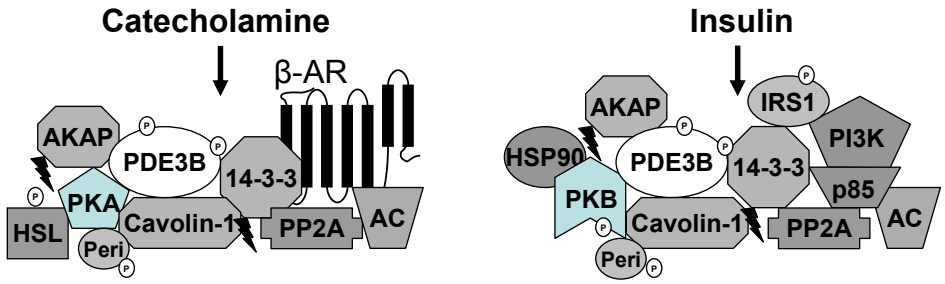

Figure 11: PDE3B in macromolecular complexes
PDE3B is a component of macromolecular signaling complexes formed after insulin and catecholamine stimulation in adipocytes. IRS; insulin receptor substrate, PI3K; phosphatidylinositol 3-kinase, PKB; protein kinase B, PDE; cyclic nucleotide phosphodiesterase, AR; adrenergic receptor, AC; adenylyl cyclase, PKA; protein kinase A, PDE; cyclic nucleotide phosphodiesterase, HSL; hormone sensitive lipase, AKAP; A-kinase anchoring proteins, PP; protein phosphatase, peri; perilipin, ; cholesterol.

important signaling proteins are essential for the insulin and CL-316243 induced formation of macromolecular complexes with PDE3B in adipocytes. Two indirect effects of cholesterol reduction are reduced caveolin-1 expression in the PM [53] and changes in the membrane composition such as reduced caveolae or lipid raft formations. These effects could perhaps explain the reduced formation of macromolecular complexes in response to atorvastatin.

The studies on PDE3B complex formation in this thesis illustrate that PDE3B and PKB interact in internal membranes after insulin stimulation of 3T3-L1 adipocytes. Interestingly PKB has been implicated in insulin-induced phosphorylation and activation of PDE3B [183, 187, 188] and therefore we wanted to further characterize the interactions between membrane-associated PDE3B and PKB. After incubation of 3T3-L1 adipocytes with insulin, PDE3B and PKB co-immunoprecipitated from solubilized membranes with antibodies against PDE3B in a PI3K dependant manner (paper III, figure 6). To identify structural determinants involved in the interaction between PDE3B and PKB, we tested the ability of wild type and truncated mutant PDE3B recombinants to co-immunoprecipitate with PKB. As shown in paper III figure 8, truncated recombinant PDE3B in which the first 1-302 (M3BΔ302) or 1-604 amino acids (M3BΔ604) were deleted did not co-immunoprecipitate with pPKB, while recombinant PDE3B in which the first 1-196 amino acids were deleated (M3BΔ196) co-immunoprecipitated with pPKB, but less efficiently than WT MPDE3B. The results suggest that the regulatory domain (1-302aa) of PDE3B may be critically important for its interaction with pPKB. We also incubated

FLAG-tagged, recombinant MPDE3B with recombinant PKB, pPKB and p-ΔPKB (PH domain deleted, active PKB) and immunoprecipitated the recombinant PDE3B and PKB. As seen in paper III figure 9 more pPKB than PKB co-immunoprecipitated MPDE3B and PDE3B co-immunoprecipitated only with pPKB and not PKB or p-ΔPKB. The results indicated that activated pPKB interacts with PDE3B via its PH domain. This was also supported from results showing that pPKB, not PKB or p-ΔPKB co-eluted with MPDE3B after gel filtration purification (paper III, figure 10). Together the results suggest that phosphorylated PKB directly interacts with PDE3B through PDE3B's regulatory domain and PKB's PH-domain, which might be important for the activation of PDE3B in response to insulin.

Concluding remarks

From the results presented in this thesis regarding the regulation of PDE3B there are obvious connections between the studies of PDE3B phosphorylation, localization and protein interactions in adipocytes. To start with some general conclusions from the PDE3B localization and interaction studies; PDE3B is localized in two different compartments, i.e. caveolae and ER. Insulin specifically activates PDE3B in the ER compartment and insulin also generates macromolecular complexes containing PDE3B and several insulin signaling molecules. The studies on complex formation in response to insulin were preformed on adipocyte fractions containing internal membranes (i.e. without any PM), which strongly suggests that the insulin stimulated PDE3B containing macromolecular complexes are localized to ER where insulin-induced activation of PDE3B takes place. A β_3-AR agonist on the other hand stimulates caveolae localized PDE3B and generates macromolecular complexes containing PDE3B and several cAMP related signaling molecules. The studies on complex formation in response to a β_3-AR agonist were performed on adipocyte total membrane fractions containing both internal membranes and PM. The β_3-AR agonist stimulated PDE3B macromolecular complexes may therefore in theory be located in ER, but is more likely located in caveolae since virtually all of the β_3-AR agonist-induced PDE3B activation and phosphorylation is located in the caveolae. Furthermore, PKA-R which is specifically a part of the β_3-AR agonist stimulated PDE3B macromolecular complex translocates only to the PM compartment and not to ER. In figure 12 a hypothesis is presented which summarize all these conclusions. β_3-AR agonist stimulation of PDE3B is clearly dependant on caveolae and caveolin-1 in adipocytes. Thus caveolae seem to be a signaling platform for the cAMP related signaling. If caveolae are central signalling platforms for insulin

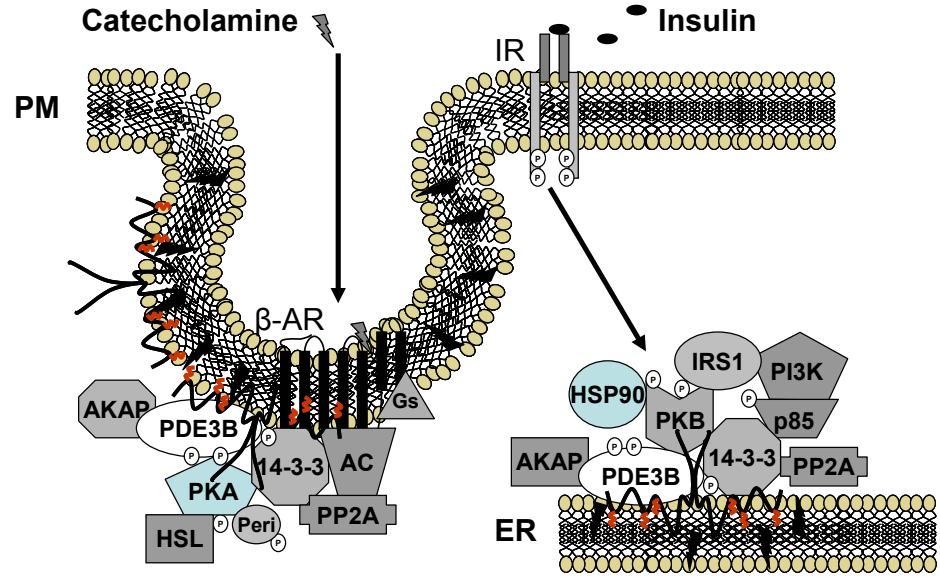

Figure 12: Summarized hypothesis of PDE3B's regulation in adipocytes
PM; plasma membrane, ER; endoplasmic reticulum, IRS; insulin receptor substrate, PI3K; phosphatidylinositol 3-kinase, PKB; protein kinase B, PDE; cyclic nucleotide phosphodiesterase, AR; adrenergic receptor, G_s; stimulatory G-protein, AC; adenylyl cyclase, PKA; protein kinase A, PDE; cyclic nucleotide phosphodiesterase, HSL; hormone sensitive lipase, AKAP; A-kinase anchoring proteins, PP; protein phosphatase, ; cholesterol, ; caveolin-1, peri; perilipin,.

stimulation of PDE3B in adipocytes remains to be further elucidated. Insulin stimulation of PDE3B activity and complex formation in the ER implys that caveolae might not be the central platform. The insulin induced activation of PDE3B in the ER does however not exclude the possibility that caveolae are important for the insulin induced activation of PDE3B. One possibility is that whole caveolae containing activated insulin signaling proteins, caveolin-1 and cholesterol are internalized after insulin stimulation to the ER. The activated proteins can then phosphorylate and activate PDE3B in the ER and perhaps other signaling proteins. A second possibility is that insulin activates the PI3K signaling cascade in caveolae which forms a complex that diffuses to the ER and PDE3B. A third possibility is that the caveolae and ER have direct physical contact. These suggestions are in line with studies summarized by Cohen et al [69] which show that caveolae and caveolin proteins have a role in insulin signaling and diabetes. On the other hand according to Pilch et al [89] there are

so far not enough experimental data to be sure that caveolae are essential for the insulin signal to propagate in adipocytes. However, caveolin-1 and cholesterol are certainly important for normal insulin signaling [70, 201, 202]. We have demonstrated that PDE3B expression and activity is dependent on both cholesterol and caveolin-1 and that the insulin stimulated PDE3B complex formation in the ER is dependent on caveolin-1 and possibly also on cholesterol. Caveolin-1 is probably, as demonstrated by our results, one of the central scaffolding molecules in the insulin stimulated complex and thereby essential for the insulin signal. Cholesterol and caveolin-1 might also be part of raft structures in the ER which could be important for the ability of molecules in the insulin stimulated PDE3B macromolecular complex to interact with and/or find each other.

The results of this thesis serve as a starting point for further studies on the exact role for each of the phosphorylation sites of PDE3B. One interesting aspect to highlight is the connection between PDE3B phosphorylation sites and PDE3B interactions. There are two different possibilities in this regard. One possibility is that PDE3B after stimulation first is phosphorylated and then becomes part of the complex. This means that certain phosphorylation sites of PDE3B act as docking sites for different interacting proteins, which makes PDE3B part of the stimulated macromolecular complex. This supported by the fact that only phosphorylated PDE3B, especially phosphorylated on S273 and S296, interact with 14-3-3 proteins [191]. Another possibility is that PDE3B after simulation first becomes part of the complex and then is phosphorylated, which means that the interactions are not dependent on the PDE3B phosphorylations. This later possibility could involve the following speculations. Basal state PDE3B interact with different molecules such as AKAP and AS47 (shown to interact with PDE3B [193]), which is supported by that the fact that PDE3B in the basal state elutes as in a complex around 700 kDa from a gelfiltration column. After catecholamine/insulin stimulation signaling proteins interact with each other and form a complex which then interacts with PDE3B either through direct interactions (not likely since PDE3B is not the central scaffold according to our results) or through indirect interactions for example via AKAP or AS47. The formed macromolecular complexes contain different kinases such as PKA or PKB which will phosphorylate PDE3B. Since PDE3B is multisite phosphorylated in response to insulin and catecholamines both of the possibilities mentioned above might be important and can generate a phosphorylation/interaction cycle partly illustrated in figure 9. Additional investigations are however needed to get a full picture. Inversigations needs to be done on the role for each PDE3B phosphorylation site for PDE3B interactions with other proteins. In addition more needs to be done regarding the interactions of PDE3B both in the stimulated and unstimulated state. This

could be done for example with proteomics analysis of the PDE3B complexes. Another approach would be to more specifically study the interactions for example between AKAP or AS47 and PDE3B.

When I look at the results from the localization and interaction studies a picture appears regarding important kinases responsible for the phosphorylation of PDE3B. In the localization studies the results show that PKB translocates to the ER/PM specifically in response to insulin and PKA translocates to the PM specifically in response to CL-316243 as illustrated in figure 11. In the interaction studies the results show that PKB is part of the macromolecular complex containing PDE3B only in response to insulin while PKA is part of the formed macromolecular complex containing PDE3B only in response to CL-316243. Although other kinases than PKA and PKB might be involved in the phosphorylation of PDE3B, the results support the hypothesis that catecholamines mediate the phosphorylations on S273, S296 and S421 in PDE3B via PKA and insulin mediate the phosphorylations of S273, S296 and possibly S421 via PKB. More investigations are needed to confirm this hypothesis or find other possible PDE3B kinases.

Populärvetenskaplig sammanfattning

I min avhandling har jag undersökt hur fosfodiesteras 3B (PDE3B) i fettväven styrs vid svar på två typer av hormoner nämligen, insulin och katekolaminer. Det senare innefattar hormoner så som adrenalin och nor-adrenalin. Varför är då detta intressant att veta? I Sverige och världen har mängden människor med fetma under de senaste åren ökat lavinartat, vilket är en stor hälsofara. En av anledningarna till att det är en stor hälsofara är att risken för att utveckla diabetes är mycket stor hos människor med fetma, ca 10 % av alla människor som lider av fetma har diabetes. En viktig koppling mellan fetma och diabetes ligger till stor del i att fettväven som främst består av fettceller inte kan lagra allt fett i kroppen och att det därmed till viss del istället cirkulerar i blodet i form av fria fettsyror som hamnar i lever och muskler. För att kunna förhindra att fettsyror hamnar i blodet och att fett lagras i muskler och i lever är det viktigt att förstå hur fettcellerna styrs till att bygga upp och bryta ned sitt lager av fett. Det skulle ju vara bra att kunna öka fettcellernas kapacitet att lagra fettet så att det inte hamnar på fel ställe i kroppen där det kan ha ohälsosamma effekter. Det enzym som jag har studerat, nämligen PDE3B är med och styr denna lagring av fett genom att stoppa nedbrytningen av fett i fettcellen till fettsyror i blodcirkulationen. Detta betyder att då PDE3B är aktiverat minskar mängden fett som hamnar i vårt blodomlopp. Två typer av hormon som är särskilt viktiga för att styra aktiviteten av PDE3B är insulin och katekolaminer. Katekolaminer binder till dess receptorer på fettcellens yta vilket leder till aktivering av signalvägar som består av olika proteiner och signalmolekyler som aktiveras eller bildas genom att påverka varandra och slutligen leda stimulering av fettlagrets nedbrytning. För att inte nedbrytningen av fettlagret skall bli allt för stor och hållas i schakt aktiveras även PDE3B av en parallell signalväg igångsatt av samma hormon. Insulin som frisätts till blodomloppet efter en måltid har motsatt effekt jämfört med katekolaminer vad gäller fettinlagring. När insulin binder dess receptor på fettcellens yta aktiveras andra signalvägar som leder till att PDE3B aktiveras och stoppar fettlagrets nedbrytning och minskar därmed de fria fettsyrorna i blodomloppet.

Jag har studerat hur PDE3B aktiveras i fettceller vid svar på insulin och katekolaminer utifrån tre olika infallsvinklar som alla är viltiga för att få en helhetsbild. Dessa infallsvinklar är; 1) fosforylering av PDE3B, 2) lokalisationen av PDE3B och 3) PDE3Bs interaktioner med olika proteiner och kolesterol. När det gäller fosforylering hade det tidigare visats att insulin och katekolaminer kan aktivera PDE3Bs enzymatiska kapacitet genom att sätta fart på andra proteiner att sätta fosfor på PDE3B. I frågan om PDE3Bs lokalisation hade det då jag initierade mina studier visats att PDE3B var stationerat vid det endoplasmatiska retikelet (ER) som är en struktur inne i fettcellen. I princip

fanns det dock ingen kunskap om rollen för denna lokalisation vid aktivering av PDE3B. Slutligen beträffande PDE3Bs interaktioner med molekyler fanns det indikationer på att PDE3B befann sig i ett komplex tillsammans andra proteiner. Vilka proteiner det kunde vara och vilken roll interaktionerna med PDE3B i komplexet kunde ha för aktiveringen av PDE3B var dock inte känt. I mina studier har jag genererat ytterliggare kunskap för att få en bild av hur PDE3B aktiveras vid svar på insulin och katekolaminer. Främst har jag kommit fram till följande med utgångspunkt i de tre olika infallsvinklarna.

1. PDE3B i fettceller blir fosforylerat på många olika ställen vid svar på både insulin och katekolaminer. Dessutom har jag identifierat 6 olika fosforyleringssäten på PDE3B i fettceller.

2. PDE3B finns på två olika ställen i fettcellen nämligen i en speciell struktur i ytskiktet runt cellen som kallas caveolae och på ett ställe längre in i cellen som kallas endoplasmatiskt retikulum (ER). PDE3B i caveolae stimuleras endast av katekolaminer medan PDE3B i ER endast stimuleras av insulin.

3. PDE3B interagerar med andra proteiner i ett stort komplex vid svar på insulin och katekolaminer i fettcellen. Många av dessa proteiner har identifierats och ett protein, caveolin-1 har visats vara särskilt viktigt.

I mina studier har jag därmed tillfört stor kunskap till styrningen av PDE3B och fettcellernas fettlagring. I diabetessammanhang skulle det vara bra att förstärka inlagringen av fett dvs. förstärka insulinsignalen och aktiveringen av PDE3B i fettcellerna. Ett läkemedel med en fettcellsspecifik PDE3B aktivator skulle antagligen ha gynnsamma effekter eftersom det skulle leda till minskad mängd fettsyror i blodcirkulation och därmed minskad fettlagring i lever och muskler. I mina studier finns det en del nya perspektiv till hur en sådan PDE3B aktivator skulle kunna se ut.

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