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Studies in beta cells and adipocytes in the context of obesity and T2D - focusing on PDE3B, OPN and SCFAs

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Studies in β-cells and adipocytes in the context of obesity and T2D

-focusing on PDE3B, OPN and SCFAs

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Abstract

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Key words Obesity, type 2 diabetes, islets of Langerhans, β-cells, adipocytes, PDE3B, OPN, SCFAs

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Till Philip, Nåmi och Zelda

”Man är flitig och stark, löper hela dagen och är snäll.”

Emilia Heimann, 8 år.

Abstract

Type 2 diabetes (T2D) is a heterogeneous disease characterized by altered lipid parameters and elevated glucose levels, as a direct consequence of impaired insulin signaling in target tissues and reduced insulin exocytosis from pancreatic β-cells. Obesity, which dramatically increases worldwide, is associated with insulin resistance and T2D. In this thesis, we elucidate the effects of hormones and nutrients on biological responses and regulatory mechanisms in pancreatic β-cells and adipocytes. The information is scarce with regard to the regulation of cyclic nucleotide phosphodiesterase 3B (PDE3B) in pancreatic β-cells. We show that PDE3B is activated in response to glucose, insulin and forskolin, which is coupled to a decrease, no apparent change or an increase in total phosphorylation of the enzyme in rat pancreatic β-cells. Furthermore, PDE1A, PDE3, PDE4C, PDE5A, PDE7A, PDE7B, PDE8A, PDE10A and PDE11A are detected in human pancreatic islets. Islets from RIP-PDE3B mice, exhibiting β-cell specific overexpression of the cAMP-degrading enzyme phosphodiesterase 3B (PDE3B) and dysregulated insulin secretion, show induced OPN protein expression. In addition, *in silico* and functional approaches demonstrate that PDE3B and OPN are connected and follow a similar protein expression pattern in response to e.g. cAMP-elevating agents and insulin. Little is known regarding the direct effects of short-chain fatty acids (SCFAs), produced through bacterial fermentation of dietary fibers, on glucose and lipid metabolism in adipocytes. We show that the SCFAs propionic acid and butyric acid inhibit cAMP-stimulated lipolysis, a mechanism that is not dependent on the cAMP-degrading enzyme PDE3B. Moreover, both SCFAs inhibit basal and insulin-stimulated *de novo* lipogenesis, which is associated with increased phosphorylation of acetyl CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis. Propionic acid and butyric acid are also able to potentiate insulin-stimulated glucose uptake.

In summary, we demonstrate that agents relevant for β-cell function regulate PDE3B activity and phosphorylation levels. Based on several strategies, we demonstrate a connection between PDE3B and OPN, the latter having a protective role in pancreatic β-cells. Further investigations are required to identify downstream targets of PDE3B that are involved in the regulation of insulin exocytosis and also to elucidate the relationship with OPN. Moreover, several PDEs are present in human pancreatic islets. The majority of these PDEs have been described as insulin secretagogues in animal models and it is thus promising to also confirm their presence in humans, as it can be advantageous for the

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Contents

List of papers

Papers included in this thesis

I. **Expression and regulation of cyclic nucleotide phosphodiesterases in human and rat pancreatic islets.** Emilia Heimann*, Helena A. Jones*, Svante Resjö, Vincent C. Manganiello, Lena Stenson, Eva Degerman. PLoS ONE, 2010 Dec 1;5 (12). *These authors contributed equally to this work.

II. **Cyclic nucleotide phosphodiesterase 3B is connected to osteopontin and protein kinase CK2 in pancreatic β-cells.** Emilia Heimann, Amitabh Sharma, Nalini Raghavachari, Vincent C. Manganiello, Lena Stenson, Eva Degerman. JBiSE, vol 6, no 5A, May 2013.

III. **Propionic acid and butyric acid inhibit lipolysis and *de novo* lipogenesis and increase insulin-stimulated glucose uptake in primary rat adipocytes.** Emilia Heimann, Margareta Nyman, Eva Degerman. Submitted to Adipocyte.

Additional paper not included in the thesis

I. **Reduced insulin secretion correlates with decreased expression of exocytotic genes in pancreatic islets from patients with type 2 diabetes.** Andersson SA, Olsson AH, Esguerra JL, Heimann E, Ladenvall C, Edlund A, Salehi A, Taneera J, Degerman E, Groop L, Ling C, Eliasson L. Mol Cell Endocrinol. 2012 Nov 25;364 (1-2):36-45.

Abbreviations

AC	adenylyl cyclase
ACC	acetyl CoA carboxylase
ADA	adenosine deaminase
ALBP	adipocyte lipid-binding protein
AMPK	AMP-protein kinase
APS	adaptor protein with pleckstrin homology
AQP7	aquaporin 7
AS160	Akt substrate of 160 kDa
ATGL	adipose triglyceride lipase
cAMP	cyclic adenosine monophosphate
CGI-58	comparative gene identification-58
cGMP	cyclic guanosine monophosphate
ChREBP	carbohydrate responsive element-binding protein
CK2	casein kinase 2
cN	cyclic nucleotide
DAG	diacylglycerol
DAVID	database for annotation, visualization and integrated discovery
DGAT	diacylglycerol acyltransferase
ECL	enhanced chemiluminescence
ECM	extracellular matrix
Epac2	exchange protein directly activated by cAMP 2
Eta-1	early T lymphocyte activator-1
FA	fatty acid
FAT	fatty acid translocase

Ffar	free fatty acid receptor
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide 1
GLUT	glucose transporters
GPAT	glycerol phosphate acyltransferase
GPCR	G-protein coupled receptor
GSIS	glucose-stimulated insulin secretion
GSV	GLUT4 storage vesicle
HSL	hormone-sensitive lipase
IL-1β	interleukin 1β
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
IR	insulin receptor
IRS	insulin receptor substrate
ISO	isoproterenol
I2D	interologous interaction database
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPL	lipoprotein lipase
MAG	monoacylglycerol
MGAT	monoacylglycerol acyltransferase
MGL	monoglyceride lipase
MODY	maturity-onset diabetes of the young
mTORC2	mTOR complex 2
NO	nitric oxide
OPN	osteopontin
PDE	cyclic nucleotide phosphodiesterase
PDK-1	pyruvate dehydrogenase lipoamide kinase isozyme 1
PLIN1A	Perilipin A
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate

PI3-kinase	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PP	polypeptide-producing cell
PPAR	peroxisome proliferator-activated receptor
PPI	protein-protein interaction
PP2A	protein phosphatase 2A
RIP	rat insulin promoter
RIM	Rab3-interacting molecule
RRP	readily releasable pool
SCFA	short-chain fatty acid
SIBLING	small integrin-binding ligand N-linked glycoprotein
SNARE	soluble NSF factor attachment protein receptor
SPP1	secreted phosphoprotein-1
STZ	streptozotocin
SUR1	sulfonylurea receptor 1
TAG	triacylglycerol
T2D	type 2 diabetes
UCP-1	uncoupling protein 1
VDCC	voltage-dependent calcium channel

Populärvetenskaplig sammanfattning

Typ 2-diabetiker: söt som socker och rik på fett

Det finns flera olika varianter av diabetes och den mest förekommande är typ 2-diabetes (T2D). T2D kallades förr i dagligt tal åldersdiabetes, men numera drabbas individer i alla åldrar. Sjukdomen kännetecknas av förhöjda nivåer av socker och fett i blodet, vilket beror på att kroppens celler har en nedsatt förmåga att tillgodose sitt energibehov. Bakomliggande orsaker förknippas med olika faktorer, till exempel genetisk bakgrund, ohälsosamma kostvanor och fysisk inaktivitet. Individer med fetma har en ökad fettansamling, som i vissa fall är förknippad med allvarliga hälsoproblem, såsom nedsatt förmåga att ta upp socker i närvaro av insulin och uppkomst av T2D. Det är inte helt klarlagt vad som orsakar den starka kopplingen mellan fetma och T2D, men fettinlagring i vävnader som inte är ämnade att lagra fett, exempelvis lever och muskulatur, samt kronisk inflammation och förändrade nivåer av cirkulerande hormoner som härrör från fettväven antas vara bidragande faktorer.

Betacellen fungerar som en sensor som känner av blodsockernivån och därefter frisätter en viss mängd insulin för att justera blodsockret efter en måltid. Insulin har en livsnödvändig roll då kroppens celler tillgodoser energibehovet genom att understöjda energiupptag och lagring av socker som glykogen i lever och skelettmuskulatur, samt fett som triacylglyceroler i fettväven. Samtidigt som det sker en lagring av energi dämpar insulin nedbrytningen av de energilager som redan finns. Eftersom socker är en av de viktigaste energikällorna för kroppen, och dessutom den enda energikällan för hjärnan, är det viktigt att upprätthålla en stabil sockernivå i blodet. När nivåerna av socker sjunker i blodet återställs sockerbalansen genom att alfacellerna i bukspottkörteln utsöndrar glukagon, som stimulerar levern att frigöra lagrat socker till blodbanan. Hos diabetiker utsöndras inte insulin i tillräckliga mängder och det insulin som cirkulerar i blodbanan har en försämrad verkan i fettväv, lever och muskulatur. Den ökade frisättningen av fettsyror och den vid fetma förändrade hormonella utsöndringen från fettväven bidrar till inflammation, och till att insulinkänsligheten försämras ytterligare.

Studier i de Langerhanska öarna och i betaceller

I bukspottkörteln finns det olika sorters celler som bildar grupperingar, så kallade Langerhanska öar. Betacellen är en av flera celltyper i de Langerhanska öarna och är som tidigare beskrivits den cell som producerar insulin. I första och andra delarbetet har de Langerhanska öarna tagits ut från bukspottkörteln och använts för att bland annat studera hur ett flertal enzymer och proteiner påverkas under olika betingelser. Eftersom vi främst är intresserade av att studera betacellen, har vi även använt oss utav odlade celler som efterliknar betacellens förmåga att producera insulin.

Konsten att kontrollera PDE3B (delarbete I)

När sockernivån ökar i blodet, vilket sker efter en måltid, känner betacellerna av det, vilket resulterar i att en signaleringsväg aktiveras inne i cellen som slutligen leder till insulinutsöndring i blodet. I betacellen finns även andra signaleringsvägar som ytterligare förstärker frisättningen av insulin. cAMP är en signalmolekyl som till exempel förstärker effekten av insulinfrisättningen genom att signalera till andra molekyler i de olika signaleringsvägarna. Enzymer fungerar som katalysatorer genom att öka eller minska hastigheten på kemiska reaktioner och på så vis omvandla ett ämne till ett annat i cellen. För att kontrollera nivåerna av cAMP, finns det särskilda enzymer, så kallade fosfodiesteraser (PDE), som har till uppgift att ombilda cAMP till ett annat ämne.

Fosfodiesteraser består av en stor grupp närbesläktade proteiner som är indelade i elva olika familjer (PDE1-11). Ett särskilt fokus har tilldelats familjen PDE3 i betacellen, där den specifika familjemedlemmen PDE3B har studerats i detalj. Man har i tidigare studier konstaterat att PDE3B är involverat i regleringen av insulinutsöndringen från betacellen. Det är därför betydelsefullt att ta reda på hur PDE3B kan styras för att det i sin tur ska kunna påverka nivåerna av signalmolekylen cAMP. I de studier vi har bedrivit fann vi att PDE3B aktiverades, vilket leder till ökad ombildning av cAMP, i närvaro av bland annat socker, insulin och förhöjda nivåer av cAMP. Vi studerade vidare ifall aktivering av PDE3B var kopplat till fosforylering av enzymet, vilket innebär att en fosfatgrupp sätts fast på specifika ställen på PDE3B. Det visade sig att aktivering av PDE3B sammanföll med varierande grad av fosforylering. Som tidigare omnämnts är PDE3B i sitt aktiva tillstånd benägen att bryta ner cAMP i betacellen. Att på ett fördjupat plan förstå hur olika betingelser kan styra PDE3B till ett mer eller mindre aktivt läge är betydelsefullt, eftersom en dämpad aktivitet av PDE3B leder till en ökad frisättning av insulin. Vidare studier krävs för att utreda vilka specifika proteiner som styr PDE3B eftersom det kan ha betydelse för regleringen av cAMP och därmed insulinfrisättningen.

PDE-familjernas existens i människans Langerhanska öar (delarbete I)

Tidigare studier har visat att andra medlemmar inom de olika PDE-familjerna har benägenhet att påverka insulinfrisättningen, eftersom en hämmad aktivitet av de olika PDE-medlemmarna ger en ökad utsöndring av insulin. Men med den begränsade tillgången till humant material, är det få studier som visar existensen av PDE-familjerna i de Langerhanska öarna från enskilda individer. Genom tillgång till Langerhanska öar från det nordiska nätverket för klinisk ö-transplantation, som forskarna inom ”Lund University Diabetes Centre” (LUDC) har ett nära samarbete med, fick vi möjlighet att studera ett urval av PDE-familjerna i material från människor. Vi fann att PDE1, PDE3 och PDE4 är i aktivt läge och att de mer specifika PDE-medlemmarna PDE4C, PDE7A, PDE8A och PDE10A existerar i de Langerhanska öarna. Vidare studier krävs för att utreda hur dessa PDE-medlemmar kan styras i betacellen.

Den sociala sfären för PDE3B (delarbete II)

Den roll som PDE3B har i betacellen har tidigare studerats i en specifik mus med förhöjda nivåer av PDE3B i betacellerna, vilket leder till att en mindre mängd insulin frisätts till blodet. I musens Langerhanska öar syns också en uttalad förändring med tanke på hur de olika celltyperna är lokaliserade, vilket kan påverka frisättningen av olika hormoner, t.ex. insulin, negativt. Musen är också mer benägen att utveckla insulinresistens (sämre verkan av insulin i målvävnaderna), och den har onormalt höga nivåer av socker i blodet efter att ha fått en diet rik på fett. Vi har studerat den information som behövs för tillverkning av proteiner för att utreda hur förhöjda nivåer av PDE3B kan påverka den inre miljön i betacellen. Resultatet visade att den specifika informationen för proteinet osteopontin fanns i större mängder i möss med förhöjda nivåer av PDE3B. Osteopontin identifierades för första gången i ben (gr. osteon, ”ben” och lat. pons, ”bro”). I andra studier har man visat att osteopontin är kopplat till olika komplikationer vid diabetes, och i betacellen skyddar den till exempel mot giftiga ämnen som kan påverka vår arvsmassa negativt. Att osteopontin just är ett brobyggande protein fick vi konstaterat i arbetet, osteopontin socialiserar nämligen med PDE3B i ett stort nätverk av proteiner. Vi fann även att mängden PDE3B och OPN följdes åt i olika miljöer. Vidare studier krävs för att utreda hur väl PDE3B och OPN samverkar i betacellen.

Studier i fettceller

I kroppen finns det olika sorters fettdepåer, som antingen är belägna under huden (underhudsfett) eller kring de inre organen och i bukhålan (visceralt fett). Fettdepåer utgör både ett isolerande och mekaniskt skydd samt tillgodogör kroppen med den energi som finns lagrad i fettcellerna (även kallade adipocyter). I delarbete III har vi tagit ut fettdepån runt bitestikeln från råtta och isolerat fettceller för att studera hur olika komponenter från födan kan påverka kroppens energireserv.

Rätt fett gör dig nätt (delarbete III)

Överviktiga med ökade nivåer av fettsyror i blodbanan har ofta en försämrad verkan av insulin vid sockerupptag till cellerna, och löper därför större risk att utveckla T2D. Men med förbättrade levnadsvanor, där en balanserad kost och aktivt liv står i fokus, kan man förbättra hälsan avsevärt och till och med i vissa fall utesluta medicinering och tillförsel av insulin.

Det är till exempel väl etablerat att ett högt kostfiberintag förbättrar ämnesomsättningen genom att till exempel sänka socker- och kolesterolnivåerna i blodet. Kostfiber består av kolhydrater som inte är tillgängliga för nedbrytning i tunntarmen. I tjocktarmen däremot tas kostfiber väl om hand av bakterier, vilket resulterar i att kortkedjiga fettsyror produceras. Det finns ett flertal varianter på kortkedjiga fettsyror, men de vanligaste är ättiksyra, propionsyra och smörsyra. Ett flertal studier indikerar att det är de korta fettsyrorna som har de hälsobringande effekterna på kroppen, snarare än kostfiber i sin helhet. Det har till exempel visats sig att de kortkedjiga fettsyrorna skyddar mot djurfetma, vilket även åtföljs av lägre nivåer av socker och fett i blodbanan. Huruvida de kortkedjiga fettsyrorna påverkar olika målvävnader, såsom muskulatur och fettväv i kroppen är inte helt klarlagt. I delarbete III har vi därför studerat effekterna av propionsyra och smörsyra på fettcellens förmåga att hantera sockerupptag och mobilisera energi i form av fettsyror. Våra fynd visar att kortkedjiga fettsyror hämmar både produktion av långa fettsyror och nedbrytning av triacylglyceroler, vilket i det långa loppet skulle ge lägre nivåer av fettsyror i blodbanan. Att öka förmågan hos fettcellerna att hämma nedbrytningen av fett är viktigt med tanke på att ökade nivåer av fettsyror i blodbanan försämrar kroppens insulinkänslighet och leder till fettansamling i andra organ än i fettväven. De kortkedjiga fettsyrorna förbättrade även insulinets förmåga att stimulera upptag av socker till fettcellerna, en mekanism som är försämrad hos individer med fetma och T2D. Det kvarstår att utreda hur de kortkedjiga fettsyrorna på ett mer detaljerat plan påverkar fettcellens

förmåga att lagra och mobilisera energi till kroppens övriga vävnader. De gynnsamma effekterna som uppvisats för de kortkedjiga fettsyrorna inger däremot en förhoppning att i framtiden kunna utveckla läkemedel för att förhindra uppkomst av övervikt och T2D.

Sammanfattning

Sammanfattningsvis har studier bedrivits för att utreda uttryck och reglering av olika PDE-medlemmar samt för att finna kopplingar mellan PDE3B och andra proteiner som inverkar på betacellens funktion, vilken är att upprätthålla en fungerande insulinfrisättning. Vidare gjordes studier i fettceller för att utreda kortkedjiga fettsyrornas effekter på mobilisering och lagring av energi. Detta är framför allt aktuellt för många överviktiga och diabetiker, eftersom de är i allra högsta grad ”söta som socker och rika på fett” och därmed i första hand behöver förbättra sitt energiupptag.

Introduction

Obesity and type 2 diabetes

Type 2 diabetes (T2D) is a worldwide epidemic[1]. It is a heterogeneous disease characterized by altered lipid parameters and elevated glucose levels (hyperglycemia), due to impaired insulin signaling in target tissues and/or reduced insulin secretion from pancreatic β-cells[2]. Obesity has dramatically increased worldwide, in parallel with increasing incidence of T2D[3].The pathogenesis of T2D is considered to be a cause of multiple factors, including a strong genetic predisposition combined with environmental factors[2, 4]. Obesity is generally caused by an imbalance in energy intake and consumption, but genetic predisposition is also considered important for the onset of the disease[2]. As a consequence of excess energy intake, the adipose tissue mass expands by hyperplasia (increase in cell number) and/or hypertrophy (expansion in cell size) to enable an increased storage capacity for triacylglycerols[5-7]. Individuals with obesity have thus excessive fat accumulation, which is associated with the development of severe health issues, such as insulin resistance and T2D[3]. On the other hand, there are considerable parts of the obese community that are metabolically healthy and do not develop T2D[8]. It is not exactly known what causes the strong association between obesity and T2D, but fat accumulation in non-adipose tissue (ectopic), chronic low-grade inflammation and altered levels of circulating hormones derived from adipose tissue are believed to be contributing factors[3, 8]. Genome-wide association studies have identified around 50 genetic loci associated with glucose or lipid-related traits as well as genes associated with obesity and T2D[9].

Insulin resistance

Insulin is an anabolic hormone with a central role in regulating nutrient utilization and promoting storage of glycogen in the liver and skeletal muscles as well as triacylglycerols in adipose tissue[10, 11]. Moreover, insulin also acts to suppress the breakdown of triacylglycerols and subsequently the release of fatty acids[11] as well as to inhibit glucose production[11]. Even though the major proportion of glucose is utilized by the liver and skeletal muscles, glucose uptake and activation of carbohydrate responsive element-binding protein (ChREBP) is also essential for

maintaining whole body homeostasis[12, 13]. Abel *et al*[14] has for example shown that lack of the glucose transporter (GLUT) responsible for insulin-dependent glucose uptake, namely GLUT4, in adipose tissue of mice leads to impaired glucose tolerance and insulin resistance. An insulin resistant state develops when tissues do not respond properly to insulin, resulting in reduced capability to utilize glucose and fatty acids that consequently gives rise to abnormally elevated glucose and lipid levels in blood[11].

Obese white adipose tissue

Obese adipose tissue shows a number of structural, morphological and functional alterations[7]. For example, the expandability of the adipose tissue involves remodeling of the extracellular matrix (ECM) around the tissue and vascularization for nutrient supply[15]. ECM consists of a network of collagen fibres, adhesion molecules and ECM proteins that have structural properties as well as facilitates interaction between cells and matrix[15]. Structural components of ECM, e.g. different types of collagen fibres, either inhibit or promote angiogenesis (blood vessel development). Hypoxia can arise with insufficient oxygen supply due to reduced capillary density, as observed in obese humans and animal models[15, 16]. Obesity-induced hypoxia facilitates remodeling of ECM through the generation of fibrotic components, factors that might induce death of adipocytes[15, 17]. Thus, the pathological changes associated with adipose tissue remodeling in obesity might contribute to the deteriorated fat storage capacity and attraction of inflammatory cells, e.g. macrophages.

The consequences of adipocyte dysfunction lead to leakage of fatty acids that accumulates in peripheral tissues, resulting in lipotoxicity, tissue damage and eventually insulin resistance[7]. Moreover, infiltration of macrophages and T lymphocytes generates cytokine-mediated inflammatory responses that mainly occurs in visceral depots of obese individuals[7]. For example, the cytokine tumour necrosis factor alpha (TNF-α) produced by macrophages and adipocytes, contributes to the elevated free fatty acids (hyperlipidemia) in the circulation that promote inflammation and insulin resistance in peripheral tissues[18]. Besides TNF-α, there are also other cytokines and chemokines involved in the inflammatory process[18].

In normal circumstances the adipocyte-derived adipokines are involved in maintaining energy homeostasis, but with dysfunctional adipocytes abnormal production of adipokines occurs along with altered cytokine and chemokine production[7, 19]. Plasma levels of the adipokine leptin increase with adiposity and can cause a leptin resistant state, which influences ectopic lipid accumulation as a consequence of reduced ability to inhibit food intake and increase fatty acid oxidation in skeletal muscles[19-21]. Another important adipokine is adiponectin,

which normally enhances insulin sensitivity by improving glucose and lipid metabolism[22], but in obese and T2D individuals insulin resistance prevail together with lower plasma levels of adiponectin[19]. There are also other adipokines with altered secretion patterns involved in promoting insulin resistance[19].

β-cell dysfunction

In an attempt to cope with an increased demand of insulin, due to suppressed insulin responses in target tissues and hyperglycemia, the pancreatic β-cells compensate by increasing its capacity to secrete insulin[11]. This is achieved by hypertrophy and/or hyperplasia, leading to increased β-cell mass and insulin content[23]. The ability of the pancreatic β-cell to compensate reflects upon genetic predisposition and mechanisms contributing to T2D, such as hyperglycemia and hyperlipidemia that might have deleterious effects on β-cell function[11, 24]. β-cell dysfunction is thus a consequence of nutritional overload and glucolipotoxicity within the cell, leading to β-cell exhaustion and initiation of T2D[11, 25]. Insulin resistance and increased adiposity have also been suggested to be associated with β-cell dysfunction[26]. The decline in insulin secretion capacity is associated with reduced β-cell function and mass (cell death) as well as insulin[23, 27], involving a number of contributing factors (e.g. oxidative stress) implicated with nutrient overload, cytokine-mediated inflammation[23] and insulin resistance[28]. With β-cell loss, there are also an implication for islet dysfunction and impaired or abnormal hormonal secretion from the other cell types within the islet. With impaired insulin secretion, T2D subsequently develops[27].

This thesis covers studies conducted in pancreatic β-cells and adipocytes. The network of cyclic nucleotide phosphodiesterase 3B (PDE3B), involving upstream regulators as well as targets associated with a β-cell specific overexpression of PDE3B has primarily been in focus for the investigations related to pancreatic islets and β-cells. Finally, the effect of the short-chain fatty acids on glucose and lipid metabolism has been studied in primary adipocytes.

Scientific background

Islet of Langerhans

Pancreas is located behind the stomach in close proximity to the duodenum and functions as both an exocrine and an endocrine gland (**Figure 1**)[29]. The exocrine part of the pancreas, consisting of acinar cells, is closely connected with a network of ducts that eventually falls into the larger pancreatic duct[29]. Acinar cells secrete alkaline pancreatic juice and several different types of enzymes into the ducts that subsequently reach the small intestine for digestion of nutrients[29, 30]. The islets of Langerhans (hereafter defined as pancreatic islets) comprise the endocrine glands, which consist of hormone-secreting cell clusters that are enclosed in the exocrine portion of the pancreas[29].

Pancreatic islets consist typically of five cell types: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing (PP) cells and ghrelin-producing ε-cells (**Figure 1**)[31]. The cytoarchitecture of islets differs between rodents and humans[32]. In rodent islets, it is well described that β-cells are in the core of the islet, including a peripheral region with α-, δ-cells and PP-cells[31, 33, 34]. For human pancreatic islets, there is no consensus for a specific cellular pattern[33]. It has for example been described that β-cells are surrounded by α-cells forming a sandwich-like structure[35] as well as that the endocrine cells are more dispersed throughout the islet[32]. Referring to both human and rodent pancreatic islets, β-cells constitute the most abundant cell type, tightly followed by α-cells, and other cell types are in minority[32].

The pancreatic islets are surrounded by a dense capillary network[31, 34]. With regard to mice, which are often used as a model for describing islet architecture[36], each pancreatic islet is penetrated by small blood vessels that connect to the capillary network[37]. The vasculature of human pancreatic islets is, however, less well described, but appears to differ from mice, as more smooth muscle cells have been observed in blood vessels of human pancreatic islets[38]. There is also species differences with regard to innervation, e.g. human pancreatic islets are sparsely innervated compared to mouse pancreatic islets, as described by Caicedo *et al*[37]. The observed differences in cellular distribution within mouse and human pancreatic islets might have an impact on cell-to-cell communication, occurring

via autocrine and paracrine signaling[37], and might influence insulin and glucagon secretion, hormones essential for regulating glucose homeostasis[39, 40].

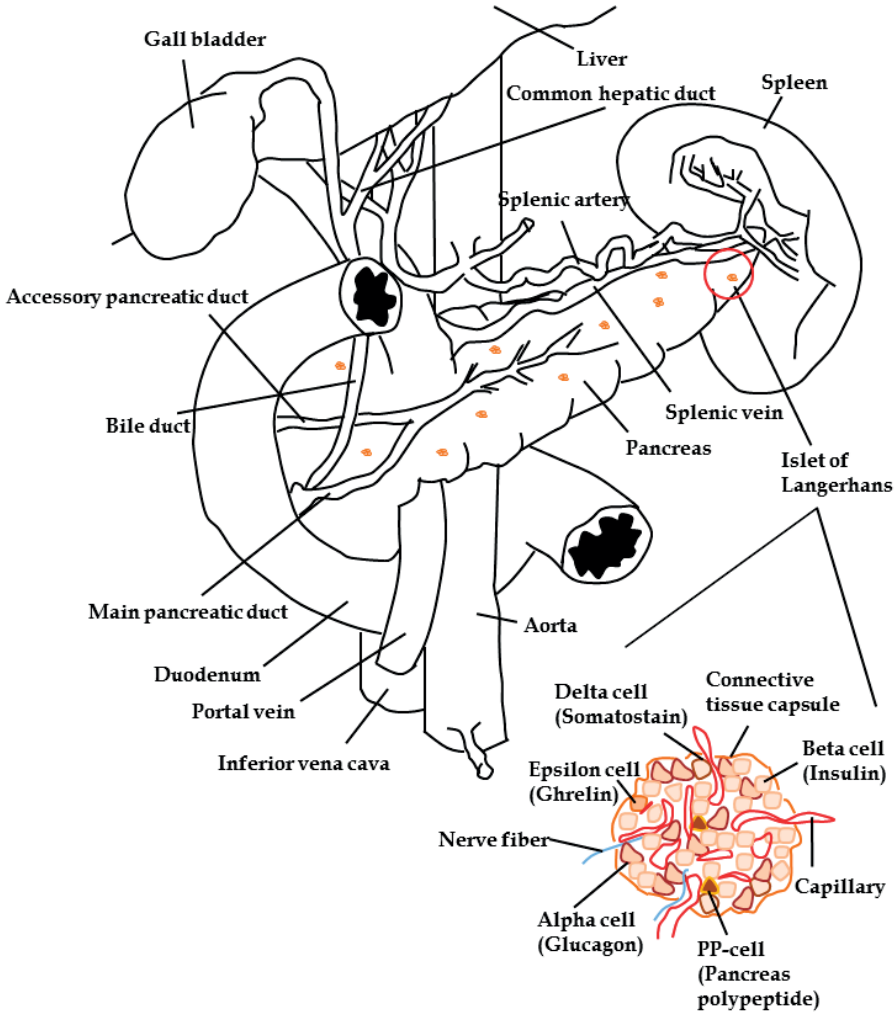

Figure 1. Pancreas and the islets of Langerhans. The illustration depicts the location of the pancreas in the abdominal cavity of the human body. Pancreas lies behind the stomach (not shown in the illustration) and connects to the duodenum via the pancreatic ducts. The endocrine part of the pancreas is known as the islets of Langerhans, which are defined as hormone-secreting cell clusters scattered throughout the pancreas. One of the islets of Langerhans is encircled in red and enlarged to emphasize the different cell types: insulin-producing beta (β) cells, glucagon-producing alpha (α) cells, somatostatin-producing delta (δ) cells, pancreatic polypeptide-producing (PP) cells and ghrelin-producing epsilon (ε) cells. The capillary network surrounding the islet ensures for nutrient supply and transport of hormones to target tissues. Nerve fibers are present in a sparse manner in humans pancreatic islets and contribute to paracrine signaling.

Calcium and cAMP: essential second messengers for insulin secretion

The pancreatic β-cells act as glucose sensors and secrete insulin in response to nutrients, hormones and neurotransmitters (**Figure 2**)[41]. In a postprandial state, increased levels of glucose is sensed by glucose transporter 2 (GLUT2), which is well established in rodents[42]. GLUT2 is present in human pancreatic β-cells, but previous studies describe a more prominent role for GLUT1 and GLUT3 in facilitating glucose entry into the human β-cell[43, 44]. The triggering pathway involves metabolization of glucose, which generates an increase in intracellular ATP, followed by a closure of the ATP-sensitive K_{ATP} channels[41]. This closure results in membrane depolarization, opening of voltage-dependent Ca^{2+} channels and influx of Ca^{2+}, the primary signal triggering exocytosis of insulin granules[41]. There is also the amplifying pathway where glucose and/or metabolized intermediates stimulate insulin secretion independently of the ATP-sensitive K_{ATP} channel[45]. The first phase of insulin secretion releases the readily releasable pool (RRP) of insulin granules that are already docked or primed to the plasma membrane whereas the second phase releases the storage-granule pool of insulin granules that are transported to the plasma membrane, generating a more sustained insulin release[46]. The fusion of granules to the plasma membrane involves interaction with the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex (syntaxin, soluble NSF attachment protein, and synaptobrevin), comprising the minimal exocytotic machinery[47], and other proteins, such as the small G proteins of the Rab family, members of the synaptotagmin family, RIM1 (Rab3-interacting molecule 1) and piccolo, involved in the Ca^{2+}-dependent exocytosis of insulin[47-49].

In addition to calcium as a triggering signal, the intracellular second messenger cyclic AMP (cAMP) is also involved in regulating the insulin secretory process[50, 51]. Both Ca^{2+} and cAMP oscillate, which is critical for provoking pulses of insulin secretion[50, 52, 53]. Recently it has been shown that cAMP potentiates glucose-stimulated insulin secretion (GSIS)[52]. The potentiating effect on GSIS by for example the incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)[52], released from intestinal cells after ingestion of a meal, is dependent on elevated cAMP levels[51]. This effect is achieved when the incretins bind to G-protein coupled receptors, subsequently activating adenylyl cyclase (AC) to produce cAMP[52]. cAMP can potentiate insulin secretion through enhancing Ca^{2+}-levels or activating protein kinase A (PKA)[54] as well as the exchange protein directly activated by cAMP 2 (Epac2)[55]. For example, Epac2, through interaction with RIM isoforms, mediates cAMP- and Ca^{2+}-dependent insulin exocytosis[56].

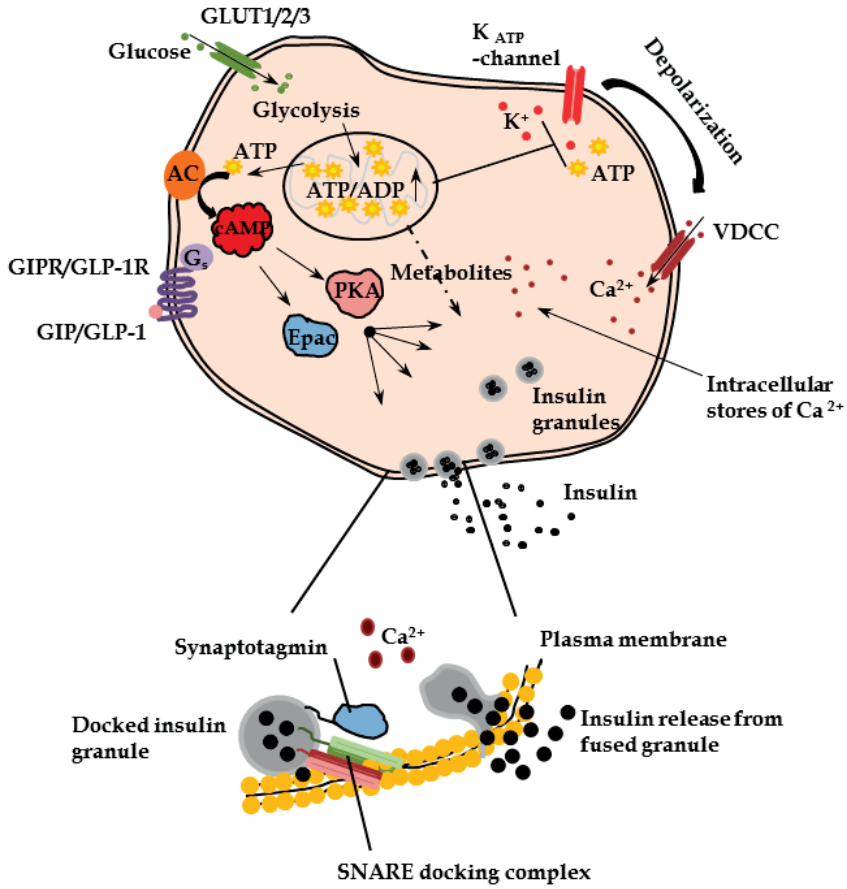

Figure 2. Glucose-stimulated insulin secretion. The β-cell senses elevated glucose levels in the circulation, leading to glucose uptake via glucose transporters (GLUT) (e.g. GLUT2 in mice and GLUT1/2/3 in humans). Metabolization of intracellular glucose (glycolysis) gives rise to an increase in intracellular ATP. With the subsequent increase in ATP/ADP ratio, the triggering pathway is activated, involving closure of the K_{ATP}-channels and depolarization of the β-cell that opens voltage-dependent calcium channels (VDCC). The subsequent influx of extracellular calcium (Ca^{2+}) and involvement of exocytotic proteins, e.g. synaptotagmin and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex potentiates the release of insulin from insulin granules. Glucose and/or metabolites also stimulate insulin secretion independently of the K_{ATP}-channel, via the amplifying pathway. The incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) potentiate glucose-stimulated insulin secretion via cAMP-dependent mechanisms, involving protein kinase A (PKA) and exchange protein directly activated by cAMP 2 (Epac2).

Adipose tissue

The white adipose tissue is an organ located under the skin (subcutaneous depot) and surrounding the inner organs (visceral depot)[57], which differ in functionality depending on location[58]. Within the loose connective tissue of the adipose tissue primarily adipocytes reside together with other cell types, e.g. pre-adipocytes, endothelial cells, erythrocytes, T lymphocytes and macrophages that comprise the stromal vascular fraction (**Figure 3**)[59, 60]. Regarding the functionality of adipose tissue, there are two main attributes that the adipose tissue occupies. First of all, it is the major site for storage of energy in the form of triacylglycerols (TAG), and in energy-deprived situations TAG are hydrolyzed and fatty acids are released and utilized as energy substrates by other tissues[5]. The other important attribute is that it functions as an endocrine organ, releasing adipokines (e.g. leptin and adiponectin) and cytokines (e.g. TNF-α), involved in e.g. regulation of whole body energy homeostasis and inflammatory responses[21]. In addition to white adipocytes, brown and possibly also brite adipocytes are believed to have important roles[59, 61]. Morphological and functional comparisons of these cells show a number of differences[59]. For example, the white adipocytes store TAG in a single lipid droplet, which takes up approximately 90% of the cell volume[58]. The brown adipocytes contain several lipid droplets and have a unique role in regulating thermogenesis with increased oxidative capacity and high expression of uncoupling protein 1 (UCP-1) in mitochondria[58, 61]. There is also accumulating evidence for a third phenotype, an intermediate of white and brown adipocytes, called brite adipocyte[61]. However, the current definition for the brite adipocyte, which has brown adipocyte-like properties and suggested to be predominantly found in white adipose tissue, is under debate[62].

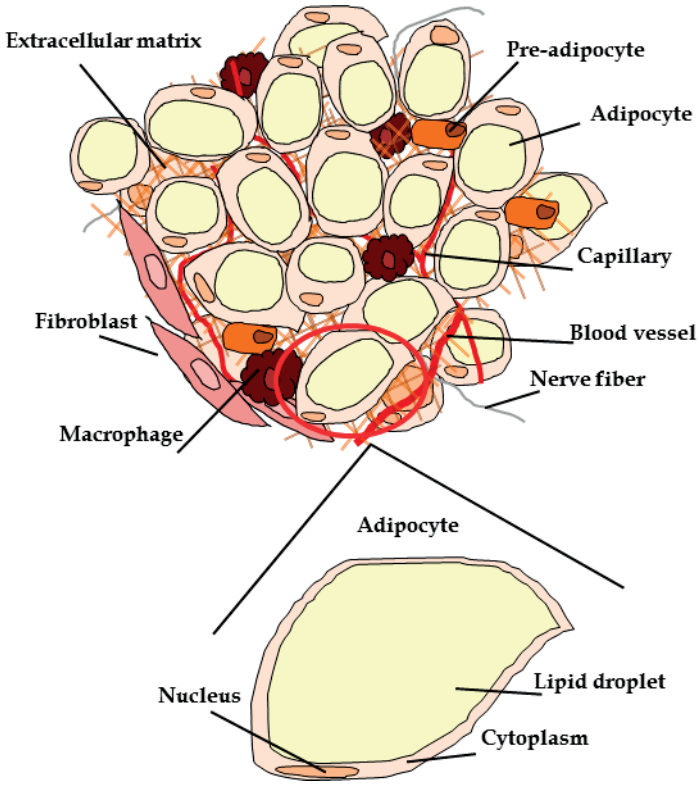

Figure 3. The adipose tissue. The adipose tissue consists of extracellular matrix, adipocytes, nerves and the stromal vascular fraction, which includes e.g. pre-adipocytes, fibroblasts, macrophages, endothelial and vascular cells. With a closer look at the adipocyte, it is apparent that the lipid droplet takes up most of the area, encircled by a thin rim of cytoplasm containing the flattened nucleus.

White adipocyte function in fasted and fed state

In energy-deprived situations, such as during fasting and exercise, the adipocyte provides other tissues with energy by hydrolyzing TAG into free fatty acids and glycerol, a process known as lipolysis (**Figure 4**)[5]. Upon activation of β-adrenergic receptors by catecholamines, such as noradrenalin and adrenalin, the activation of G-stimulatory proteins leads to adenylyl cyclase (AC) activation, generating elevated intracellular levels of cAMP and activation of protein kinase A (PKA)[63]. PKA phosphorylates and activates hormone sensitive lipase (HSL), leading to translocation of HSL from the cytosol to the lipid droplet[63]. Perilipin A, a protein coating the lipid droplet[64], is subsequently phosphorylated by PKA, which enables HSL to possess its hydrolase activity on TAG, as perilipin A is no longer blocking the access to the lipid droplet[5]. Hormone sensitive lipase (HSL) has a multifunctional role with hydrolase activity primarily for diacylglycerol (DAG)[65], but also for TAG, cholesterol ester and retinyl ester[65, 66]. Another lipase, the adipose triglyceride lipase (ATGL), also known as desnutrin, is involved in hydrolyzing TAG into DAG. The co-activator of ATGL, comparative gene identification-58 (CGI-58), is known to enhance the hydrolyzing activity of ATGL[67]. The final step of lipolysis is not regulated by hormones and generates free fatty acids and glycerol from the breakdown of monoacylglycerols, catalyzed by monoglyceride lipase (MGL)[65].

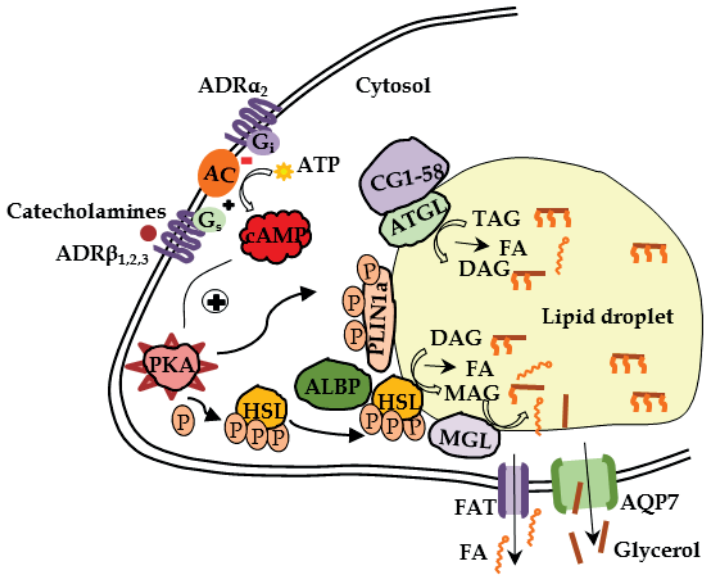

Figure 4. cAMP-mediated lipolysis in adipocytes. In an energy-deprived state, catecholamines bind to the adrenergic receptor coupled to $G_{\alpha s}$, leading to activation of adenylyl cyclase (AC) and production of cAMP. Elevated intracellular cAMP levels activate protein kinase A (PKA), which subsequently phosphorylates and activates hormone-sensitive lipase (HSL), leading to translocation of the enzyme from the cytosol to the lipid droplet. PKA also phosphorylates perilipin A (PLIN1a), which leads to a conformational change of PLIN1a and lipases gain access to the lipid droplet. The hydrolyzing activity of adipose triglyceride lipase (ATGL), HSL and monoglyceride lipase (MGL) generate diacylglycerol (DAG) and monoacylglycerol (MAG). Comparative gene identification-58 (CGI-58) is a co-activator of ATGL whereas adipocyte lipid-binding protein (ALBP) interacts with HSL and facilitates intracellular transport of fatty acids (FA). Fatty acid translocase (FAT) and aquaporin 7 (AQP7) are transporters for FA and glycerol, respectively.

In the postprandial state, insulin inhibits lipolysis through binding to its receptor leading to autophosphorylation and subsequently phosphorylation and activation of downstream targets, such as protein kinase B (PKB), the kinase responsible for phosphorylation and activation of the cyclic nucleotide phosphodiesterase 3B (PDE3B)[68]. The activated PDE3B exerts its effect on cAMP, leading to reduced levels and inhibition of cAMP-dependent activation of lipolysis (**Figure 5A**)[68]. Thus, insulin promotes energy storage by inhibiting the hydrolysis of TAG. Other processes for insulin-induced energy storage in the postprandial state are stimulation of dietary-derived lipid uptake (**Figure 5B**)[69], glucose uptake and subsequently fatty acid synthesis, known as *de novo* lipogenesis[66, 70]. Dietary lipids transported with chylomicrons are hydrolyzed by lipoprotein lipases (LPL), which is stimulated by insulin[66, 69, 71], and the generated fatty acids are taken up via diffusion[72] or fatty acid transporters, e.g. fatty acid translocase (FAT)[73]. The fatty acids are then converted to fatty acyl-CoA and utilized together with glycerol-3-phosphate in the triacylglycerol synthesis, which for example involves the catalyzing activity of different acyltransferases, e.g. diacylglycerol acyltransferase (DGAT) involved in the final step of triacylglycerol synthesis[74].

De novo lipogenesis, which is the synthesis of fatty acids from glucose intermediates or amino acids, is used to a minor extent in adipocytes[66]. Insulin mediates an increase in acetyl CoA carboxylase (ACC)[70], which regulates the rate-limiting step of fatty acid synthesis, catalyzing the production of malonyl-CoA from two acetyl-CoA moieties[75]. Two isoforms of ACC exist, ACC1 and ACC2, and it has been observed that these isoforms produce different pools of malonyl-CoA, subsequently used as substrate for fatty acid synthesis or acting as a negative regulator of β-oxidation, respectively[75]. Malonyl-CoA elongates to longer chains of fatty acids is subsequently converted to monounsaturated fatty acid to be used as substrate in triacylglycerol synthesis[70].

Even though only a minor proportion of glucose is taken up by adipocytes, it has a substantial role for whole body glucose homeostasis[76]. For example, an adipose-selective reduction of GLUT4 in mice leads to impaired glucose tolerance and insulin resistance[14]. Insulin-mediated glucose uptake by glucose transporter 4 (GLUT4) is initiated by the binding of insulin to its receptor (insulin receptor, IR) that provokes autophosphorylation of IR as well as recruitment and phosphorylation of adaptor proteins, such as insulin receptor substrates (IRS) (**Figure 5B**)[77]. IRS proteins are then able to interact with and activate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase)[77] that eventually leads to phosphorylation of PKB and Akt substrate of 160 kDa (AS160), involved in regulating GLUT4 translocation to the plasma membrane[77, 78]. There is also a PI3-kinase independent pathway in the trafficking of GLUT4, involving the adaptor protein with pleckstrin homology (APS)[76, 79]. After glucose uptake, glucose is utilized, which generates metabolic intermediates, e.g. dihydroxyacetone phosphate and acetyl-CoA, used as energy substrate or for production of e.g.

glycerol-3-phosphate and fatty acyl-CoA subsequently used for triacylglycerol synthesis[5]. In addition, glucose and/or metabolites activate ChREBP, a transcription factor that for example regulates expression of lipogenic enzymes[12, 13].

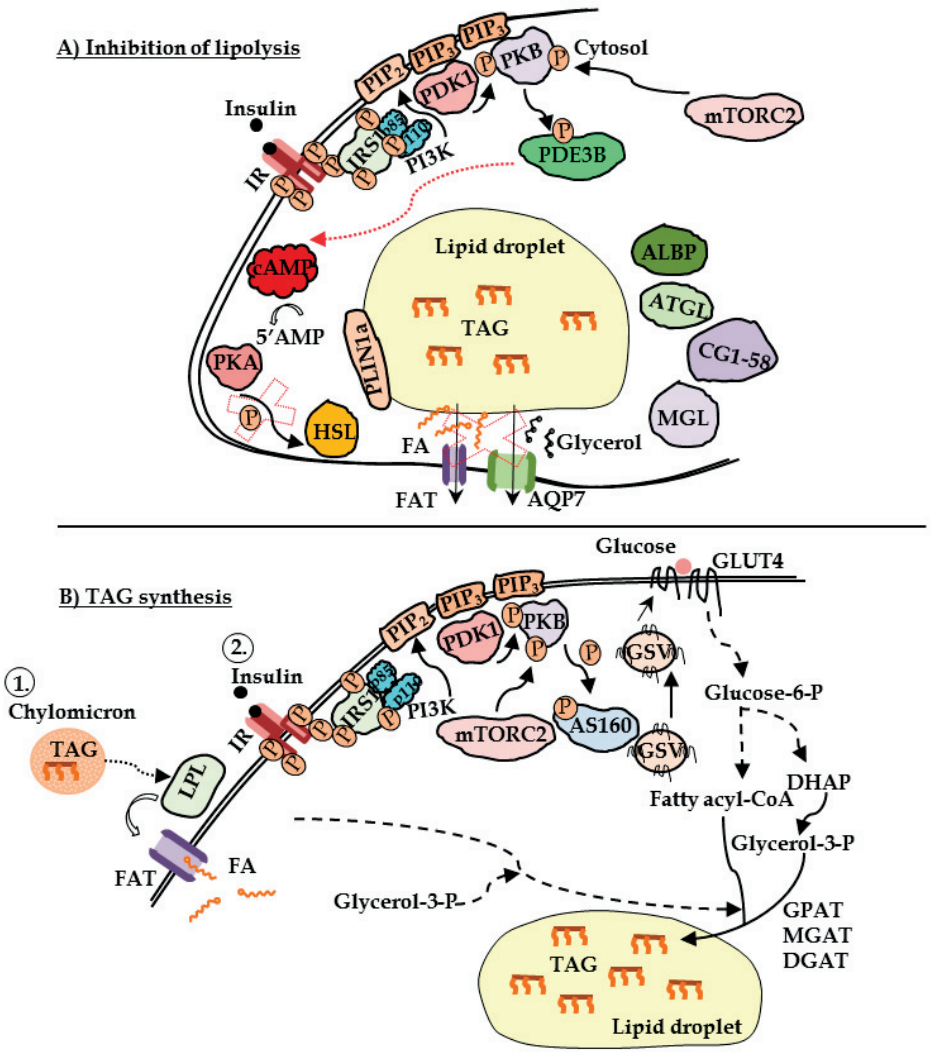

Figure 5. Insulin signaling in the postprandial state. A) Insulin mediates inhibition of cAMP-mediated lipolysis. Insulin binding to the insulin receptor (IR) induces conformational changes and activation of its tyrosine kinase activity, leading to recruitment of insulin receptor substrate 1 (IRS-1). The receptor tyrosine kinase phosphorylates IRS-1 at specific sites, which leads to the recruitment of SH2-containing phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). The subsequent activation of PI3K leads to production of phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 binds to the pleckstrin homology domain of protein kinase B (PKB), which gives rise to translocation and binding of PKB to the plasma membrane. PKB is phosphorylated and activated by pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK-1) and mTOR complex 2 (mTORC2). PKB phosphorylates and activates cyclic nucleotide phosphodiesterase 3B (PDE3B). PDE3B hydrolyzes cAMP, leading to the inhibition of cAMP-mediated lipolysis. B) Insulin promotes fatty acid and glucose uptake for energy storage. 1. Chylomicrons transport dietary-derived TAG from the intestine to the adipose tissue. Insulin mediates activation of lipoprotein lipase (LPL), located in the endothelial wall of capillaries surrounding the adipose tissue. LPL hydrolyzes TAG into fatty acids (FA), which enter the adipocyte via fatty acid translocase (FAT) or diffusion across the plasma membrane. FA together with glycerol-3-phosphate re-esterifies into TAG. 2. Insulin mediates activation of the PI3K-dependent pathway, as described in detail above. PKB phosphorylates Akt substrate of 160 kDa (AS160), leading to inhibition of the GTPase activity of AS160. This facilitates translocation of GLUT4 storage vesicle (GSV) to the plasma membrane for insulin-dependent glucose uptake. Metabolized glucose generates intermediates, such as fatty acyl-CoA and glycerol-3-phosphate, which are used as substrates for glycerol phosphate acyltransferase (GPAT), monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) in TAG synthesis.

The families of cyclic nucleotide phosphodiesterases

Cyclic nucleotide (cN) phosphodiesterases (PDE) comprise eleven families (PDE1-11) with a total of 21 gene products and 100 resulting mRNA products, resulting in a wide range of protein products[80, 81]. The PDE families are structurally related and share a conserved catalytic site[80] . With distinct domain properties, the PDEs differ in the mechanisms whereby they are regulated[80]. PDEs share the properties to hydrolyze cyclic AMP (cAMP) and cyclic GMP (cGMP) to generate AMP and GMP[80]. Five PDE families have hydrolyzing activity for both cAMP and cGMP (PDE1, 2, 3, 10 and 11) whereas the other PDE families hydrolyze cAMP (PDE4, 7 and 8) or cGMP (PDE5, 6 and 9)[80]. The tissue and cellular distribution of PDE families varies, some are widely distributed whereas others are more restricted to certain cell types[80]. It is well described that PDEs compartmentalize to specific sites of the cell, either in cytosolic compartments and/or in membranes, which implicates a unique functional role for certain PDEs as they target specific signalosomes, e.g. containing a cAMP pool with a protein complex of scaffolds and cAMP-effectors[80, 82]. PDEs are activated by different regulators, e.g. calcium, hormones and neurotransmitters[80]. Altered PDE activities are seen in several diseases or metabolic conditions[80, 83]. In some cases, specific inhibitors targeting selective PDE families are being used in the clinic for the treatment of diseases[84], e.g. intermittent claudication (PDE3 inhibitors), pulmonary hypertension (PDE5 inhibitors) and erectile dysfunction (PDE5 inhibitors)[85-87]. Studies in animals lacking specific PDEs and use of tools that diminish the activity of selective PDE families have provided information regarding the function of different PDE isoforms[80, 83]. The effect of PDE3 on whole body metabolism has for example been studied in animal models selectively lacking the PDE3 family[88] or having enhanced activity of PDE3 in pancreatic β-cells[89] as well as using PDE3 inhibitors, e.g.[68].

The family of cyclic nucleotide phosphodiesterase 3

The PDE3 family consists of two isoforms, PDE3A and PDE3B, derived from two distinct genes[80]. These isoforms have similar structures with a conserved catalytic site, containing a 44-amino-acid insertion that differs between PDE3A and PDE3B[90]. Both PDE3A and PDE3B have high affinity for cAMP and cGMP[91]. The N-terminal portion contains hydrophobic membrane-association domains and several consensus sites for protein kinase A (PKA), protein kinase B (PKB) and binding sites for 14-3-3[92]. PDE3A is expressed in cardiac myocytes, platelets and vascular smooth muscle cells[93] whereas PDE3B is expressed in hepatocytes, adipocytes and pancreatic β-cells, cells having a central role in energy homeostasis[68]. In this thesis, PDE3B (**Figure 6**) is in focus and will be described in the following sections.

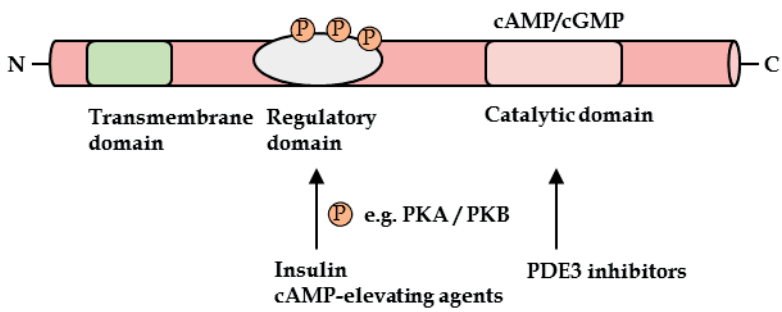

Figure 6. Structure of PDE3B. The conserved catalytic domain hydrolyzes both cAMP and cGMP and is a target for selective PDE3 inhibitors, e.g. OPC3911. In the N-terminal portion are hydrophobic regions located, which are predicted segments for attachment to membranes (transmembrane domain). The regulatory domain contains several serine residues that are phosphorylated in response to insulin and cAMP-elevating agents in isolated adipocytes, 3T3-LI adipocytes and H4II hepatoma cells. Some of these sites are located within consensus sequences for phosphorylation by PKA and PKB.

The role of PDE3B in liver, adipose tissue and pancreatic β-cells

The importance of PDE3B has been revealed in a number of studies where the expression or activity level has been altered to address its role in specific physiological functions and whole body metabolism, e.g.[88, 89, 94-100]. Furthermore, in various tissues of obese and diabetic animal models, an altered PDE3 activity has been observed[101-103]. For example, PDE3B mRNA, protein and enzymatic activity are reduced in adipose tissue of obese and diabetic animal models[102, 104, 105] as well as obese individuals[106]. A number of metabolic alterations are associated with the lack of PDE3B, as indicated in for example the PDE3B-null mice[88, 94]. PDE3B has a central role in regulating lipolysis, as it is the key enzyme in the anti-lipolytic action of insulin[99]. The PDE3B-null mice display enhanced cAMP-stimulated lipolysis, which is consistent with increased serum fatty levels[88]. Moreover, other alterations in the PDE3B-null mice were also associated with insulin resistance, such as increased serum insulin levels as well as dysregulated glucose homeostasis, involving increased gene expression of enzymes having a role in glucose production in the liver[88, 97]. An increased glucose output from the liver[88] was compatible with the role of PDE3B and related cAMP pools in glycogenolysis and gluconeogenesis[107-109]. Thus, the lack of PDE3 activity has negative consequences on glucose production and lipolysis, contributing to enhanced glucose and fatty acid levels in the circulation[94, 110].

Interestingly, the use of selective PDE3 inhibitors or by knocking down PDE3B in mice lead to increased mitochondrial activity and gene expression promoting brown adipogenesis in adipose tissue[94]. The increased protein level of uncoupling protein-1 also indicates a brown adipocyte phenotype[94] with uncoupling of the respiratory chain to produce heat instead of ATP of oxidized fatty acids[111]. Thus, the PDE3B-null mice might be able to neutralize to some extent the effects of enhanced lipolytic rate through increased energy dissipation.

Furthermore, the glucose-stimulated insulin secretion (GSIS) and the potentiating effect seen by GLP-1 were enhanced in PDE3B-null mice[88]. Thus, this indicates that PDE3B has an important role in regulating cAMP-mediated insulin secretion, which will be further described in the following section to emphasize the focus on PDE3B in pancreatic β-cells in this thesis.

PDE3B participates in insulin secretion in pancreatic β-cells

PDE1, PDE3, and PDE4 constitute the major PDE families in pancreatic β-cells and inhibition or siRNA-mediated silencing of the isoforms PDE1C, PDE3B and PDE4C potentiates glucose-stimulated insulin secretion (GSIS) in rodent models, e.g.[95, 112-114]. In human pancreatic islets, inhibition of PDE3 has been shown to potentiate insulin secretion[115]. With regard to PDE3B, extensive studies have revealed a functional role for PDE3B in pancreatic β-cells[89, 95, 96, 100]. PDE3B localizes not only to plasma membrane, but also to membranes of insulin granules and is suggested to regulate first and second phase of Ca^{2+}-stimulated insulin secretion[100].

Härndahl *et al*[89], generated a transgenic mouse model (RIP-PDE3B) with β-cell specific overexpression of PDE3B (full-length mouse Pde3b cDNA) under the control of the rat insulin promoter (RIP). Two transgenic lines of the RIP-PDE3B mouse model were established: one with a 2-3 fold (RIP-PDE3B/2) and another with a 7-10 fold (RIP-PDE3B/7) increase in PDE3 activity. The overexpression of β-cell PDE3B impaired GSIS and GLP-1 potentiated insulin secretion in RIP-PDE3B mouse islets[89]. On a long-term basis, the augmented levels of PDE3B cause β-cell dysfunction associated with deranged cellular organization and altered hormonal content within the islets[89]. The impairment is even more pronounced when the RIP-PDE3B mouse model is challenged by a high-fat diet, as these mice are more prone to develop hyperglycemia and peripheral insulin resistance[96].

More recent studies show evidence for the presence of other isoforms belonging to the families PDE2, PDE5, PDE8, PDE9, PDE10 and PDE11, in most cases detected at the mRNA level[113, 116]. Of the newly identified PDEs, PDE8B and PDE10A have been implicated in β-cell function, since diminished activity of PDE8B[113, 116] as well as inhibition of PDE10A[117] potentiated GSIS in rat pancreatic islets.

As it is not known how PDE3B is regulated in pancreatic β-cells, it led us to study activation and phosphorylation of the enzyme in response to stimuli of relevance for β-cell function. As a continuation, the long-term regulation of PDE3B protein in response to stimuli of relevance for insulin secretion was also studied. To further characterize the islets from the transgenic RIP-PDE3B mice, we did an attempt to identify gene targets that might be essential for their phenotypes and/or having some kind of connection with PDE3B. Finally, it is well recognized that studies made in animal models are not always comparable with findings in human individuals. With the scarce information that is available regarding PDEs in humans, we aimed to study the activity and expression pattern of selected PDE isoforms in pancreatic islets from human donors.

Osteopontin

Ostepontin (OPN), first identified as a secreted phosphoprotein, was given its name for the role in bone (osteon), where it acted as a bridge (pons) between cells and hydroxyapatite crystals in bone mineralisation[118]. OPN was also named secreted phosphoprotein 1 (SPP1)[118] and early T lymphocyte activator-1 (eta-1)[119], which reflects the diverse roles of the protein. OPN belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family consisting of several bone-derived proteins, e.g. bone sialoprotein, comprising the non-collagenous part of the extracellular matrix[120]. The proteins within the SIBLING family share some characteristics, e.g. exon structure and motifs, but differ with regard to distribution and function[120]. OPN expression has been detected in a variety of tissues and cells[118]. There are three different splice variants of OPN, OPN-a, -b and -c in humans[121]. OPN-a constitutes the full-length variant and alternative splicing of OPN-a give rise to OPN-b and -c, which lack 14 and 28 amino acids, respectively[121]. Through alternative splicing, alternative translation, post-translational modification as well as thrombin cleavage, different OPN proteins are formed and can thus exert different effects[118]. OPN forms are found intracellularly, in both cytoplasm and nucleus, and extracellularly in extracellular matrix and in body fluids, e.g. blood and urine[118]. The different forms of OPN have been ascribed different functions in cell migration, cell attachment, cell signaling and chemotaxis[118]. Also, the ability of OPN to interact with several integrins, especially members of the integrin family αvβ and αβ as well as CD44 is a necessity for a wide array of cellular functions[118]. Despite the fact that OPN is ubiquitously expressed and being involved in many processes, OPN deficient mice display a fairly normal physiology[122-124]. However, in pathological situations OPN either acts as a protectant or is involved in the progression of the disease[124].

Osteopontin in obesity and type 2 diabetes

OPN seems to be the link between obesity-induced inflammation and insulin resistance associated with T2D[125, 126]. In humans and animal models of T2D, OPN expression is elevated in kidney and arteries and is associated with nephropathy, a complication associated with late progression of diabetes[127-129]. OPN expression is also elevated in liver and adipose tissue as well as in plasma in individuals or animal models with diet-induced obesity, insulin resistance or T2D[125, 126, 130-136]. The increased OPN plasma levels observed in T2D individuals are reduced when patients are treated with peroxisome proliferator-activated receptor (PPAR)-α agonists for ameliorating dyslipidemia[130]. In addition, OPN mRNA expression is also suppressed in adipose tissue from obese rats and humans given PPAR-γ

agonist, mainly used for improving insulin responsiveness[134]. Thus, OPN appears to have a role in obesity and insulin resistance.

Osteopontin: the bridge between obesity and insulin resistance

The residing macrophages within the adipose tissue display the highest expression of OPN compared to the other cell types and seem to be the cell type responsible for the induced expression of OPN in murine obesity[125]. OPN is induced in monocytes that are in the progress to differentiate into macrophages[137] and mediates migration of macrophages via integrin α4 and α9[126, 138]. Accumulation of macrophages in adipose tissue is associated with obesity and insulin resistance, as a consequence of increased production of pro-inflammatory cytokines and local inflammation[139]. Obese mice lacking OPN or obese mice subjected to anti-OPN treatment display improved insulin sensitivity and decreased accumulation of macrophages in adipose tissue and liver[126, 133, 134, 140]. Moreover, mice lacking OPN are also protected from developing hepatic steatosis and lipids are only stored in white adipose tissue[133], without causing hypertrophy in adipocytes or insulin resistance in liver and skeletal muscle[134]. In the context of obesity and insulin resistance, OPN exacerbates the inflammatory state through recruitment of macrophages that upon activation secrete cytokines e.g. TNF-α and interleukin 6 (IL-6), known to cause impaired insulin signaling that consequently leads to insulin resistance[139, 141].

Osteopontin: a protectant in pancreatic β-cells

In contrast to the role of OPN in obesity and insulin resistance, OPN acts as a protectant in pancreatic islets and β-cells[142-145] in type 1 and type 2 diabetes[146, 147]. OPN is increased in response to several cytokines, e.g. interleukin 1β (IL-1β), in rat islets and in a β-cell line[143]. IL-1β mediates impaired glucose-stimulated insulin secretion (GSIS), but in combination with OPN, GSIS is restored in rat pancreatic islets[143]. In addition, β-cell OPN is induced in response to increased production of nitric oxide (NO) mediated by IL-1β and has a role in protecting the islets and β-cells from cytotoxicity through negatively regulating NO by inhibiting inducible nitric oxide synthase (iNOS) synthesis[143]. In this thesis, OPN has been studied in the context of β-cells in a possible crosstalk with PDE3B.

The generation of short-chain fatty acids

It is well established that dietary fibers are good for health[148] and several studies indicate that a high fiber intake improves metabolic parameters in obese and T2D individuals[149-151]. Dietary fibers consist of non-digestible carbohydrates, such as different starches and polysaccharides[152] that are not accessible for breakdown by enzymes in the small intestine[153]. The bacteria residing in the colon are responsible for the fermentation of dietary fibers, resulting in the generation of the major end products short-chain fatty acids (SCFAs). SCFAs are mainly produced from indigestible carbohydrates, but also from fatty acids and amino acids derived from proteins and peptides[154, 155]. The most abundant SCFAs are acetic acid, propionic acid and butyric acid and the majority is formed in caecum and ascending colon[156, 157]. The formation and proportion of SCFAs is dependent on several factors, such as amount and type of indigestible carbohydrate and its accessibility in diet, fermentation capability of the residing bacterial populations and gut transit time[154, 157]. Only a minor proportion of SCFA is excreted in feces, the remaining part is rapidly absorbed throughout the colon, by both passive and active transport mechanisms[158]. Butyric acid is mostly utilized by colonocytes as energy substrate[159], whereas propionic acid and acetic acid are used as metabolites in the liver and in peripheral tissues, respectively[155, 160]. Measures have been done in sudden-death victims and all three SCFAs have been estimated to be in the micromolar range concentration in the systemic circulation[155, 156, 161].

Targeting obesity with short-chain fatty acids

There are indications for a role of SCFAs in contributing to the health beneficial effects of dietary fibers on metabolism, as is described below (**Figure 7)**. Studies indicate that SCFAs, either given through the diet or administrated orally or rectally, foremost improve glucose and lipid parameters in animal models and human individuals[162-167]. For example, it has been shown that rodents given acetic acid, propionic acid or butyric acid are protected from diet-induced obesity[168-170]. Furthermore, obese mice fed a normal diet supplemented with butyric acid improved metabolic parameters and displayed reduced fat content, consistent with a reduction in body weight and improved insulin tolerance, suggesting a role for butyric acid in treatment of diet-induced obesity[168]. Even though SCFAs have been ascribed a favorable role in metabolism, it is important to elucidate the direct mechanisms that contribute to the beneficial effects of SCFAs on target tissues.

Effects of short-chain fatty acids in liver, adipose tissue and pancreatic islets

SCFAs are energy substrates and are not only produced through bacterial fermentation but also through endogenous production, either by increased fatty acid oxidation or by metabolized amino acids and glucose[171]. The liver has an important role in lipid and cholesterol metabolism[172] . Studies have suggested that propionic acid reduces triacylglycerol and cholesterol content in liver and has cholesterol-lowering effects in serum of rat[163, 166]. Consistent with this is the observation that propionic acid inhibits cholesterol and fatty acid synthesis as well as generation of triacylglycerol in isolated rat[173, 174] and human hepatocytes[175]. Acetic acid and butyric acid are on the other hand used as a substrate for generating fatty acids, cholesterol and triacylglycerol in isolated hepatocytes from rat[173, 174] and human individuals[175]. These observations indicate that the different SCFAs can have diverse roles in lipid and cholesterol metabolism in the liver.

The adipose tissue is the major site for energy storage and is also an endocrine organ, releasing adipokines involved in the regulation of whole body energy homeostasis[5, 21]. In rodent adipocytes, acetic acid and propionic acid inhibit lipolysis and induce differentiation of fibroblasts into 3T3L1- adipocytes, the latter confirmed by the adipocyte differentiation marker PPAR-γ2 and formation of oil droplets[176-178]. There is also evidence for acetic acid to suppress free fatty acids in plasma from mice[177] and humans[179-181]. In another study, it was observed that acetic acid inhibits insulin-stimulated glucose and fatty acid uptake in primary murine adipocytes[182]. Explants of human omental adipose tissue treated with propionic acid have on the other hand increased levels of GLUT4 mRNA[183]. Adipokines are released from adipocytes and adipose tissue, a secretion pattern that is altered in obesity[7]. The adipokine leptin is involved in regulating food intake and energy expenditure[19]. In recent studies it has been observed that SCFAs induce leptin production in adipocytes and adipose tissue in mice[178, 184]. The stimulatory effect of propionic acid on leptin production has also been shown in omental and subcutaneous adipose explants from non-diabetic individuals[185]. Thus, these studies suggest that SCFAs are able to modulate adipocyte function and might therefore be important mediators in regulating whole body energy homeostasis.

SCFAs might contribute to the beneficial effects on glucose metabolism by for example targeting the hormone-secreting cells in pancreas. In a previous study, it was shown that propionic acid inhibits glucose-stimulated insulin secretion in isolated rat pancreatic islets[186]. A stimulatory role was, however, shown in a study by Brockman *et al*[187], where propionic acid and butyric acid infused intraportally increased both insulin and glucagon secretion in sheep. Moreover, acetic acid and propionic acid have the ability to enhance the secretion of the incretin glucagon-like peptide 1 (GLP-1) from intestinal L-cells and can thus indirectly potentiate

insulin secretion via the incretin effect[188]. There is obviously a role for SCFAs in regulating hormonal secretion from pancreatic islets, but whether SCFAs have a stimulatory or inhibitory role on hormone-secreting cells needs to be established.

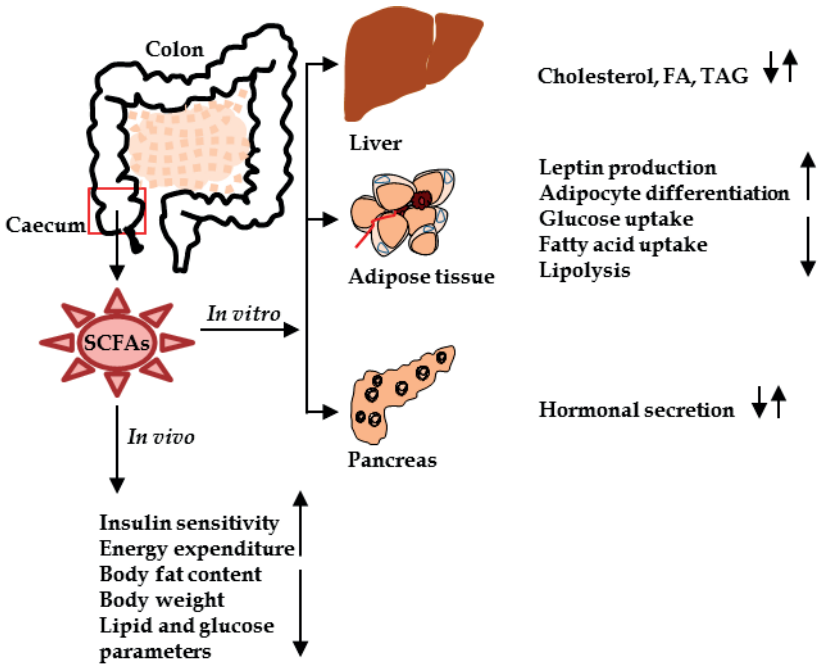

Figure 7. The effect of short-chain fatty acids on metabolism. Fermentation of dietary fibers by bacteria in caecum produces short-chain fatty acids (SCFAs), mainly acetic acid, propionic acid and butyric acid. *In vivo* studies in rodents, as illustrated above, give evidence for a protective role for SCFAs against diet-induced obesity. However, the direct effects of SCFAs on target tissues, such as pancreas, adipose tissue and the liver, are still inconsistent, as is clearly indicated in the illustration.

Short-chain fatty acids and their receptors

SCFAs mediate effects via their receptors, the free fatty acid receptor (Ffar) 2 and 3, also known as G protein coupled receptor (GPCR) 41 and 43, respectively[171]. These receptors are activated by SCFAs with different specificity[171]. Ffar2 and 3 utilize G proteins comprised of different α subunits for intracellular signaling; Ffar2 and 3 couple to $G_{\alpha i/\alpha o}$ whereas Ffar2 also couples to $G_{\alpha q}$[189, 190]. The Ffar receptors are found in many tissues, e.g. liver, pancreatic islets and adipose tissue[171]. Referring to adipose tissue, which is within the scope of this thesis, Ffar2 and 3 have been reported to be expressed in both human and mouse adipose tissue as well as in mouse adipocytes by some researchers[176, 178, 184, 191], whereas others state that only Ffar2 is detected in adipocytes[176, 178]. Mouse models with deficiency in either Ffar2 or 3 have been generated to study the effect on whole body homeostasis, although with rather contradicting findings[169, 182, 192-194]. To assess the importance of Ffar2 in adipose tissue, Kimura *et al*[182] generated a mouse model that specifically overexpressed Ffar2 in adipose tissue. The Ffar2-adipose tissue specific model was lean and when challenged by a high-fat diet, these mice were protected against development of obesity and showed improved insulin sensitivity[182].

In summary, from *in vivo* studies it seems that SCFAs have anti-obesity properties. However, the information is scarce with regard to the direct effects of SCFAs on target tissues involved in regulating whole body homeostasis, such as adipose tissue, pancreatic islets and the liver. The mechanisms behind the health beneficial properties of SCFAs are important to elucidate to be able to develop a strategy for preventing obesity and T2D. In this thesis, the effects of propionic acid and butyric acid on glucose and lipid metabolism have been studied in primary adipocytes. The study contributes with new aspects related to lipolysis and to our knowledge, effects of propionic acid and butyric acid on the *de novo* lipogenesis and glucose uptake, have not been demonstrated previously.

Aims

The general aim of this thesis was to elucidate the effects of hormones and nutrients on the regulatory mechanisms and functions in β-cells and adipocytes.

The specific aims were to:

I. Study phosphorylation and activation of PDE3B in response to glucose, forskolin and insulin.

II. Examine the expression and activity of selected PDEs in human pancreatic islets.

III. Identify a connection between genes important for the phenotypic characteristics of the RIP-PDE3B islets and PDE3B by *in silico* and functional approaches.

IV. Study the effect of short-chain fatty acids on basal and hormone-regulated lipid and glucose metabolism in primary adipocytes.

Methodology

This section summarizes the general methods used in this thesis.

Animal models

Mice overexpressing the full-length mouse Pde3b cDNA under the control of the rat insulin promoter (RIP) have been used as a model. RIP-PDE3B/2 and RIP-PDE3B/7 exhibit a 2-3 fold and a 7-10 fold increase in PDE3 activity, respectively[89]. These two RIP-PDE3B mouse models (paper II) have been used for collection of pancreatic islets to identify genes associated with their typical phenotypic characteristics (impaired insulin secretion and deranged cellular morphology). The wild type mice C57Bl/6 (paper II) and the Sprague Dawley rat (paper I and III) have been used for collection of pancreas and collection of pancreatic islets as well as for collection of epididymal adipose tissue.

Primary Adipocytes

Epididymal adipose tissue was dissected from Sprague Dawley rats, 36 days of age. Primary adipocytes were isolated from adipose tissue by a collagenase digestion technique[195]. The advantage of using primary adipocytes instead of an adipocyte-like cell line is that freshly isolated adipocytes can be obtained from specific adipose depots and from animals with different age. They also resemble a more physiological model than immortalized cell lines. However, primary adipocytes are not long-lived in culture and need to be handled with care during experimental setups.

In paper III, primary adipocytes were used to study the effect of short-chain fatty acids on glucose and lipid metabolism.

Pancreatic islets

Pancreatic islets obtained from healthy human donors were provided by the Nordic Network for Clinical Islet Transplantation (directed by Olle Korsgren, Uppsala University) and were characterized by the Human Tissue Laboratory at Lund University. It is desirable to conduct research in human pancreatic islets as it enables an insight in human biology. Unfortunately, the availability of human pancreatic islets is scarce and it is thus more convenient to conduct research in animal models. Freshly isolated pancreatic islets from both mice (8-12 weeks old wild type C57Bl/6 and transgene RIP-PDE3B) and rat (5-6 weeks old Sprague Dawley) were utilized. A collagenase digestion technique described by Lacy and Kostianovsky[196] was used to isolate pancreatic islets. In brief, the pancreas was filled with a collagenase solution, removed and pancreatic islets were collected under a stereomicroscope. To use freshly isolated pancreatic islets is an advantage, as it resembles a more physiological model with the β-cells in their native milieu, enabling cell-to-cell interaction with intact paracrine signaling. One disadvantage is, however, the limited lifespan of isolated islets, as they are derived from sacrificed animals and cannot be maintained in culture for longer time period.

Within the scope of paper I and II, pancreatic islets derived from human, rat and mice have been used to study mRNA (microarray), protein and activity levels of selected PDEs and OPN.

INS-1 (832/13) cells

The insulinoma cell line (832/13) was originally derived from rat and is a single β-cell population with genotypic and phenotypic uniformity when cultured in an *in vitro* condition[197]. The use of a cell line is more cost efficient and less time-consuming than to work with primary cells isolated from tissue. One reason for this is that a cell line is immortalized and can be used for several experimental setups as it is subcultured regularly. It also minimizes the requirement of sacrificing animals for use in experimental setups. The rat insulinoma cell line INS-1 (832/13), stably transfected with the human proinsulin gene, is a clone of the INS-1 cell line consisting of well differentiated β-cells[197]. They mimic the normal function of pancreatic β-cells and are stable in the sense of responsiveness to secretagogues such as glucose, the main trigger for insulin secretion, glucagon-like peptide 1 (GLP-1) and palmitate[197]. It has also been demonstrated that INS-1 (832/13) contains both K_{ATP}-dependent and independent pathways, which makes it a useful tool for understanding the precise molecular mechanisms behind glucose-stimulated insulin secretion[197].

In paper I and II, we have used INS-1 (832-13) cells to study acute regulation (activation and phosphorylation) of PDE3B as well as to study long-term regulation of PDEs and OPN in response to e.g. glucose, insulin and cAMP-elevating agents.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE and immunoblot analysis have been used in order to separate and detect proteins of particular interest with antibodies raised against a specific peptide or phospho-peptide. The procedure for SDS-PAGE and immunoblotting involves several steps and to acquire experimental data that is as accurate as possible, method optimization is a necessity to avoid unspecific binding and weak detection signals. Enhanced chemiluminescence (ECL) was used for detection of the specific proteins and the reaction that follows with ECL produces luminescence that is related to the amount of protein detected with an imager.

This method has been used throughout this thesis to study protein and phosphoprotein expression levels in pancreatic islets or β-cell as well as adipocytes.

PDE assay and selective PDE inhibitors

The family-selective PDE inhibitors for PDE1, PDE3 and PDE4 were present or absent in the enzymatic assay to be able to measure particular PDE activities. Enzymatic measurements of PDEs were conducted using ^{3}[H]-labelled cAMP as a substrate[198]. In brief, ^{3}[H]-cAMP, was hydrolyzed to ^{3}[H]-AMP by the particular PDE in the sample. The final product, ^{3}[H]-adenosine produced through dephosphorylation of ^{3}[H]-AMP, is measured using liquid scintillation counting and represents the PDE enzymatic activity/ies in the sample[198].

In paper I, we have studied the activation of PDE3 in response to glucose, insulin, forskolin and agents that modulates the K_{ATP}-channel to be in its active or close state in rat pancreatic islets and INS-1 (832/13) cells. We have also studied the activity of PDE1, PDE3 and PDE4 using family-selective PDE inhibitors in human pancreatic islets. In paper II, INS-1 (832/13) cells were incubated overnight with family-selective PDE inhibitors (PDE1/3/4) or with the general PDE inhibitor IBMX to study the effect on the expression of PDEs.

Studying phosphorylation of PDE3B

Previously, attempts have been made to develop phosphospecific antibodies against serine residues known to be phosphorylated on PDE3B in intact adipocytes in response to insulin and isoproterenol, but without a success in generating high-quality antibodies against a specific site. Thus, to be able to detect changes in phosphorylation pattern, we used a radiolabeling method that enables subsequent quantitative analysis. We overexpressed PDE3B using an adenoviral system, to acquire sufficient amounts for detecting a phosphorylation pattern in response to a certain stimulus. Thus, cell were first pre-labeled with radioactive phosphate, P^{32}, to label the cellular ATP pool and then stimulated with various stimuli, as indicated below. Immunoprecipitation enables isolation of a particular protein in a crude lysate with use of specific antibodies prepared against a certain peptide of the protein of interest[199]. The advantages with immunoprecipitation are that it allows antibody recognition of an antigen in its native conformation and enables a more concentrated form of the protein in the solution prior to separation and quantification of particular proteins. Membrane fraction containing PDE3B was prepared by a 175 000 g centrifugation step. PDE3B was then immunoprecipitated from detergent-solubilized membranes. Separation of the immunoprecipitated PDE3B through SDS-PAGE, immunoblot analysis and subsequently autoradiography enabled detection and visualization of emitted [^{32}P] bound to PDE3B.

In paper I, we have studied total phosphorylation of PDE3B in response to glucose, insulin and forskolin in INS-1 (832/13) cells.

Affymetrix Genechip

The Affymetrix genechip monitors the relative levels of expression of thousands of genes simultaneously and provides a quantitative measure of the abundance of a particular mRNA sequence. However, using microarrays as a tool for identifying gene expression alterations involves several steps, including tissue harvesting, sample preparation, and microarray performance with labeling and hybridization, which are sources of variability and can thus influence the final results. Thus, to minimize experimental errors, the Genomics Core at National Heart, Lung, and Blood Institute (National Institutes of Health, USA) with standardized setups performed the microarray and initial analysis. The Swegene Centre for Integrative Biology at Lund University also performed extensive data analysis to evaluate and approve the quality of the microarray data.

In paper II, we have performed gene expression analysis to identify changes in gene expression in RIP-PDE3B pancreatic islets. Islets from C57Bl/6 mice were used as controls in the experimental setup.

Bioinformatics databases

The interologous interaction database (I2D), a database of predicted and experimentally validated protein-protein interactions (PPIs)[200, 201], was used to derive all existing protein interactions for the mouse interactome. Within the biological network, the PPIs consist of nodes (proteins) and edges (interactions) that connect the nodes with each other[202]. Certain candidate genes, herein defined as seed genes, were selected to find possible connections between them in the PPI network that contained the integrated differential expression data. With the use of NetworkX in Python (http://networkx.lanl.gov/) a function was implemented to return all shortest paths between the seed genes. In the search of minimal route connecting the seed genes, we restricted to path length of 1, 2 and 3. The path length describes the nearest route from protein A to protein B and path length 1 is defined as the nearest route with a direct interaction between protein A and B. Cytoscape, an open-source platform for complex network analysis[203], was used to visualize the constructed PPI network.

The RIP-PDE3B gene list, consisting of the genes that are altered compared to gene expression levels in wild type mice, was submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) and through the functional annotation clustering tool, pathway analysis results were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG), the bioinformatics resource that displayed most gene hits associated with diseases[204].

In paper II, we have constructed a protein-protein interaction (PPI) network with integrated RIP-PDE3B expression data using a systems biology approach. The RIP-PDE3B gene lists for both RIP-PDE3B/2 and RIP-PDE3B/7 islets have also been used to extract genes associated with diseases and related pathways (unpublished findings).

Glucose and lipid metabolism in primary adipocytes

In paper III, we have studied the effect of propionic acid and butyric acid on basal and stimulated lipolysis, *de novo* lipogenesis and glucose uptake in primary rat adipocytes.

Lipolysis is the hydrolysis of triacylglycerol into glycerol and free fatty acids[5]. In the lipolysis assay[205], lipolysis is measured as the release of glycerol into the cell-free medium. More specifically, glycerol is converted to glycerol-3-phosphate by glycerol kinase and glycerol-3-phosphate dehydrogenase in the presence of ATP and NAD, respectively. The subsequent measure of NADH, the reduced form of NAD^+, at optical density of 340 nm determined the rate of lipolysis.

Fatty acids are synthesized from intermediates of glycolysis (metabolization of glucose) in a process known as *de novo* lipogenesis[66]. The produced free fatty acids are then subsequently re-esterified with glycerol-3-phosphate in the synthesis of triacylglycerols[66]. In the assay for lipogenesis[206], a toluene-based scintillation liquid containing non-water soluble scintillators was used to measure the incorporation of [^{3}H]-labelled glucose metabolites into lipids by scintillation counting.

Glucose uptake is facilitated by the glucose transporter (GLUT), where GLUT1 and GLUT4 are responsible for the basal and insulin-dependent glucose uptake, respectively, in adipocytes[207]. In the assay for glucose uptake[208], the incorporation of [^{14}C]-labelled glucose into adipocytes was determined using liquid scintillation counting. Cytochalasin B, an inhibitor of glucose uptake by both GLUT1 and GLUT4[209], was utilized to determine the rate of facilitated uptake of [^{14}C]-labelled glucose.

Results and Discussion

Cyclic nucleotide phosphodiesterase 3B (PDE3B) is expressed in adipose tissue, liver, pancreatic β-cells and hypothalamus and has been found to regulate many metabolic events central to whole body energy homeostasis[68]. It is well established that PDE3B localizes to different compartments in the cell, e.g. invaginations of the plasma membrane, known as caveolae, in adipocytes and hepatocytes as well as in plasma membrane and insulin granules in pancreatic β-cells, which is evident from localization studies[95, 97, 98, 100]. PDE3B is involved in regulating glucose and lipid metabolism in adipocytes[98, 99] and hepatocytes[88, 97], as well as insulin secretion in pancreatic β-cells[89, 95, 96, 100, 113]. The following sections for paper I and II, will describe findings referring to β-cell PDE3B.

The first paper contributes with findings regarding phosphorylation and activation of PDE3B and expression of PDEs in human and rat pancreatic islets. In the second paper, we show the connection between PDE3B, osteopontin (OPN) and protein kinase CK2 in pancreatic β-cells.

Regulation of PDE3B in pancreatic β-cells and expression of PDEs in human pancreatic islets (paper I)

cAMP has an established role as a potentiator of insulin secretion through intracellular mechanisms. PDEs, enzymes with the function to hydrolyze cAMP, thus have a potential function as insulin secretory regulators in β-cells. Previous studies have shown that β-cell PDE3B has an important role in the regulation of glucose-stimulated insulin secretion (GSIS) and cAMP-potentiated GSIS in rat and mouse[89, 95, 100, 113]. In the following sections, we describe the phosphorylation and activation of PDE3B in response to stimuli having a role for insulin secretion and the presence of selected PDEs in human pancreatic islets.

Activation of PDE3B in rat pancreatic islets and INS-1 (832/13) cells

Glucose, the main trigger of insulin exocytosis, was used in the present investigation to study activation of PDE3 in rat pancreatic islets and β-cells. We found that glucose activate PDE3B (**Figure 8A**). Diazoxide, a K_{ATP}-channel activator that hyperpolarizes the β-cells and prevents insulin secretion even in the presence of glucose[41], was used in the presence of high glucose to elucidate if glucose-mediated activation of PDE3B is dependent on endogenously produced insulin. Diazoxide did not inhibit glucose-mediated activation of PDE3B, indicating that glucose is not mediating its effect via endogenously produced insulin (**Figure 8B**).

Elevated K^+ concentration depolarizes the β-cell and triggers Ca^{2+} influx and insulin secretion, thus bypassing glucose metabolism[41]. The finding that high K^+ induces activation of PDE3B (**Figure 8C**) suggests that glucose-induced activation of PDE3B is mediated downstream of the metabolization of the sugar, maybe at the level of Ca^{2+} influx. It is known that increased Ca^{2+} gives rise to elevated cAMP levels via activation of Ca^{2+}-dependent adenylyl cyclases (AC)[210, 211]. Thus, it is possible that glucose mediates its effect on PDE3B via a cAMP-dependent mechanism[54].

The second messenger cAMP is involved in regulating glucose-stimulated insulin secretion (GSIS) in a fine-tuned interplay with calcium[52]. cAMP is also essential for pancreatic β-cells with its role in cell differentiation, growth and survival[52]. The finding that the cAMP-elevating agent forskolin induced activation of PDE3B (**Figure 8D**) is in agreement with previous findings demonstrating activation of PDE3B in response to cAMP-increasing hormones in adipocytes and hepatocytes[212, 213].

Insulin appears to have both inhibitory and stimulatory roles in events that confer to β-cell function, e.g. apoptosis, insulin biosynthesis, and insulin secretion[28]. The ability of insulin to activate PDE3B (**Figure 8E**) in rat pancreatic islets and β-cells is in agreement with the finding that insulin activates PDE3B in rat and mouse adipocytes[92, 214] and hepatocytes[92, 97]. Thus, the insulin action on pancreatic β-cell might be of importance for mediating an autocrine effect on insulin exocytosis[28].

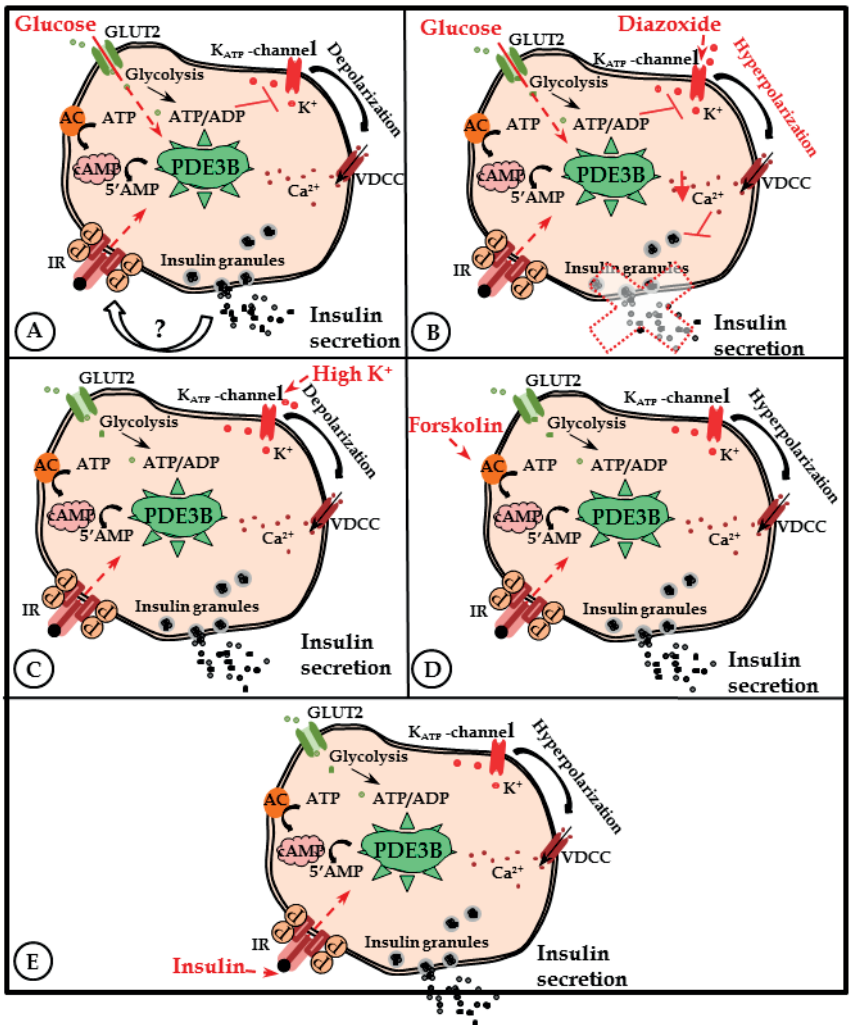

Figure 8. Activation of PDE3B in pancreatic islets and β-cells. PDE3B is activated in response to glucose, forskolin and insulin in isolated rat pancreatic islets (A-E) and β-cells (A and C-E). High glucose (A) involves uptake and metabolization of glucose, which generates an increase in ATP and closure of the K_{ATP}-channel. This closure results in depolarization, opening of voltage-dependent Ca^{2+}-channels (VDCC) and an influx of Ca^{2+}, which triggers exocytosis of insulin granules. Diazoxide (B) is a K_{ATP}-channel activator that hyperpolarizes the β-cells and prevents insulin secretion even in the presence of high glucose. Elevated K^+ (C) concentration depolarizes the β-cell and triggers Ca^{2+} influx and insulin secretion, thus bypassing glucose metabolism. Forskolin (D) activates adenylyl cyclase (AC), leading to increased intracellular cAMP levels. Insulin (E) binding to insulin receptor (IR), results in activation of downstream targets, which involves stimulatory or inhibitory action on insulin secretion. In (C), (D) and (E), low glucose maintains the β-cell in a hyperpolarized state.

Phosphorylation of PDE3B in INS-1 (832/13) cells

Activation coupled to phosphorylation of PDE3B has been extensively studied in adipocytes[215-217] and partly in hepatocytes[92] but there are no previous reports concerning phosphorylation of PDE3B in pancreatic β-cells. To be able to study phosphorylation of PDE3B in rat pancreatic β-cells, PDE3B was overexpressed using an adenoviral system. The phosphorylation of the recombinant PDE3B was studied, as described in the methodology section. Notably, control experiments with recombinant PDE3B in INS-1 (832/13) cells showed that it could be activated by e.g. high glucose and insulin and was localized to the same intracellular compartments as the endogenous PDE3B. Further, we have previously shown that recombinant PDE3B attenuates glucose-induced insulin secretion and glucagon-like peptide 1 (GLP-1) potentiated insulin secretion[95, 100]. In agreement with results from adipocytes and hepatocytes, forskolin-induced activation of PDE3B was coupled to an increased total phosphorylation of PDE3B (**Figure 9**). However, glucose-stimulated activation of PDE3B was coupled to a decrease in total phosphorylation of PDE3B (**Figure 9**). This is the first time that an increase in PDE3B activity has been coupled to a decrease in total phosphorylation of the enzyme, which could be explained by a glucose-induced activation of a phosphatase dephosphorylating an "inhibitory" phosphorylation site in PDE3B.

Stimulation with insulin did not result in any apparent change in the amount of phosphorylation of PDE3B (**Figure 9**). However, to detect insulin-induced alterations in phosphorylation, it may be necessary to study changes in the phosphorylation of unique phosphorylation sites in PDE3B. In adipocytes, both protein kinase B (PKB) and protein kinase A (PKA) have been suggested kinases for PDE3B[215, 217-219] and several phosphorylation sites have been identified and shown to be specific for activation by, for example, insulin and isoproterenol[92, 216]. Thus, it has not been established which kinases phosphorylate PDE3B in β-cells. We therefore suggest that forskolin and insulin induce phosphorylation of PDE3B, presumably by activating PKA and PKB, respectively.

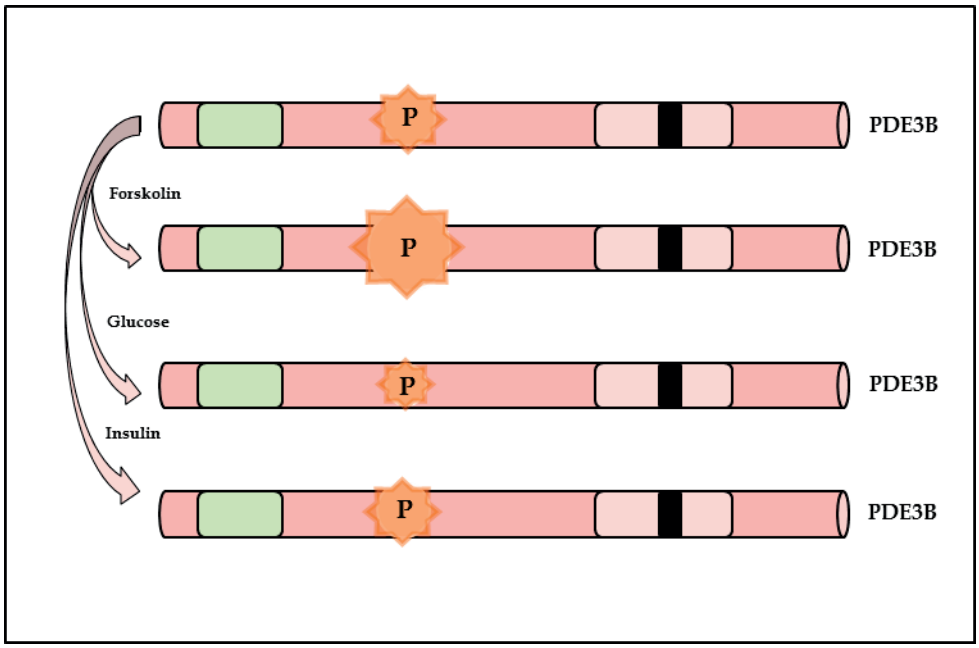

Figure 9. Phosphorylation of PDE3B in β-cells. Activation of PDE3B is associated with a change in total phosphorylation. PDE3B in the top is illustrated as being phosphorylated in the basal state to more easily describe an increase, a decrease and no apparent change in phosphorylation in response to forskolin, high (16 mM) glucose and insulin, respectively.

The presence of PDEs in human pancreatic islets

In paper I, selected members of the cyclic nucleotide phosphodiesterase (PDE) families were studied in human pancreatic islets. The activities of PDE1, PDE3 and PDE4 were detected with family-selective PDE inhibitors in enzyme assays (**Figure 10A**). Immunoblot analysis confirmed the presence of PDE4C, PDE7A, PDE8A and PDE10A in human pancreatic islets (**Figure 10B**). Unpublished results show that PDE1A, PDE5A, PDE7B and PDE11A are detected in pancreatic islets from human donors (**PDE10B**). Of four PDE4 members (PDE4A, B, C and D)[80], only PDE4C was detected, indicating that PDE4C is the major PDE4 isoform in human pancreatic islets. Members of the PDE7 family have to our knowledge not been described previously in pancreatic islets or β-cells. With regard to PDEs as potential targets for modulating insulin secretion, several studies have shown that diminished activity of PDE1C[113], PDE3B[95, 220], PDE4C[113] or PDE8B[116] potentiate glucose-stimulated insulin secretion in animal models. Inhibitors for PDE10A have also been described as insulin secretagogues[117], but others state that the effect on glucose-stimulated insulin secretion is not significant[113]. The presence of PDE1, PDE3 and PDE4 activities in human pancreatic islets has to our knowledge only been reported in one previous article, which also describes the potentiating effect of inhibiting PDE3 on insulin secretion[115]. However, as PDE3 inhibitors have adverse metabolic effects in other tissues, it is thus promising that abolished activity of other PDE isoforms potentiates glucose-stimulated insulin secretion.

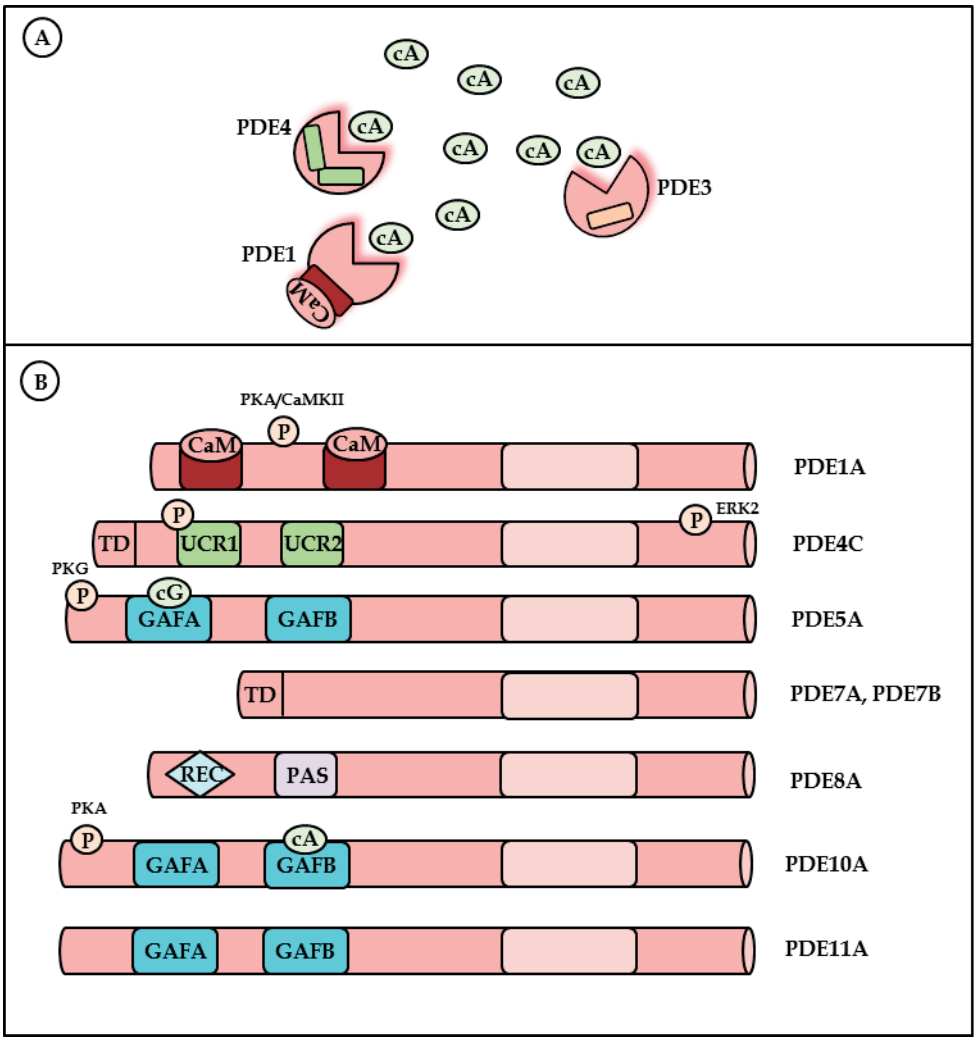

Figure 10. The presence of PDEs in human pancreatic islets. Illustrate A) the activity of PDE1, PDE3 and PDE4 as well as B) the expression of PDE1A, PDE4C, PDE5A, PDE7A, PDE7B, PDE8A, PDE10A and PDE11A in human pancreatic islets. cA, cAMP; CaM, Calmodulin; P, phosphorylation; PKA, protein kinase A; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase; TD, Targeting domain; UCR, Upstream conserved region; ERK2, Extracellular signal-regulated kinase 2; PKG, Protein kinase G; cG, cGMP; GAF, cGMP-specific phosphodiesterases, adenylyl cyclases and formate-dehydrogenlyase transcriptional activator domain; REC, Signal regulatory domain; PAS, Period circardian protein, aryl hydrocarbon receptor nuclear translocator protein and single-minded protein domain.

Cyclic nucleotide phosphodiesterase 3B is connected to osteopontin and protein kinase CK2 in pancreatic β-cells (paper II)

Transgenic RIP-PDE3B mice exhibit either a ~2-fold (RIP-PDE3B/2) or a ~7-fold (RIP-PDE3B/7) increase in PDE3 activity (Pde3b cDNA is under the control of the rat insulin promoter (RIP))[89]. It is evident from a previous study that the most typical characteristics of the RIP-PDE3B mice are deranged islet morphology and impaired insulin secretion in response to glucose and GLP-1[89]. These alterations are exaggerated with an increased activity of PDE3, as seen in the RIP-PDE3B/7 mice with the most evident change in PDE3 activity[89]. When challenged by a high-fat diet, the RIP-PDE3B/2 mice are also more prone to develop insulin resistance and glucose intolerance than wild type mice[96]. In paper II, several approaches were implemented to clarify mechanisms whereby overexpression of PDE3B in pancreatic β-cells causes phenotypic alterations in RIP-PDE3B mice. These strategies involve microarray analysis, *in silico* and functional studies. We contribute with findings regarding the connection between PDE3B and osteopontin (OPN), proteins having an essential role in pancreatic β-cells.

OPN is up-regulated in RIP-PDE3B islets

Microarray analysis revealed that several genes are altered in the islets of the RIP-PDE3B transgenic mouse models compared to islets of wild type mice. The results show that 38 and 1026 genes were altered in islets of RIP-PDE3B/2 and RIP-PDE3B/7 mice, respectively, as compared to islets of wild-type mice (**Figure 11A)**. To further evaluate the phenotypic characteristics, the gene expression profiles for RIP-PDE3B/2 and RIP-PDE3B/7 islets were compared to identify if the same genes were altered in both models. Opn, a multifunctional protein relevant in the context of β-cell function[143, 145] and suggested to be an actor in the initiation of insulin resistance[126, 140], was one of the genes displaying a "dose-dependent" alteration, when RIP-PDE3B/2 and RIP-PDE3B/7 expression levels were compared. OPN was therefore selected for further analysis. The induced level of OPN mRNA was consistent with an increased level of OPN protein in islets from the RIP-PDE3B/7 mice (**Figure 11B**).

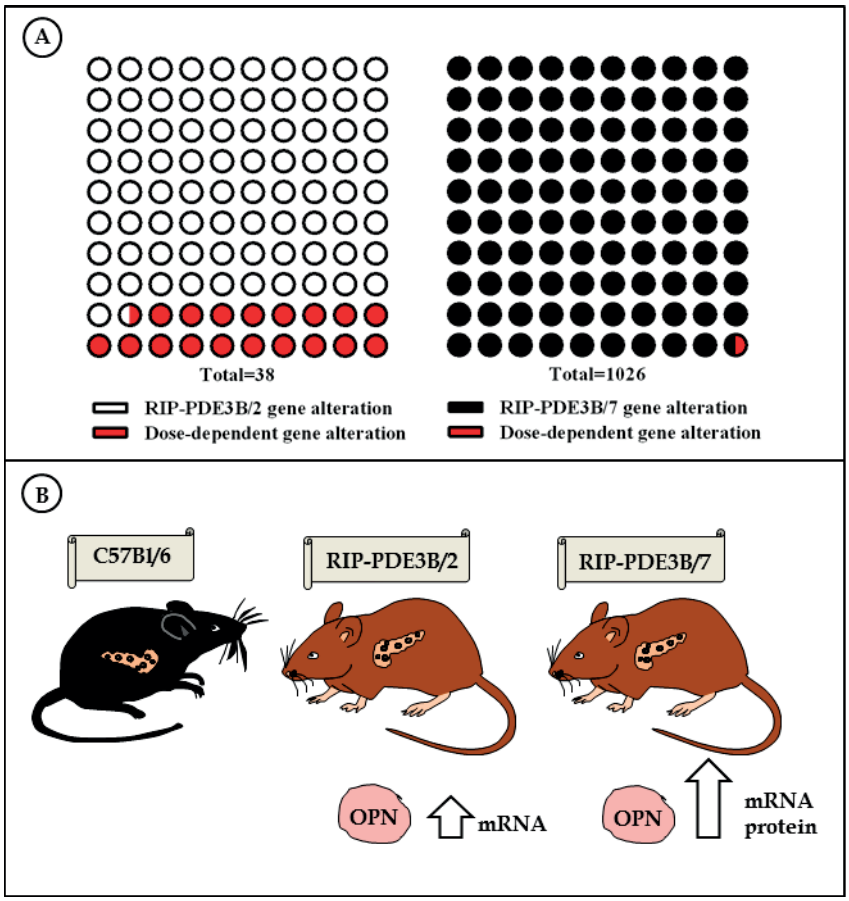

Figure 11. Alteration in mRNA levels in islets of RIP-PDE3B mice. A) 38 and 1026 genes are altered in RIP-PDE3B/2 islets (white and red circles) and RIP-PDE3B/7 islets (black and red circles), respectively. 7 genes (red circles), detected in both RIP-PDE3B/2 and RIP-PDE3B/7 islets, show a "dose-dependent" alteration that reflects upon the increase in PDE3B activity in islets of the RIP-PDE3B/2 (2-fold induction of PDE3B activity) and RIP-PDE3B/7 (7-fold induction) mice. B) OPN is one of the 7 genes that are altered in a dose-dependent manner in islets of RIP-PDE3B/2 and RIP-PDE3B/7 mice. In RIP-PDE3B/7 islets, OPN protein expression follows a similar trend as for OPN mRNA in comparison with expression levels in C57Bl/6 islets. There is also an increase in PDE3B protein expression in islets of RIP-PDE3B/7 mice.

PDE3B interacts with OPN via 14-3-3 and protein kinase CK2

In an attempt to establish a connection between PDE3B and OPN, a network consisting of predicted and experimentally validated protein-protein interactions was constructed with the use of the interologous interaction database[204]. The differentially expressed data for the RIP-PDE3B mouse models with defined seed genes (Opn, Pde3b and members of nuclear factor of activated T cells (Nfat)) was integrated into the network together with the shortest path length, which simplified describes the nearest possible route between particular proteins in the network. In the sub-network that evolved from this selection, Opn was the only differentially expressed gene that was detected and connected to Pde3b via Ywhab and Csnk2a1 (**Figure 12)**. Some mechanisms behind these interactions have been reported by others. OPN is for example phosphorylated by protein kinase CK2, a kinase that is predicted to be involved in regulating a huge proportion of the phosphoproteome[221]. With regard to 14-3-3s, studies imply that protein kinase CK2 can phosphorylate 14-3-3s[222]. In the network, Pde3b is directly connected with Ywhab, a protein belonging to the family of 14-3-3s[223]. The interaction between PDE3B and 14-3-3s has been identified[224] and "protects" PDE3B from being dephosphorylated, which has been revealed by Palmer *et al*[225]. In the present study, the utilization of a protein kinase CK2 inhibitor reduced the level of PDE3B and OPN proteins in pancreatic β-cells. Inhibition of protein kinase CK2 has previously been shown to elevate insulin secretion[226]. Thus, it is possible that the reduced PDE3B protein level contribute to this effect, as family-selective PDE3 inhibitors act as insulin secretagogues[113]. However, a direct interaction between PDE3B, OPN and protein kinase CK2 has, to our knowledge, not been studied.

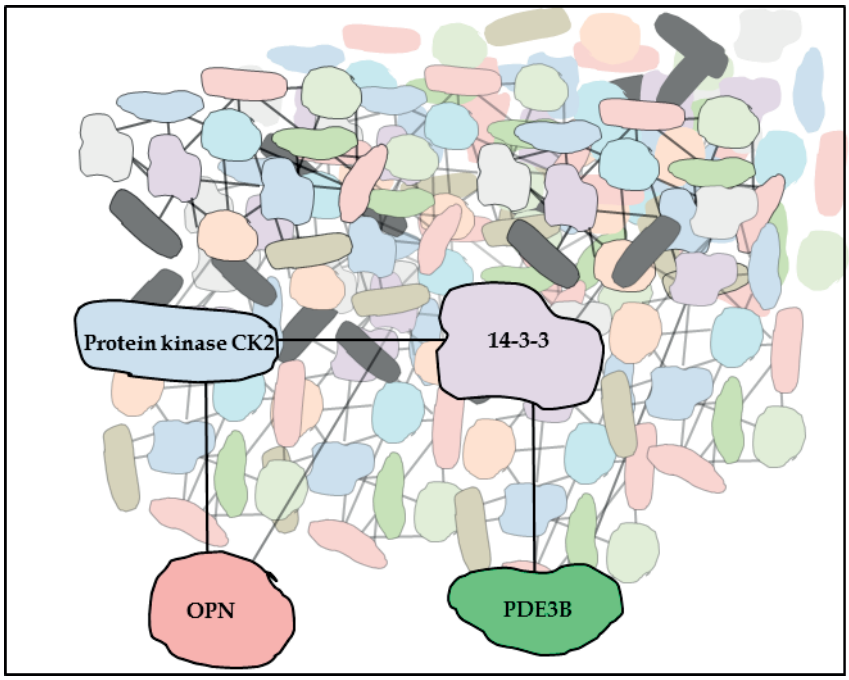

Figure 12. A connection between PDE3B and OPN in the sub-network. Path length defines the shortest distance between two nodes (proteins). For PDE3B and OPN the shortest distance is via 14-3-3 and protein kinase CK2 where 3 indicates the number of links (distance) between them (path length 3).

Long-term regulation of PDE3B and OPN in pancreatic β-cells

The sub-network with differentially expressed data for both RIP-PDE3B/2 and RIP-PDE3B/7 mice indicated that Opn connected with Pde3b via Csnk2a1. We therefore decided to study the regulation of PDE3B and OPN protein expression in response to glucose, insulin and cAMP-elevating agents.

Stimulating cells with forskolin induces activation of adenylyl cyclase (AC), leading to an increase in intracellular cAMP levels[227]. We show that forskolin induces PDE3B protein (**Figure 13**), indicating for a long-term feedback mechanism for negatively regulating cAMP levels within the β-cell. The use of selective PDE inhibitors for PDE1, 3 or 4 allow for inhibiting specific PDE families localized to different regions in the cell[80]. We found that PDE3B protein is induced by selective inhibitors for PDE1, PDE3 and PDE4 (**Figure 13**). This indicates, in the context of β-cell, that PDE1, 3 and 4 communicate in the regulation of specific cAMP pools. Indeed, all three PDEs have been implicated in the regulation of insulin secretion[100, 112, 113, 228]. The regulation of OPN protein by forskolin and PDE inhibitors follow a similar pattern as observed for PDE3B

protein (**Figure 13**). There is evidence that cAMP response elements exist in the promoter regions of both PDE3B and OPN genes[229-231]. With regard to OPN, CREB is involved in the induction of OPN expression by binding to AP-1 sites on the promoter[229]. In one of the studies related to PDE3B, stimulation with the non-selective PDE inhibitor IBMX increased phosphorylation of CREB and could thereby regulate Pde3b expression through enhancement of promoter activities[231]. cAMP has a dual role in regulating OPN mRNA and protein levels in different cell types[232-235]. As for the long-term regulation of PDE3B by cAMP, both up- and down-regulation of PDE3B have been shown, depending on cell type[236-239].

An autocrine effect of insulin can be both inhibitory and stimulatory on glucose-stimulated insulin secretion[240]. In the present study, insulin induced PDE3B and OPN protein expression to a similar extent in pancreatic β-cells (**Figure 13**). Consistent with our finding is that insulin has been shown to induce PDE3B protein in 3T3-L1 adipocytes[214]. With regard to OPN, a recent study observed that insulin stimulation increased OPN protein expression in vascular smooth muscle cells in spontaneous hypertensive rat[241]. In pancreatic β-cells, OPN is capable of improving glucose-stimulated insulin secretion[143]. Thus, insulin-induced effects on OPN protein could have an important role in mediating a positive autocrine effect on insulin exocytosis[242].

Glucose is the main trigger of insulin exocytosis in pancreatic β-cells[243]. We show that high glucose induces PDE3B protein in pancreatic β-cells (**Figure 13**). On the other hand, we were not able to detect any change in OPN protein expression in response to high glucose (**Figure 13**). However, in other studies divergent effects of glucose on OPN promoter activity, mRNA and protein in pancreatic islets and β-cells have been reported[142, 244]. It is known that the OPN promoter contains glucose-responsive elements[245] and the activity of the promoter is either induced or reduced by glucose in the β-cell line RINm5f, which is dependent on the glucose concentration and incubation time[142, 145]. In the non-obese diabetic (NOD) mice, OPN, detected at 4 weeks of age, is reduced with the onset of hyperglycemia at 16 weeks of age[142]. It thus appears that the regulation of OPN in response to glucose is rather complex.

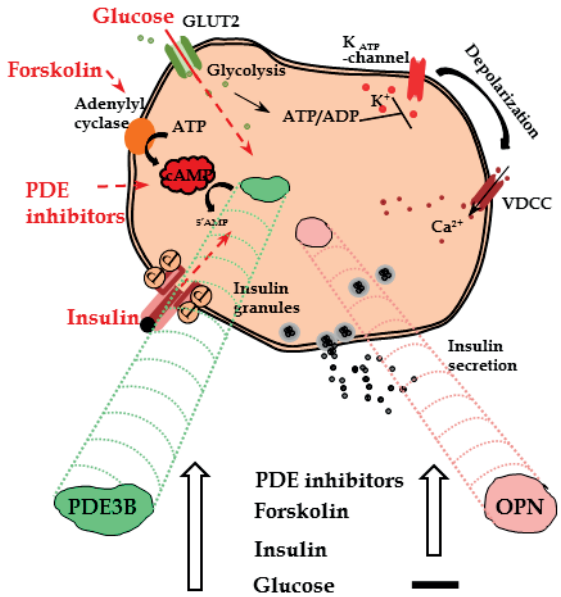

Figure 13. Long-term regulation of PDE3B and OPN in pancreatic β-cells. High (16 mM) glucose, insulin and cAMP-elevating agents, such as forskolin and selective PDE inhibitors for PDE1, 3 and 4 families, induce PDE3B protein. OPN protein expression follow a similar trend in response to insulin and cAMP-elevating agents, but no changes is observed in response to high glucose.

Genes associated with diabetes in islets of the RIP-PDE3B/7 mouse model (unpublished results)

Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway analysis enables extraction of genes associated with different diseases and metabolic pathways[204]. To further evaluate the altered RIP-PDE3B gene expression, KEGG was used to find genes associated with diabetes. The gene hits in islets of the RIP-PDE3B/2 mouse model were associated with glycerolipid metabolism, NOD-like receptor signaling pathway and focal adhesion, but there were no indication for an association with diabetes. For the RIP-PDE3B/7 mouse model, there were a number of pathways associated with the altered gene expression and three of those pathways were related to diabetes: maturity-onset diabetes of the young (MODY), insulin signaling pathway and T2D (**Figure 14**).

Of the genes associated with MODY and T2D, GLUT2 (Slc2a2) was one of the genes that was down-regulated (**Figure 15**). Reduced expression of GLUT2, the glucose transporter that predominates in rodent pancreatic β-cells[42], is associated with β-cell dysfunction[246] and might contribute to the impairment of glucose-stimulated secretion (GSIS) in islets of RIP-PDE3B mouse models[89]. In both RIP-

PDE3B/2 and RIP-PDE3B/7 islets, GLUT2 is irregularly distributed and shows weak immunostaining in the plasma membrane[89, 96].

An up-regulation of insulin (Ins2) was also detected in the RIP-PDE3B/7 islets and as indicated in **Figure 15**, involved in MODY, T2D and insulin signaling. However, in the two RIP-PDE3B mouse models, insulin content was equal compared to wild type after normalization for total islet protein, but displayed irregular insulin immunostaining in RIP-PDE3B islets, e.g.[89]. Islet amyloid peptide, associated with MODY, was up-regulated in the RIP-PDE3B/7 mouse model (**Figure 15**). In response to glucose, insulin is released together with islet amyloid polypeptide, which in T2D contributes to the formation of amyloid deposits, relevant for the associated decline in β-cell mass[247].

The two transcription factors forkhead box protein A2 (Foxa2) and neurogenin 3 (Neurog3) are associated with MODY and were up-regulated in the RIP-PDE3B/7 islets (**Figure 15**). Studies describe the importance of forkhead box protein A2 and neurogenin 3 in pancreatic β-cells, e.g. increased protein levels of forkhead box protein A2 impair glucose-stimulated insulin secretion[248] whereas a deficiency in neurogenin 3 has devastating effects on endocrine cell development, as the generation of pancreatic cell types is totally abolished[249].

The gene for Abcc8 encodes for the sulfonylurea receptor 1 (SUR1) subunit of the K_{ATP}-sensitive channel[250] was associated with T2D and showed reduced mRNA levels in the RIP-PDE3B/7 mouse model (**Figure 15**). In the RIP-PDE3B mouse model, the first phase of insulin secretion is specifically affected[89], but whether this impairment is due to fewer or less functional K_{ATP}-sensitive channels in the plasma membrane has not been investigated. Moreover, the insulin signaling pathway revealed that Prkar1a encoding protein kinase A (PKA), involved in regulating insulin secretion[54], was down-regulated. Thus, down-regulation of SUR1 and PKA can be likely explanations for the impairment of GLP-1 to potentiate glucose-stimulated insulin secretion in RIP-PDE3B islets[89].

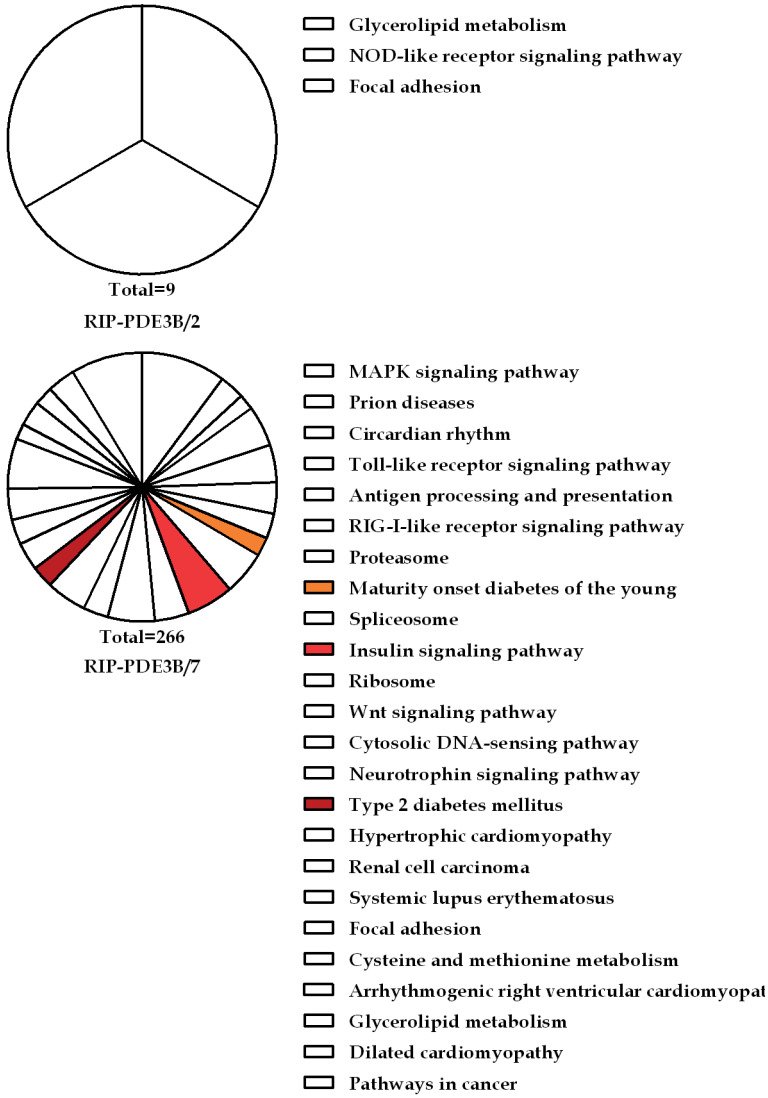

Figure 14. Extraction of genes associated with pathways in RIP-PDE3B islets. The gene list for RIP-PDE3B/2 and RIP-PDE3B/7 islets was submitted to DAVID and pathway analysis results were obtained from KEGG. 9 (RIP-PDE3B/2) and 266 (RIP-PDE3B/7) genes were associated with diseases or distinct pathways. DAVID uses modified Fisher Exact P-Value, EASE Score Fisher for gene enrichment analysis (P-Value = 0 represents perfect enrichment). P-values ranged from 0.01 to $8.2*10^{-4}$.

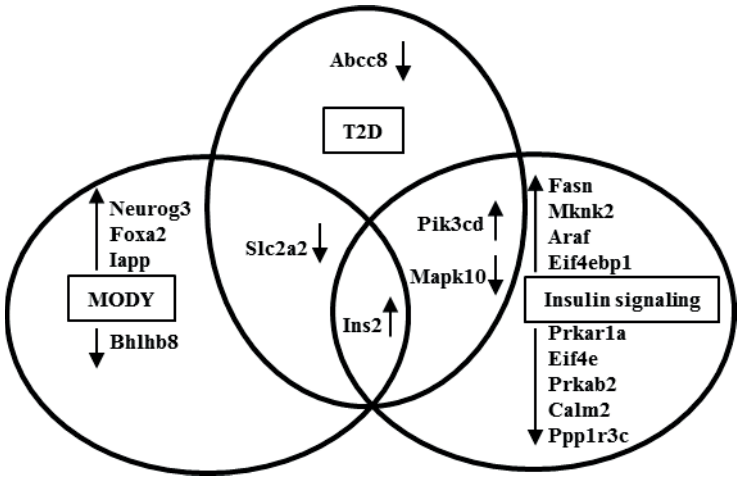

Figure 15. Genes associated with diabetes in RIP-PDE3B/7 islets. There were a minor proportion of genes in the RIP-PDE3B/7 gene list that were enriched in maturity onset diabetes of the young (MODY), type 2 diabetes (T2D) and insulin signaling. Genes in the intersections are enriched in two or three pathways. The gene expression is presented as up- or down-regulated, which is based on fold change of normalized values for RIP-PDE3B/2 and RIP-PDE3B/7 compared to C57Bl76 mice.

In summary, the altered genes that were associated with diabetes and related signaling mechanisms might contribute to the exaggerated phenotype of the RIP-PDE3B/7 islets. Notably, KEGG could only extract a minor proportion of the genes with altered expression and there are indeed other genes that might contribute to the phenotypic characteristics of the RIP-PDE3B mouse models.

Propionic acid and butyric acid inhibit lipolysis and *de novo* lipogenesis and increase insulin-stimulated glucose uptake in primary adipocytes (paper III, unpublished)

Fermentation of dietary fibers by colonic microbiota generates SCFAs, e.g. acetic acid, propionic acid and butyric acid that have beneficial effects on health in the context of energy metabolism[251]. Adipocytes within adipose tissue store energy and through the release of fatty acids and adipokines they play a role in regulating whole body energy homeostasis[5]. The importance of healthy adipocytes is seen in obesity, where decreased ability to store energy in adipocytes leads to increased circulating levels of free fatty acids and accumulation of lipids in non-adipocyte cells, which is associated with insulin resistance and increased risk of developing T2D[7]. Studies have shown that propionic acid and butyric acid protect against development of insulin resistance and obesity in mice challenged by a high-fat diet[168, 169]. The finding that these propionic acid and butyric acid have anti-obesity properties is highly relevant for the development of drugs for the treatment of obesity and thus also T2D. Hence, it is highly relevant to study the direct effect of SCFAs on adipocytes. Thus, paper III contributes with findings regarding the effect of propionic acid and butyric acid on glucose and lipid metabolism in primary rat adipocytes.

Propionic acid and butyric acid inhibit lipolysis

In adipose tissue, the hydrolysis of triacylglycerols into free fatty acids and glycerol, a process known as lipolysis, is often dysregulated in obesity, leading to excessive release of free fatty acids to the plasma and reduced insulin sensitivity[252]. To be able to identify the effects of propionic acid and butyric acid on lipolysis, we stimulated primary adipocytes with or without different lipolytic agents in the presence or absence of SCFAs and measured glycerol release from the cells. The lipolytic agents, isoproterenol (ISO) and adenosine deaminase (ADA), stimulate lipolysis by increasing intracellular cAMP levels through activation of G_s via ISO and inhibition of G_i via the removal of adenosine using ADA[253]. In the present study, we show that both SCFAs inhibit ISO and ADA-stimulated lipolysis (**Figure 16A**). We also show that lipolysis stimulated by suppression of the cAMP-degrading enzyme PDE3B is inhibited by both SCFAs (**Figure 16A**). The ability of SCFAs to inhibit lipolysis when PDE3B is suppressed indicates that SCFAs do not mediate their anti-lipolytic effects via enhanced degradation of cAMP, which is the case for insulin[254]. In previous studies, propionic acid and acetic acid were shown to inhibit basal and ISO- or

ADA-stimulated lipolysis in 3T3-LI adipocytes and primary mouse adipocytes[176-178]. Thus, inhibition of lipolysis by SCFAs could have an important role in preventing elevation of circulating free fatty acids, associated with lipid accumulation and lipotoxicity seen in obese and T2D individuals.

Propionic acid and butyric acid inhibit *de novo* lipogenesis

De novo lipogenesis is an insulin-stimulated process generating fatty acids from e.g. glucose intermediates that are subsequently used for synthesis of triacylglycerols[66]. The effect of propionic acid and butyric acid on basal and insulin-stimulated *de novo* lipogenesis was studied in primary rat adipocytes. In both conditions, propionic acid and butyric acid significantly inhibited *de novo* lipogenesis (**Figure 16B)**. We also show, which is consistent with the inhibition of *de novo* lipogenesis, that ACC1 is phosphorylated at serine 79 in the presence of propionic acid and butyric acid (**Figure 16B**), leading to inhibition of the rate-limiting enzyme of *de novo* lipogenesis[66]. In other studies, it has been shown that propionic acid inhibits whereas butyric acid stimulates fatty acid synthesis in rat hepatocytes[173, 174]. During *de novo* lipogenesis, malonyl CoA, the natural inhibitor of carnitine palmitoyltransferase controlling the transport of fatty acid into the mitochondria, is produced from acetyl CoA[75]. The ability of SCFAs to diminish *de novo* lipogenesis might have an important role to lower the production of malonyl CoA and to increase β-oxidation, a beneficial effect in the context of obesity and insulin resistance.

Propionic acid and butyric acid potentiate insulin-induced glucose uptake

Even though only a minor proportion of glucose is utilized by adipose tissue, the insulin-stimulated glucose uptake in adipocytes is favorable for whole body glucose homeostasis. A study by Abel *et al*[14] exemplifies that an adipocyte-specific knockdown of glucose transporter 4 (GLUT4) in mice develop insulin resistance in skeletal muscles and the liver, consequently leading to glucose intolerance and hyperinsulinemia. Thus, the effect of SCFAs on insulin-stimulated glucose uptake was studied. We found that both propionic acid and butyric acid potentiated insulin-induced glucose uptake in a dose-dependent manner (**Figure 16C**). In contrast to our findings, Kimura *et al*[182] observed that acetic acid inhibits insulin-stimulated glucose uptake in primary mouse adipocytes. Thus, the finding that propionic acid and butyric acid augment insulin-induced glucose uptake might be beneficial in those circumstances when glucose homeostasis is disturbed.

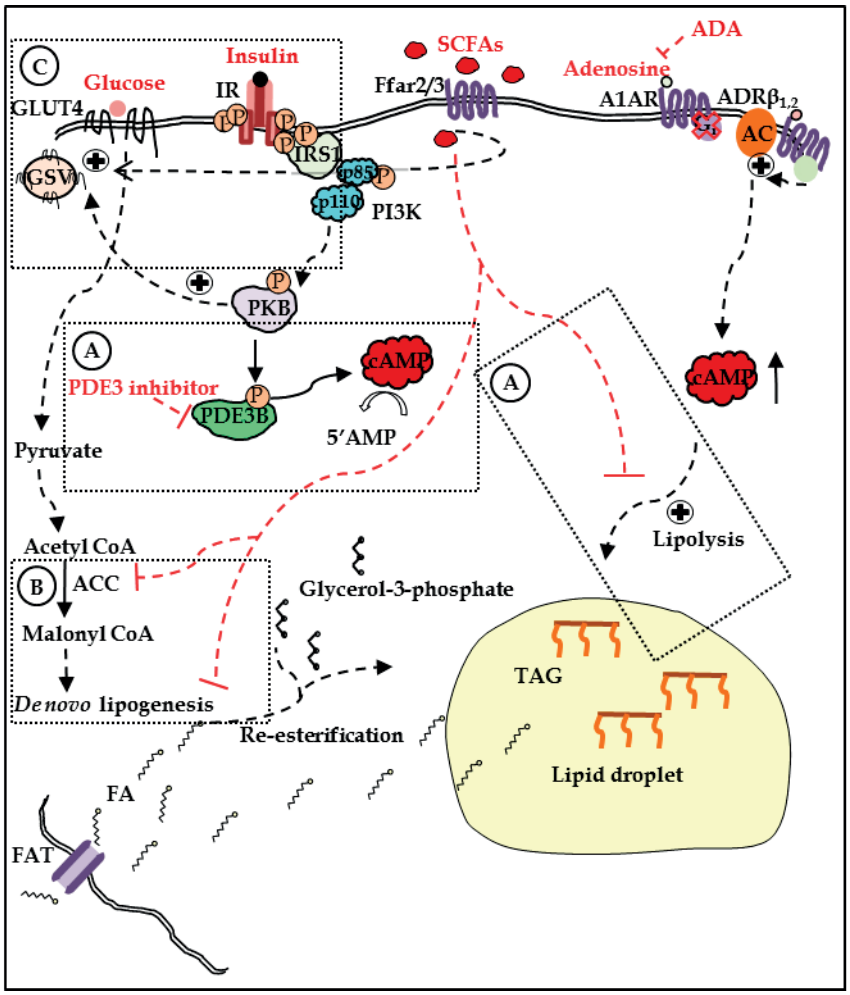

Figure 16. Effect of SCFAs on glucose and lipid metabolism in adipocytes. **A)** SCFAs (propionic acid and butyric acid) inhibit cAMP-potentiated lipolysis stimulated by adenosine deaminase (ADA), isoproterenol (ISO) or ISO in combination with a PDE3 inhibitor. ADA deaminates adenosine and prevents inhibition of adenylyl cyclase (AC), which leads to production of cAMP. ISO induces receptor-coupled activation of G_s and production of cAMP via activation of AC. Inhibition of PDE3B by a family-selective PDE3 inhibitor, results in elevated intracellular levels of cAMP. **B)** Propionic acid and butyric acid inhibit basal and insulin-stimulated *de novo* lipogenesis. Glucose is taken up by adipocytes through glucose transporter (GLUT) 1 (basal state, not illustrated) and 4 (insulin-stimulated state) and is eventually converted to acetyl CoA. Acetyl CoA carboxylase (ACC), the rate-limiting enzyme of *de novo* lipogenesis, catalyzes the production of malonyl CoA from two acetyl CoA moieties, the first step in *de novo* lipogenesis. FAs are then used in triacylglycerol synthesis. **C)** Propionic acid and butyric acid potentiate insulin-stimulated glucose uptake. Insulin binding to insulin receptor (IR) induces conformational changes and receptor activation, leading to autophosphorylation and recruitment of insulin receptor substrate 1 (IRS-1). In brief, IRS-1 signaling events lead to phosphorylation of PKB that mediates insulin-stimulated GLUT4 translocation to the plasma membrane and subsequently glucose uptake. Ffar, Free fatty acid receptor.

Conclusions

Within the scope of this thesis, investigations have been made in both pancreatic β-cells and primary adipocytes. The following conclusions are drawn:

Paper I

I. Activation of PDE3B in response to glucose, forskolin and insulin is associated with a decrease, an increase as well as no apparent change in phosphorylation of the enzyme, respectively.

II. PDE1, PDE3, PDE4C, PDE7A, PDE8A and PDE10A are expressed in human pancreatic islets.

Paper II

I. Based on *in silico* and functional approaches, PDE3B and OPN are connected via 14-3-3 and protein kinase CK2 in the protein-protein interaction network.

II. PDE3B and OPN protein levels are co-regulated in response to insulin, forskolin and the family-selective inhibitors for PDE1, PDE3 and PDE4 in pancreatic β-cells.

III. A minor proportion of the altered genes in the RIP-PDE3B/7 mouse model are associated with diabetes.

Paper III

In primary rat adipocytes, propionic acid and butyric acid:

I. inhibit cAMP-potentiated lipolysis.

II. do not mediate their anti-lipolytic effect via PDE3B, which is the case for insulin.

III. inhibit basal and insulin-stimulated *de novo* lipogenesis.

IV. increase phosphorylation of acetyl CoA carboxylase 1, which lead to reduced activity of the rate-limiting enzyme of the *de novo* lipogenesis.

V. inhibit basal and potentiate insulin-stimulated glucose uptake.

Concluding remarks and future perspectives

With regard to β-cell dysfunction, reduced cAMP content or impaired cAMP responses, associated with reduced insulin secretion, has been observed in pancreatic islets of diabetic animal models[255, 256] and more recently also in human subjects[257]. In the clinic, glucagon-like peptide 1 (GLP-1) receptor agonists have been developed to potentiate cAMP-dependent insulin secretion in diabetic individuals[54, 258]. It is well established that cAMP potentiates insulin secretion[51] and within the scope of this thesis, investigations have been made to elucidate the regulation of PDE3B, an important regulator of distinct cAMP pools[68]. Evidently, PDE3B is located close to the exocytotic machinery and is involved in insulin secretion[95, 100].

The finding that the activity of PDE3B is modulated by stimuli of relevance for insulin secretion is applicable for finding downstream targets of PDE3B. A previous study related to PDE3B has described its location to insulin granules[100], a cellular compartment comprised of several different proteins participating in insulin exocytosis[47]. However, a possible interaction between PDE3B and particular exocytotic proteins has not been investigated previously and would therefore be an interesting path to follow for future investigations. To search for proteins in the genetically modified mouse model with β-cell specific increase of PDE3 activity[89], is an attempt for identifying proteins that assumably are affected by intracellular changes in specific cAMP pools relevant for insulin exocytosis.

With the investigation of islets from the RIP-PDE3B mouse models (increased activity of β-cell PDE3) we might have identified gene targets that influence insulin secretion. For example, we show that the sulfonylurea receptor 1 (SUR1) subunit of the K_{ATP}-sensitive channel is reduced at the mRNA level in the RIP-PDE3B/7 mice. In the RIP-PDE3B mouse model, the first phase of insulin secretion is specifically affected[89], but whether this impairment is due to fewer or less functional K_{ATP}-sensitive channels in the plasma membrane has not been investigated. Besides for SUR1, there were also other genes associated with diabetes that can be conferred to future investigations. Another finding was the markedly up-regulated expression of OPN in islets of the RIP-PDE3B mouse models. OPN is ascribed a protective role and improves glucose-stimulated insulin secretion by reducing nitric oxide production in diabetic rat islets[143]. With several

different strategies, we described a possible connection between PDE3B, OPN and protein kinase CK2, proteins with important functions in pancreatic β-cells. Further research is needed to elucidate if there is a direct association between these proteins.

We confirm and present new data regarding the presence of selected PDEs (PDE1A, 3, 4C, 5A, 7A, 7B, 8A, 10A and 11A) in human pancreatic islets. This finding confirms consistency in expression pattern between man and rodents. However, the expression of PDE5A, PDE7A, PDE7B and PDE8A observed in human pancreatic islets has to our knowledge not been described in previous research conducted in animal models, which implies species differences.

With the exception for the scarce knowledge regarding PDE7 and PDE5, it is apparent that abolished activity of particular members of the above mentioned PDE families potentiates glucose-stimulated insulin secretion[113, 116, 117]. Further investigations are needed to elucidate the regulation of these PDEs, as they might be targets for the treatment of T2D. In relation to T2D, a divergent role has evolved for OPN, both suggested as a participant in the development of insulin resistance and local inflammation in adipose tissue[126, 134, 140] as well as a protectant in cytokine-mediated β-cell destruction[143]. OPN is thus a suitable candidate for understanding the mechanisms behind the development of T2D.

In the final work of this thesis, the effect of SCFAs on glucose and metabolism in rat adipocytes was investigated. The importance of having healthy adipocytes is seen in obese individuals with disturbed metabolic parameters, as for example indicated by an elevation of circulating free fatty acids in the plasma[7]. SCFAs exerted an anti-lipolytic effect independently of the cAMP-degrading enzyme PDE3B. This finding is highly relevant as it is known from previous studies that PDE3 activity is reduced in adipocytes from obese and T2D animal models[102, 104, 105] as well as obese individuals[106]. The suggested overall anti-lipolytic effect that SCFAs possess might prevent elevated plasma levels of free fatty acids, associated with lipotoxicity and lipid accumulation in non-adipocytes as seen in obese and T2D individuals. SCFAs diminish *de novo* lipogenesis, the synthesis of triacylglycerols from other intermediates than dietary lipids[5]. We assumed that this resulted in a lower production of malonyl CoA, consequently leading to reduced inhibition of β-oxidation. This needs to be confirmed experimentally, but indicates an increased oxidation of energy intermediates that would be beneficial in the context of obesity and insulin resistance. We also observed that SCFAs potentiate insulin-stimulated glucose uptake. The finding that these SCFAs augment insulin-stimulated glucose uptake is promising for T2D individuals with a disturbed ability to maintain whole body glucose homeostasis. However, the precise mechanisms behind the effects exerted by SCFAs on glucose and lipid metabolism have not been elucidated and are goals for future studies.

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