Pathophysiological regulation of white adipocyte exocytosis of different adiponectin molecular forms

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Cover illustration by Marina Kalds Said: Depiction of the different adiponectin molecular forms in the circulation.

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To my brother and parents

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ABSTRACT

In this thesis we have identified mechanisms involved in the exocytosis of different adiponectin molecular forms in health and in metabolic disease. We have also studied similarities and differences in depot-specific adipocyte adiponectin release. In **paper I** we show that the physiological regulation of subcutaneous white adipocyte adiponectin exocytosis involves β3 adrenergic receptors (β3ARs) and Exchange Protein directly Activated by cAMP, isoform 1, (Epac1) signalling. Furthermore, we show that adiponectin secretion is disturbed in obesity/type 2 diabetes induced catecholamine resistance due to reduced abundance of the key proteins β3ARs and Epac1. This condition of *catecholamine resistance* is further associated with a ~50% reduction of circulating high-molecular weight (HMW) adiponectin. In **paper II** we show that β3AR-activation rapidly triggers the release of HMW adiponectin-containing vesicle whereas insulin induces release of smaller molecular forms, with delayed time-kinetics. We moreover demonstrate that both catecholamine-triggered exocytosis of HMW adiponectin and the insulin-induced secretion of smaller adiponectin forms is entirely diminished in adipocytes from obese/type 2 diabetic mice. The equivalent regulation of secretion of different adiponectin molecular forms by catecholamines and insulin was confirmed in human adipocytes, thus defining a novel role of β3ARs in human adipocyte function. In **paper III** we propose that adiponectin exocytosis is regulated by sympathetic nerve endings, co-releasing noradrenaline and ATP within the adipose tissue. Secretion measurements confirmed that noradrenaline (elevates cAMP), like adrenaline in paper I, triggers adiponectin exocytosis. Extracellular ATP was shown to augment the exocytotic process, largely due to its elevation of intracellular Ca^{2+}. We also show that defect purinergic signalling together with reduced white adipose tissue noradrenaline content likely aggravates the catecholamine resistance observed in paper I and II. Finally we describe regulation of mouse visceral adipocyte adiponectin secretion in **paper IV**. As demonstrated in subcutaneous adipocytes (paper I-III), visceral adipocyte adiponectin secretion is also stimulated by activation of β3AR and Epac1. In obese/diabetic conditions, visceral adipocytes are likewise unresponsive to stimulation with catecholamines, but the underlying molecular defect does not involve reduced levels of neither β3AR nor Epac1, thus differing from observations in subcutaneous adipocytes. In conclusion, our results suggest that secretory defects in obesity/type 2 diabetes, attributed to catecholamine resistance, underlie the reduced levels of HMW adiponectin in metabolic disease.

Keywords: White adipocytes, adiponectin exocytosis/secretion, health and metabolic disease

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SAMMANFATTNING PÅ SVENSKA

Övervikt och fetma är ett alltmera alarmerande hälsoproblem, som i förlängningen kan leda till utveckling av typ 2-diabetes och hjärt-kärlsjukdomar. I dagsläget dör fler av överviktsrelaterade sjukdomar än av undervikt. Under metabolt hälsosamma omständigheter lagras fett i kroppens stora energiförråd – den vita fettväven. Den vita fettväven delas ytterligare in i två grupper baserat på anatomisk placering; subkutant fett (under huden) och visceralt fett (i bukhålan). På senare år har forskningen visat att vit fettväv även kan frisätta biologiskt aktiva molekyler direkt till blodet, som möjliggör kommunikation med kroppens många andra organ.

Adiponektin som frisätts från vita fettceller (adipocyter) är ett så kallat anti-diabetisk hormon, som minskar risken för att utveckla typ 2-diabetes genom att bland annat öka glukosupptaget från blodet och därmed förbättra insulinkänsligheten. Vid fetma eller typ 2-diabetes sjunker nivåerna av adiponektin i blodet. Framställning av exogent adiponektin har visat sig komplicerat då hormonet kan frisättas till blodet i olika komplexa molekylära former. Senare tids forskning indikerar att dessa olika molekylära former kan ha varierande fysiologiska effekter. Den mest komplexa strukturen, hög-molekylärt adiponektin har visats ha mest fördelaktiga egenskaper gällande den metabola hälsan. Trots flera studier om effekter av adiponektin är kunskapen om vad som reglerar frisättningen av hormonet relativt okänd. Vår grupp har tidigare visat att signalmolekylen cAMP, via aktivering av proteinet Epac, triggar snabb frisättning av adiponektin från subkutana vita adipocyter.

I denna avhandling visar vi för första gången att frisättning av hög-molekylärt adiponektin från subkutana adipocyter, stimuleras av adrenerg β3-signalering och Epac1. Vidare, visar vi även hur denna stimulering sannolikt sker till följd av påverkan av närliggande sympatiska nerver som frisätter noradrenalin (aktiverar β3-receptorn) och ATP, som vi spekulerar potentierar adiponektin-frisättningen. Tidigare utförda studier pekar på att bukspottskörtelhormonet insulin även kan öka mängderna frisatt adiponektin. I motsats till insulin, som cirkulerar högt i blodet efter målintag, så är blodnivåer av noradrenalin istället ökade vid fasta.

Ur ett fysiologiskt perspektiv är sannolikheten liten att såväl både cirkulerande insulin och noradrenalin skulle påverka samma hormon. Våra resultat demonstrerar att insulin endast ökade frisättningen av de mindre molekylära formerna utan någon påverkan på frisatt högmolekylärt adiponektin.

Obesa/diabetiska möss hade oförändrade cirkulerande nivåer av adiponektin med avseende på alla molekylära former men kraftigt sänkta nivåer av hög-molekylärt adiponektin. Subkutana fettceller, isolerade från obesa/diabetiska möss, kunde inte svara på adrenerg-stimulering till följd av en nedreglering av β3-receptorn och Epac1 men även minskat innehåll av noradrenalin i fettväven. Studier på viscerala fettceller visar att frisättningen av adiponektin, likt fynd från subkutana fettceller, också kan stimuleras av adrenerg stimulering. Dessutom såg vi att viscerala fettceller från obesa/diabetiska möss frisatte mindre adiponektin till följd av adrenerg stimulering jämfört med metabolt friska möss. Den defekta frisättningen orsakades dock inte av minskat genuttryck av varken β3-receptorn eller Epac1.

Sammantaget, indikerar våra resultat att adiponektinfrisättningen regleras via liknande mekanismer i subkutana och viscerala fettceller. Den uppvisade defekta frisättningen av adiponektin vid fetma/diabetes tros dock ha skilda bakomliggande molekylära orsaker. Våra fynd bidrar till en ökad kunskap om de cellulära mekanismerna som reglerar frisättningen av olika former av adiponektin vid hälsa och metabol sjukdom. Vi föreslår att defekt adrenerg stimulerad frisättning av högmolekylärt adiponektin ligger till grund för de uppvisade sänka nivåerna vid metabol sjukdom så som fetma eller typ 2-diabetes.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Komai AM*, Musovic S*, Peris EF, Alrifaiy A, El Hachmane MF, Johansson M, Asterholm IW, Olofsson CS. White adipocyte adiponectin exocytosis is stimulated via β3-Adrenergic signaling and activation of Epac1: catecholamine resistance in obesity and type 2 diabetes.
Diabetes. 2016; 65(11):3301-3313

* The authors contributed equally and their names appear in alphabetical order.

II. Musovic S, Komai AM, Banke EN, Noor UA, Asterholm IW, Olofsson CS. Epinephrine and insulin stimulates white adipocyte secretion of diverse adiponectin forms: evidence for blunted exocytosis of high-molecular weight adiponectin in diabesity-induced catecholamine resistance. *Manuscript*

III. Musovic S, Komai AM, Micallef P, Wu Y, Asterholm IW, Olofsson CS. Sympathetic innervation and purinergic signaling in regulation of white adipocyte adiponectin secretion. *Manuscript*

IV. Musovic S, Olofsson CS. Adrenergic stimulation of adiponectin secretion in visceral mouse adipocytes: blunted release in high-fat diet induced obesity. *Submitted*

Publications not included in this thesis

- Komai AM, Brannmark C, Musovic S, Olofsson CS. PKA-independent cAMP stimulation of white adipocyte exocytosis and adipokine secretion: Modulations by Ca^{2+} and ATP. *J Physiol. 2014; 592(23):5169-5186*

- Brannmark C, Lovfors W, Komai AM, Axelsson T, El Hachmane MF, Musovic S, Paul A, Nyman E, Olofsson CS. Mathematical modeling of white adipocyte exocytosis predicts adiponectin secretion and quantifies the rates of vesicle exo- and endocytosis. *J Biol Chem. 2017;292(49):20032-20043*

TABLE OF CONTENT

ABBREVATIONS

$[Ca^{2+}]_i$	Intracellular levels of Ca^{2+}
AC	Adenylyl cyclase
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADR	Adrenaline
AR	Adrenergic receptor
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
Chow adipocytes	Primary adipocytes isolated from chow-fed mice
CL	CL316,243
CSF	Cerebral spinal fluid
DEXA	Dexamethasone
Epac	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FFA	Free fatty acids
FSK	Forskolin
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLUT4	Glucose transporter 4
GTP	Guanosine triphosphate
GWAT	Gonadal white adipose tissue
HFD	High-fat diet
HFD adipocytes	Primary adipocytes isolated from HFD-fed mice

HMW	High-molecular weight
IBMX	3-isobutyl-1-methylxanthine
IWAT	Inguinal white adipose tissue
LMW	Low-molecular weight
MMW	Middle-molecular weight
NA	Noradrenaline
NBCS	Newborn calf serum
PDE3B	Phosphodiesterase 3B
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PPAR	Peroxisome proliferator activated receptor
SAT	Subcutaneous adipose tissue
siRNA	Small interfering RNA
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNS	Sympathetic nervous system
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TH	Tyrosine hydroxylase
TZD	Thiazolidinedione
VAT	Visceral adipose tissue
VAMP	Vesicle-associated membrane protein
WAT	White adipose tissue
WHO	World Health Organisation

INTRODUCTION

World Health Organisation (WHO) reported in 2016 that over 1.9 billion adults were overweight whereof 650 million were classified as obese. Global prevalence of overweight and obesity has nearly tripled since the seventies. Formerly considered a western world health issue the occurrence of overweight and obesity has reached epidemic proportions with increased numbers now in low- and middle income countries. Overweight and obesity is characterised by an excessive expansion of the white adipose tissue (WAT), an organ traditionally considered a passive energy reservoir. However, discoveries from the last two decades have established WAT as an important metabolic organ with essential functions in whole body physiology, conveyed by the secretion of bioactive molecules termed *adipokines* (Kershaw & Flier, 2004). The growing epidemic caused by obesity and obesity-associated comorbidities such as type 2 diabetes (T2D) calls for an increased understanding of the role of adipose tissue in regard to metabolic health.

White adipose tissue

Adipose tissue is divided into white and brown adipose tissue that differ both in terms of morphology and functionality. In humans, WAT is further divided into regional depots; subcutaneous adipose tissue (SAT) is situated underneath the skin and visceral adipose tissue (VAT) is found surrounding the internal organs in the abdominal cavity (Trujillo & Scherer, 2006). The distinction between subcutaneous and visceral depots is attributed to inherent differences in cellular composition, metabolic properties and microenvironment. Distribution of WAT is highly individual and depends on factors such as age, nutrition, gender and genetics (Wajchenberg *et al.*, 2002). For example, men are more inclined to store fat in visceral compartments while premenopausal women have a greater tendency to accumulate fat in the SAT depots (Karastergiou *et al.*, 2012). In rodents, WAT is also a multi-depot organ as in humans, nonetheless there are some differences. For instance, rodents have a visceral fat pad located in the perigonadal region called gonadal white adipose tissue (GWAT), which is absent in humans (Chusyd *et al.*, 2016).

Cellular composition of WAT

Primary white adipocytes contain one large lipid droplet, accounting for ~95% of total cell volume. Due to their high lipid-storage capacity, white adipocytes are heterogeneous in size and can vary from 25-200 μM (Meyer *et al.*, 2013). Adipose tissue can enlarge either as a results of bigger adipocyte size due to increased lipid storage (hypertrophy) or higher differentiation rate of preadipocytes to mature adipocytes (hyperplasia; Rutkowski *et al.*, 2015). WAT consists of not only adipocytes but also of other cell types which account for a significant proportion of total cell number (Fig. 1). Collectively, these nonadipocytes are called the stromal vascular fraction (SVF) and include preadipocytes, endothelial cells, pericytes, monocytes, macrophages and other cell types; all important for the maintenance of adipose tissue homeostasis. Pericytes and endothelial cells provide the surrounding cells and vasculature important growth and developmental factors while immune cells are important for clearance of apoptotic/necrotic cells (Trujillo & Scherer, 2006). Pathological conditions can have a strong impact on the cellular composition of the SVF. For example, obesity is associated with local inflammation due to increased number of immune cells (Kanneganti & Dixit, 2012).

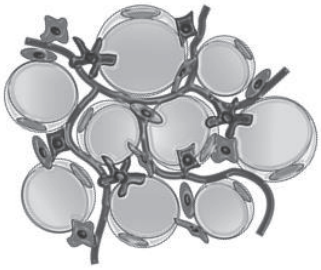

Fig. 1: Illustration of the heterogeneous cellular composition of WAT surrounded by vasculature

Lipid metabolism

Lipid mobilisation and utilisation are two fundamental processes in the white adipocyte. During times of energy deficiency (fasted state), lipids stored as triglycerides are degraded and released as free fatty acids (FFAs) in the circulation to be taken up and utilised as energy by non-adipose organs. This breakdown of lipids is termed lipolysis and several studies highlight WAT adrenergic receptors (ARs; Lafontan & Berlan, 1993) as the primary physiological regulators of stimulated lipolysis (Duncan *et al.*, 2007; Hellstrom *et al.*, 1997).

Activation of ARs results in various intracellular responses depending on receptor subtype. βAR activation elevates cytosolic cyclic adenosine monophosphate (cAMP) levels through activation of adenylyl cyclases (ACs), which catalyse the synthesis of cAMP from adenosine triphosphate (ATP). In contrast, activation of α2AR decreases cAMP through inhibition of ACs while α1AR elevate cytoplasmic calcium levels through activation of phospholipase C (Lafontan & Berlan, 1993). Lipolysis is stimulated through elevations of cAMP and further downstream activation of protein kinase A (PKA; fig 2: *left*) which phosphorylates hormone-sensitive lipase, the main enzyme that hydrolyses triglycerides to FFAs (Anthonsen *et al.*, 1998). Furthermore, decreased βAR-stimulated lipolysis has also been linked to obesity (Lonnqvist *et al.*, 1992).

Insulin is secreted by pancreatic β-cells upon elevation of blood glucose (fed state). Actions of white adipocyte insulin-signalling decreases circulating FFAs, both via stimulation of FFAs uptake and inhibition of lipolysis (Stahl *et al.*, 2002). In summary, activation of membrane bound insulin receptors induces an intracellular signalling cascade (Fig. 2: *right*) involving phosphorylation of both phosphoinositide 3-kinases (PI3Ks) and Akt/Protein kinase B (PKB). Additionally Akt/PKB activates phosphodiesterase 3B (PDE3B) which breaks down cAMP (Degerman *et al.*, 2011). PKB-signalling is also involved in the insulin-stimulated glucose uptake, increasing the presence of glucose transporter 4 (GLUT4) in the adipocyte membrane (Furtado *et al.*, 2002). Obesity is linked with an elevated basal (unstimulated) lipolysis caused by impaired insulin sensitivity.

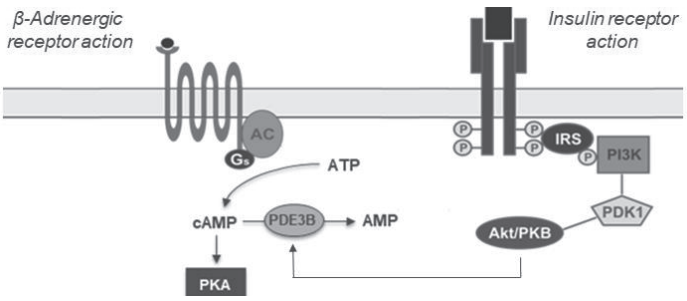

Fig. 2: Receptor-mediated signalling pathways involved in white adipocyte lipid metabolism. To the *left*, PKA-dependent βAR signalling pathway. To the *right* PKB-dependent insulin receptor action.

Sympathetic innervation of WAT

The sympathetic nervous system (SNS) impact adipose tissue physiology through actions on for example lipolysis. Evidence propose that the primary involvement of SNS in lipid metabolism is mediated via direct innervations of sympathetic postganglionic nerves (Bartness *et al.*, 2010; Youngstrom & Bartness, 1995), co-releasing noradrenaline (NA) together with ATP (Burnstock, 2007). NA has higher affinity for the β3AR than adrenaline (ADR; Tate *et al.*, 1991), which is the predominant receptor subtype in rodents. The abundance of β3AR in human WAT is lower (Berkowitz *et al.*, 1995) and the role of this receptor subtype in human adipocyte physiology is not yet completely understood, although an involvement in lipolysis has been reported (Baskin *et al.*, 2018). Effect of extracellular ATP are mediated via purinergic signalling. Numerous purinergic receptors are expressed in WAT and their roles in adipogenesis and lipid metabolism have been described (Tozzi & Novak, 2017). The possible role of parasympathetic nervous innervation has been studied less. However the anatomical closeness of the vagus nerve to the visceral fat depot indicates a possible link. Also, the autonomic nervous system is known to have impact on peripheral tissues via both branches of innervation (Bartness *et al.*, 2014).

Endocrine functions of WAT

In the late 80s, WAT started getting recognised as an endocrine organ releasing several adipokines, and it was reported that the release of some adipokines were disturbed in metabolic disease. Since then, many additional adipokines have been identified. Adipsin (complement factor D) was the first adipokine described (Cook *et al.*, 1987). It plays an important role in the immune system (Xu *et al.*, 2001) and also has several positive metabolic effects such as improvement of pancreatic β-cell function (Lo *et al.*, 2014). The discovery of the white adipocyte hormones leptin (Zhang *et al.*, 1994) and adiponectin (Scherer *et al.*, 1995) further established the importance of adipose tissue endocrine function. Leptin receptor action regulates appetite in the brain by signalling for satiety and increasing energy expenditure (Kelesidis *et al.*, 2010). Circulating levels of leptin correlates positively to body weight and adipocyte size (Lonnqvist *et al.*, 1997). Furthermore, serum levels of leptin are elevated in obese individuals.

Adiponectin

Adiponectin is a 30 kDa, 244-247 amino acid long polypeptide. Full-length (monomeric) adiponectin consists of four distinct regions; an N-terminal signalling sequence, a non-conserved variable region, a collagenous domain and a C-terminal globular domain (Fig. 3; Scherer *et al.*, 1995). Human adiponectin is encoded by the ADIPOQ gene and expression is amplified during white adipocyte differentiation. Moreover, adiponectin gene levels is regulated by several cellular transcriptional factors such as peroxisome proliferator activated receptor γ (PPARγ; Maeda *et al.*, 2001). Even though adiponectin gene expression has been detected in other cell types (Krause *et al.*, 2008; Uribe *et al.*, 2008), the possible contribution of non-adipocytes to circulating adiponectin levels seem unlikely (Wang *et al.*, 2013).

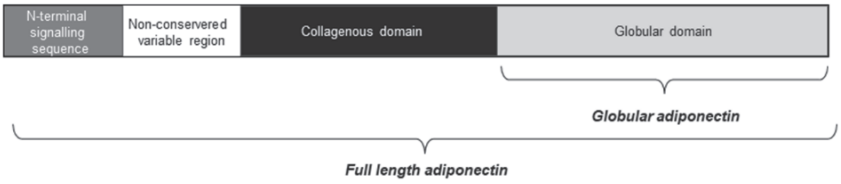

Fig. 3: The four amino acid sequence regions that full-length adiponectin (monomeric) polypeptide consists of.

Circulating levels of adiponectin are exceedingly high (~0.01% of total plasma proteins) compared to other traditional endocrine hormones such as insulin or leptin (Arita *et al.*, 1999). Serum adiponectin levels display a strong sexual dimorphism and women have higher levels than men (Combs *et al.*, 2003). As mentioned earlier, pre-menopausal females accumulate more adipose tissue in the subcutaneous regions. Some studies show that subcutaneous adipocytes secrete more adiponectin compared to visceral, thus contributing more to circulating serum levels of the adipokine (Fisher *et al.*, 2002; Meyer *et al.*, 2013). The male sex hormone testosterone has been reported to reduced adiponectin synthesis as well as interfere with cellular post-translational modifications (Xu *et al.*, 2005) and might contribute to the observed gender difference in regards to circulating adiponectin levels.

Circulating molecular forms of adiponectin

Adiponectin undergoes several post-translational modifications in the endoplasmic reticulum (ER) prior to release from the white adipocyte (Wang *et al.*, 2007). Full-length adiponectin is present in the circulation as different molecular structures, linked together with covalent disulphide bonds (Fig. 4). The different forms are in literature classified as following; trimeric low-molecular weight (LMW), hexameric middle-molecular weight (MMW) and the most complex structure which is oligomeric high-molecular weight (HMW) adiponectin (Pajvani *et al.*, 2003; Scherer *et al.*, 1995). Moreover, different physiological clearance of the diverse forms has been reported (Halberg *et al.*, 2009).

Interconversion does not occur after release into the blood stream and is thus only regulated on cell level. Disulphide bond formation occurs in the ER though oxidation. Earlier studies have shown the involvement of ER-chaperones ERp44 and Ero1-α in the regulation of higher adiponectin complex formation (Qiang *et al.*, 2007; Wang *et al.*, 2007). In addition, a third ER-resident protein, DsbA-L, has been identified to have a key role in the regulation of adiponectin multimerisation, due to its high intrinsic characteristics to form disulphide bonds. This was confirmed in studies on transgenic mice, overexpressing DsbA-L in the adipose tissue. These animals had enhanced adiponectin multimerisation, compared to wild-type littermates, as revealed by increased circulating levels and adipocyte content of HMW adiponectin (Liu *et al.*, 2012).

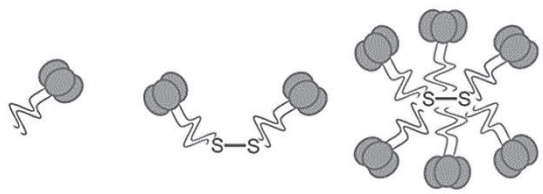

Fig. 4: Different circulating adiponectin molecular forms.

Receptor mediated physiological outcomes

Adiponectin mediates its endocrine effects through two identified receptors; Adiponectin Receptor 1 (AdipoR1) and Adiponectin Receptor 2 (AdipoR2). The two receptors display high amino acid sequence homology and are seven-transmembrane receptors, similar to traditional G-coupled receptors, however with a cytoplasmic N-terminal and an extracellular C-terminal. AdipoR1 is ubiquitously expressed and speculated to act via AMPK signalling while AdipoR2 is mainly present in the liver and proposed to regulate expression of PPARα. Reports suggest that AdipoR1 displays high affinity to the globular region of adiponectin while full-length adiponectin is required to activate AdipoR2 (Yamauchi *et al.*, 2003). Earlier studies in skeletal muscle, reveal that adiponectin increases glucose uptake and β-oxidation, thus improving muscle insulin sensitivity (Kuoppamaa *et al.*, 2008; Yamauchi *et al.*, 2002). In liver, adiponectin suppresses glucose production by downregulating rate-limiting enzymes involved in gluconeogenesis. Further actions of adiponectin in liver include stimulation of glycolysis, β-oxidation and suppression of lipid accumulation. (Combs *et al.*, 2001; Liu *et al.*, 2012). Via these overall favourable outcomes on glucose and lipid metabolism, adiponectin improves systemic insulin sensitivity.

The wide distribution of adiponectin receptors in several other tissues contributes to the extensive physiological effects of adiponectin. Basic science studies have shown adiponectin to have cardioprotective properties, by increasing angiogenesis, providing protection from foam cell formation and development of atherosclerosis (Shimada *et al.*, 2004; Wang & Scherer, 2008). Quite contradictory, recent studies suggest adiponectin as a risk factor for cardiovascular health issues (Dadson *et al.,* 2015, Holland *et al.,* 2011). In addition to the peripheral effects on insulin sensitivity, adiponectin also display central effects. In the brain, adiponectin has been reported to increase energy output with no effect on appetite. Initially, it was speculated that adiponectin does not cross the blood-brain barrier (Spranger *et al.*, 2006), however the trimeric LMW adiponectin form has been detected in human cerebrospinal fluid (CSF). Peripheral administration of adiponectin in serum results in a parallel increase in CSF, but at 1000-fold lower concentration (Kusminski *et al.*, 2007).

The T-cadherin receptor has been proposed as a third adiponectin-binding receptor. This receptor type is highly expressed in endothelial and smooth muscle cells with significant roles in cell growth and vasculature development. Yet the T-cadherin receptor shows no interactions to neither globular nor trimeric adiponectin (LMW) but has instead been proposed to specifically bind to the higher complex structures (Hug *et al.*, 2004), signifying the importance of higher order structures for the full biological function of adiponectin.

HMW adiponectin as predictor of metabolic disease

Obesity can result in disturbed WAT functions resulting in dysfunctional plasma levels of several known adipokines. Other significant characteristics of obese WAT are higher degree of inflammation and local deprivations of oxygen supply. Obesity-caused WAT expansion is often linked to insulin resistance. The relationship between adiponectin and metabolic disease has been studied in several studies. Adiponectin depleted mice have decreased insulin sensitivity (Nawrocki *et al.*, 2006), whereas transgenic mice, overexpressing full-length adiponectin are more metabolically healthy and less inclined to be affected by high-fat diet (HFD) feeding (Otabe *et al.*, 2007). Moreover, the cross-breeding of mice displaying the obese genetic phenotype ob/ob with transgenic adiponectin mice improved circulating glucose and triglyceride levels, thus improving overall metabolic health (Kim *et al.*, 2007). Nevertheless, other studies suggest that the insulin-sensitising properties of adiponectin can be attributed to the explicit effects of HMW adiponectin. Circulating HMW adiponectin levels are decreased in T2D individuals compared to healthy controls (Hara *et al.*, 2006; Tabara *et al.*, 2008; Zhu *et al.*, 2010). Pharmacological treatment with tiazolidinediones (TZDs; PPAR-γ agonists) in T2D patients, increased circulating HMW adiponectin together with improved insulin sensitivity (Pajvani *et al.*, 2004). In regards to adiponectin, observed cellular actions of TZDs on the white adipocyte include increased gene expression, secretion and multimerisation (Iwaki *et al.*, 2003; Phillips *et al.*, 2009; Yu *et al.*, 2002;).

Secretory pathways of hormone secretion

Intercellular communication is essential for mammalian multicellular systems, such as the white adipose tissue. There are two ways in which the cells can interact with other cells, either through direct cell-cell interactions or via secreted products. Due to the large size and charge of proteins they cannot be transported over the lipid bilayer via diffusion mechanisms and therefore need to relay on other transporting systems such as exocytosis. In eukaryotic cells, there are two types of exocytosis; constitutive and regulated. Constitutive exocytosis operates via unstimulated vesicular transport to the plasma membrane whereas regulated exocytosis is reliant on signal transduction pathways induced by extracellular stimulation.

Regulated exocytosis

Polypeptides, synthesised and modified in the ER pass the Golgi complex to the *trans*-Golgi where initial formation of immature vesicles occur. In endocrine cells that release hormones, mature secretory vesicles can be coarsely categorised in functional pools. Vesicles belonging to the readily releasable pool are situated close to the plasma membrane and are rapidly released upon triggering of the right stimulatory signal (Burgoyne & Morgan, 2003). Observations with electron microscopy show that this vesicle pool is rather small and quickly exhausted. Electrophysiological studies and live cell imaging of vesicle exocytosis in hormone releasing cells further reveal that stimulated exocytosis is a rapid cellular event, with fusion of single vesicles occurring within milliseconds (Huang *et al.*, 2007; Kasai *et al.*, 2012).

In most neuronal and endocrine cells, fusion of readily releasable vesicles with the plasma membrane is triggered by rises in intracellular Ca^{2+} ($[Ca^{2+}]_i$) due to influx through membrane-bound calcium channels and/or release from $[Ca^{2+}]_i$ stores (Burgoyne & Morgan, 2003). Whole-cell patch-clamp recordings of exocytotic events carried out in adrenal chromaffin cells verified the presence of a limited readily releasable pool of catecholamine-containing vesicles that upon Ca^{2+} elevations released their cargo to the circulation for systemic effects on target tissues (Heinemann *et al.*, 1994). Another typical example is the pancreatic β-cell, where insulin-containing vesicles are released in a Ca^{2+}-dependent manner when blood glucose levels rises

(Barg *et al.*, 2002; Olofsson *et al.*, 2009). When the readily releasable pool is depleted, immature vesicles from the reserve pool of vesicles need to undergo Ca^{2+}, cAMP and/or ATP dependent modifications and/or physical translocation in order to gain release competence (Burgoyne & Morgan, 2003). The replenishment of the readily releasable vesicle pool is necessary in order to maintain hormone release over longer time periods.

Soluble-N-ethylmaleimede-sensitive factor attachment protein receptor (SNAREs) are a group of membrane-associated proteins that are involved in different stages of the exocytotic process (Kasai *et al.*, 2012). SNAREs are located both on the secretory vesicle itself and on the target cell membrane. Upon signal transduction, these SNARE proteins will together form a tightly bound four helix complex, which is thought to be involved in providing the energy necessary for fusion. The specific cellular events leading up to complete fusion is not fully understood but suggested to involve direct involvement of SNAREs (Han *et al.*, 2017).

Epac-dependent cAMP signalling

The intracellular signalling molecule cAMP, plays important roles in most cell types affecting differentiation, cell growth, apoptosis, secretion and many other cellular processes. Elevations of intracellular cAMP can trigger two identified signalling pathways involving actions on either PKA or Exchange protein directly activated by cAMP (Epac; Holz *et al.*, 2006). While the role of PKA-signalling has been firmly established in the regulation of stimulated lipolysis, the significance of Epac-signalling in white adipocyte physiology is less investigated. Epacs are a novel group of cAMP-binding proteins that exists in two isoforms; Epac1 and Epac2. The two isoforms share extensive sequence homology but differ in tissue distribution. Epac1 is expressed in most cell types whereas Epac2 expression is restricted to neuroendocrine cell types (Schmidt *et al.*, 2013). cAMP plays a role in modulating the exocytotic pathway in several endocrine cell types. For instance, in pancreatic β-cells, PKA and Epac2 cAMP-signalling is essential for the regulation of insulin granule dynamics (Shibasaki *et al.*, 2007). A similar augmenting effect of cAMP on exocytosis has been defined in adrenal chromaffin cells (Novara *et al.*, 2004).

Further downstream, Epac can activate several small GTPases belonging to the Ras superfamily, recognised to be involved in vesicle trafficking and exocytosis. Epac is a guanine nucleotide exchange factor (GEF) which upon stimulation by an upstream signal catalyses the exchange of a guanosine diphosphate (GDP) to guanosine triphosphate (GTP) thus activating the small GTPases (Simanshu *et al.*, 2017).

Regulation of adiponectin secretion

The possible role of regulated adiponectin secretion was first proposed by Scherer and colleagues in 1995. In their first article about adiponectin they suggested that insulin stimulates adiponectin release, presenting evidence from data in cultured 3T3-L1 adipocytes (Scherer *et al.*, 1995). Since, several studies have shown various outcomes of insulin-treatment on adiponectin in regards to gene expression, synthesis and release. Long-term exposure (>4 h) to insulin in both cultured and isolated rodent adipocytes induced adiponectin release (Blumer *et al.*, 2008; Cong *et al.*, 2007; Lim *et al.*, 2015; Pereira & Draznin, 2005) and the effect was shown to act via PI3K- and PDE3B-dependent pathways (Cong *et al.*, 2007). Interestingly, shorter time exposure to insulin (30-120 min) has also been shown to induce adiponectin release in a PI3K-dependent manner, without contribution of increased gene expression or protein synthesis. Subcellular fractionation post insulin-exposure revealed that the highest content of adiponectin was localised close to the plasma membrane fraction (Bogan & Lodish, 1999; Lim *et al.*, 2015), suggesting that insulin affects the release-process of already synthesised and modified adiponectin.

The effect of catecholamines on adiponectin release has been investigated in a few studies. In human VAT explants, an inhibitory outcome was observed on adiponectin gene expression as a result of long-term (>8 h) exposure with βAR- or cAMP-agonists (Delporte *et al.*, 2002) and similar results have been observed in mouse adipose tissue (Delporte *et al.*, 2002; Fasshauer *et al.*, 2001). In isolated GWAT rat adipocytes, treatment with the βAR agonist isoprenaline for 4-24 h inhibited adiponectin secretion while stimulating lipolysis (Cong *et al.*, 2007). The studies described above have thus investigated long-term/chronic effects of catecholamines on adiponectin release.

In 2014, our group was first to define that white adipocyte exocytosis (measured as increase in membrane capacitance) and adiponectin secretion in both cultured and human primary adipocytes is triggered by elevations of cAMP via activation of Epac, in a PKA-independent manner (Fig. 5). The recruitment of new releasable vesicles from the reserve pool was shown to occur in a Ca^{2+}-dependent manner. Thus, in Ca^{2+}-free conditions, the readily releasable pool will be depleted and exocytosis can not sustain for longer time-periods, even in the presence of cAMP. Furthermore we have shown that a combination of Ca^{2+} and ATP potentiates adiponectin exocytosis via direct effects on the release process itself (Komai *et al.*, 2014). These findings propose an acute stimulatory effect of catecholamines on adiponectin secretion (through the activation of βARs) and suggests adrenergic signalling as a physiological regulator of adiponectin release.

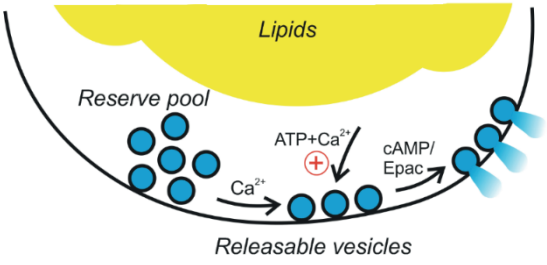

Fig 5: Model of white adipocyte exocytosis. Elevations of cAMP trigger the release of readily releasable vesicles whereas intracellular rises of Ca^{2+} and ATP augment the exocytotic event.

AIMS

The overall aim of this thesis was to define the (patho-) physiological regulation of adiponectin exocytosis/secretion as well as the depot-specific adipocyte adiponectin release.

The specific aims of the four papers included in this thesis were to:

I. Study the effects of catecholamines on subcutaneous white adipocyte adiponectin exocytosis/secretion and how adrenergically stimulated adiponectin release is affected by diet-induced diabetes/obesity.

II. Explore the similarities and differences between adiponectin secretion stimulated by catecholamines and insulin in subcutaneous white adipocytes.

III. Investigate the role of sympathetic innervation and purinergic signalling in the regulation of adiponectin exocytosis/secretion in subcutaneous white adipocytes.

IV. Define the regulation of visceral white adipocyte adiponectin secretion in health and in metabolic disease.

MATERIAL AND METHODS

The reader is referred to paper I-IV for a more detailed description of material and methods used in this thesis.

Cell culture

3T3-L1 adipocytes are a well-known *in vitro* white adipocyte model of murine cell origin and was first isolated and cloned in the seventies (Greenberger & Aaronson, 1974). The cell line is extensively used in studies of white adipocyte development, lipid metabolism and endocrine function (Fasshauer *et al.*, 2001; Scherer *et al.*, 1995). 3T3-L1 preadipocytes have a fibroblast like morphology and can within ten days be differentiated into mature lipid-storing adipocytes (Fig. 6).

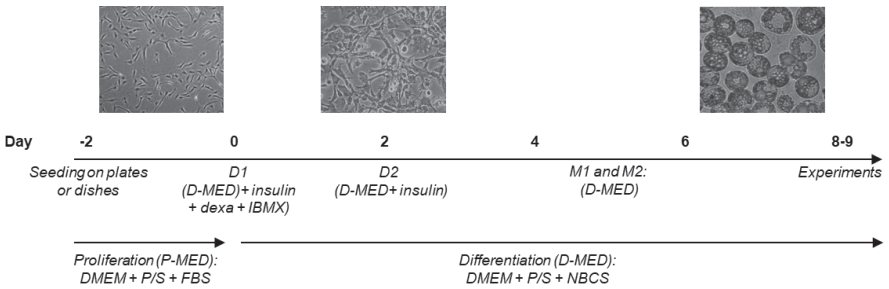

Fig. 6: Differentiation of 3T3-L1 adipocytes from fibroblast-like cells (day 0) to mature lipid-storing adipocytes (day 8-9).

3T3-L1 preadipocytes were cultured in flasks and kept at 37°C and 5% CO_2 together with high-glucose DMEM containing 10% newborn calf serum (NBCS) and 1% penicillin-streptomycin (P/S). When cells reached ~70% confluency, they were trypsinised (3 min, 37°C) and seeded on either plastic- or glass-bottom dishes or 12-well plates. Cells were grown to higher confluency prior to differentiation (~90%). Differentiation (D1) was initiated by addition of insulin (850 nM), dexamethasone (1 µM) and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM) in high glucose DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The exposure to this mixture, at early stages of cell differentiation, upregulates

essential adipocyte genes; as result both glucose uptake and triglyceride synthesis increases. After 48 hours the medium was replaced with fresh medium (DMEM; 10% FBS, 1% P/S) containing only insulin (D2). At this point, the 3T3-L1 adipocytes start to accumulate several lipid droplets. Thereafter, medium was changed every second day up until day 8-9 of differentiation, when experiments were carried out.

Isolation of primary white adipocytes

Human subcutaneous white adipocytes were isolated from adipose tissue biopsies. Mouse inguinal and gonadal white adipocytes were isolated according to established protocol (Ruan *et al.*, 2003; Fig. 7) from 8-16 week old C57BL/6J mice, either fed chow or HFD (60% kcal from fat) for 8 weeks Adipose tissue was collected and minced into small pieces and thereafter digested in collagenase type II (1 mg/mL in KRHG, 3% BSA) for 45-60 min at 37 °C. Following the incubation with collagenase, the adipose tissue suspension was poured through a 100 µM nylon mesh into a tube. Adipocytes floating on top were washed with buffer (KRHG, 1% BSA). Adipocytes were allowed to float to the surface and buffer solution beneath the adipocyte layer, containing SVF cells, was removed with a syringe. Isolated adipocytes were moved to a separate tube and allowed to float in order to facilitate removal of more excessive buffer by using a smaller syringe. Human adipocytes were incubated overnight and secretion measurements proceeded the following day. Mouse adipocytes were directly used for adipokine secretion experiments or frozen at -80°C for further analysis. All animal and human studies were approved by the regional ethical review board.

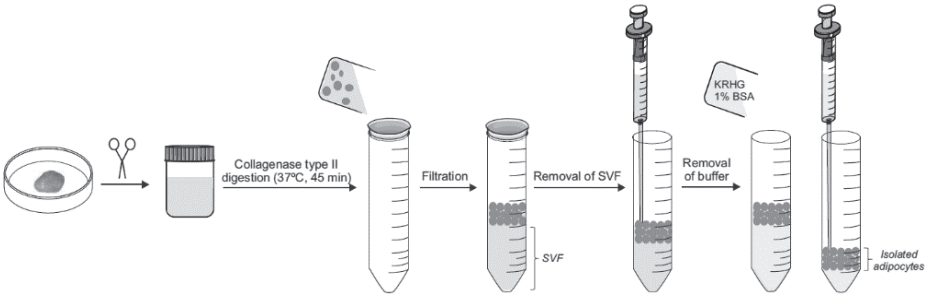

Fig. 7: Illustration depicting the procedure for isolation of primary adipocytes

Measurements of white adipocyte secretion

Isolated adipocytes (10-15% v/v) or 3T3-L1 adipocytes, cultured on 12 well plates, were incubated in 5 mM glucose extracellular solution containing test substances for 30 min at 32˚C under gentle shaking conditions. Primary cell incubation was terminated by centrifugation of cell suspension in diisononyl phthalate oil followed by snap freezing in dry ice. This allows for separation of adipocytes from media, containing the secreted product. Tubes were cut through at two levels, separating cells from media and removing the oil layer in the middle. Following incubations with 3T3-L1 adipocytes, the medium was collected and centrifuged in order to remove detached cells. Supernatant was collected following centrifugation and adipocytes were lysed in lysis buffer containing protease inhibitors. All samples were stored in -80 ˚C. Secreted adiponectin was measured with specific ELISA and normalised to total protein content obtained with the Bradford assay.

Capacitance measurements

Exocytosis was measured in 3T3-L1 adipocytes with membrane capacitance measurements using the patch-clamp method (Neher & Marty, 1982). Membrane capacitance can be calculated from equation $C_m = A \times \mathcal{E}/d$ where A is the membrane surface area. The thickness of the plasma membrane (d) and the specific membrane capacitance Ɛ are constant. Exocytosis results in an increase of the membrane area and is thus proportional to the capacitance. Hence, membrane capacitance (C_m) is proportional to the plasma membrane area (A). The rate of vesicle fusion with plasma membrane (exocytosis) was measured as capacitance over time.

Ratiometric calcium imaging

($[Ca^{2+}]_i$) levels were measured with dual-wavelength ratiometric imaging in 3T3-L1 adipocytes. The cells were loaded with the fluorescent Ca^{2+} dye fura-2-AM together with mild detergent Pluronic for 45 minutes prior to measurement. Fura-2-AM is a highly membrane permeable ester that is hydrolysed by cellular esterases once inside the cell. Fura-2 can be excited at two wavelengths depending on whether it is bound to Ca^{2+} or not. The excitation maximum for unbound fura-2 is 380 nm while it is 340 nm for Ca^{2+}-bound fura-2. The emitted light is collected at 510 nm.

The ratio (Fura-2_{bound}/Fura-$2_{unbound}$) allows for calculations of $[Ca^{2+}]i$ concentrations over time. Ratiometric imaging reduces the impact variations in fura-loading as well as declined fluorescence signal due to bleaching between experiments. $[Ca^{2+}]i$ was calculated using the equation 5 of Grynkiewicz et al 1985 and a Kd of 224 nM. (Grynkiewicz *et al.*, 1985).

Gene expression analysis

Quantitative real-time RT-PCR can be used to measure expression of genes of interest. The method requires complementary DNA (cDNA) as a starting material, which can be made from mRNA with the enzyme reverse transcriptase. Assessment of RNA purity, following isolation, is validated through spectrophotometrical absorbance measurements at 260 and 280 nm. Amplifications of cDNA sequences of interest can be detected with fluorescent labelling dyes. In this thesis, the fluorescent dye SYBR green was used which binds to newly synthesised double-stranded DNA (see Fig. 8 for more details). The amount of synthesised cDNA is directly proportional to fluorescence and can be detected over time.

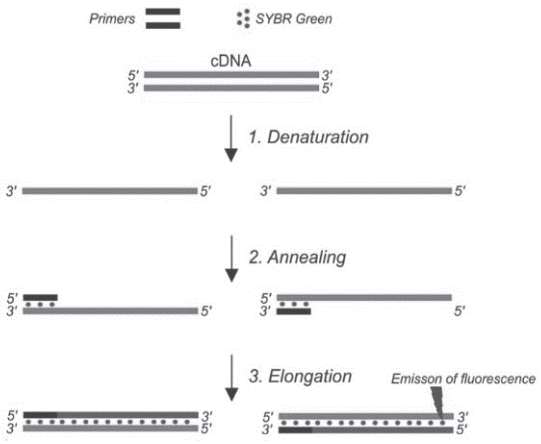

Fig. 8: Each PCR-cycle include three steps that are repeated 40-45 times. The reaction is initialised with an increase in temperature, which results in the separation of double-stranded DNA into two single-stranded DNA (*denaturation*). In the next step, temperature is reduced allowing for primers to bind to denatured target DNA (*annealing*). In the last step, a new complementary DNA-strand is synthesised by DNA polymerase (e*longation*).

siRNA transfection

Intracellular incorporation of small interfering RNA (siRNA) can be used to silence/suppress the expression of a specific gene in order to study its function. In this thesis we used siRNA to silence the expression of β3AR and Epac1 in cultured adipocytes. 3T3-L1 adipocytes were transfected with Silencer Select siRNA (80 nmol/L) at day 6 of differentiation using transfection reagent Lipofectamine 2000. Medium was changed eight hours post transfection. Further studies of gene expression and secretion were carried on 60 hours after transfection with siRNA. Knockdown of protein of interest was confirmed with gene expression analysis. Adiponectin release after 30 minutes at 32°C was measured in order to study the functionality of the knockdown. In these experiments scramble siRNA was used as a negative control.

Data analysis

Results included in this thesis are presented as mean values ± SEM, expressed either in fold change over basal (unstimulated conditions) or in absolute concentrations. Statistical significance was calculated with OriginPro (OriginLab Corporation, USA). Student's *t*-test (unpaired or paired as appropriate) was used to determine significance between two experimental groups. Analysis of variance (ANOVA) was used to determine statistical significance between two or more independent groups and/or conditions.

RESULTS AND DISCUSSION

Paper I

In paper I of this thesis we investigated the effects of ADR and the highly selective β3-agonist CL 316,243 (CL) on white adipocyte adiponectin exocytosis using a combination of electrophysiological and biochemical measurements. Effects on adiponectin release were studied in both cultured 3T3-L1 adipocytes and in primary subcutaneous adipocytes isolated from lean or obese/diabetic mice. The current study builds on our previously published work, showing that cAMP elevations trigger white adipocyte release of adiponectin-containing vesicles via Epac-dependent signalling pathways (Komai *et al.*, 2014).

Gene expression of adrenergic receptor subtypes and Epac isoforms

In order to confirm the presence of ARs in the adipocytes used in this study (Lafontan & Berlan, 1993), we measured gene expression of all five ARs in undifferentiated and differentiated 3T3-L1 cells. In differentiated adipocytes, β3AR expression was highly abundant while significantly lower expression was obtained for the other receptor subtypes. We measured mRNA levels of the cAMP-binding proteins Epac isoforms 1 (*Rapgef3*) and 2 (*Rapgef4*). Epac1 was expressed in differentiated 3T3-L1 adipocytes while Epac2 could not be detected. Interestingly mRNA levels of Epac1 were 60% higher in undifferentiated cells. This is in agreement with studies that show that cAMP-stimulated adipogenesis involves both PKA- and Epac-dependent signalling pathways (Jia *et al.*, 2012; Petersen *et al.*, 2008). Equivalent gene expression ratios of ARs and Epac isoforms were obtained in primary mouse adipocytes isolated from inguinal white adipose tissue (IWAT).

Adrenergic stimulation of adiponectin in white adipocytes occur via activation of β3ARs and Epac1

White adipocyte exocytosis was measured as increase in membrane capacitance with the patch-clamp method. In differentiated 3T3-L1 adipocytes, infused with a non-stimulatory pipette solution (lacking cAMP), extracellularly applied ADR (5 μM) and

β3-agonist CL (1 μM) triggered a similar magnitude of exocytosis. To study the role of Epac in adrenergically stimulated adipocyte exocytosis, adipocytes were pretreated with the membrane-permeable competitive Epac inhibitor ESI-09 (10 μM) for 30 min prior to addition of ADR. Pretreatment with ESI-09 completely abolished the exocytosis triggered by ADR (Fig. 9: *left*). Previously published results from our group demonstrate that the cAMP-produced membrane capacitance increase in 3T3-L1 adipocytes largely represent the release of adiponectin-containing vesicles (Komai *et al.*, 2014; El Hachmane *et al.*, 2015). In agreement with the capacitance data, incubation of 3T3-L1 adipocytes with ADR or CL for 30 min stimulated adiponectin secretion (~1.8-fold with either secretagogue). ADR can bind to several ARs with different affinity (Lafontan & Berlan, 1993). Although the similar effects on adiponectin release by ADR and CL suggest that adrenergically stimulated adiponectin release is chiefly mediated via β3ARs. We further validated the role of Epac1 in isolated IWAT adipocytes. In agreement with capacitance measurements in 3T3-L1 cells, inhibition of Epac in IWAT adipocytes abolished adiponectin secretion stimulated by ADR or CL (Fig. 9: *right*).

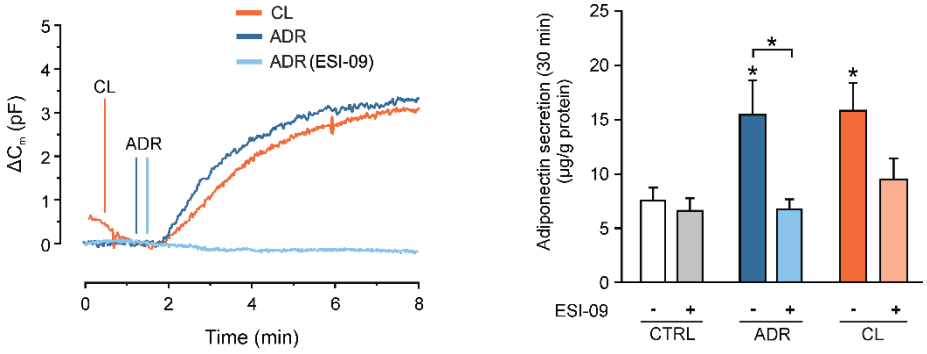

Fig. 9: ADR and β3AR-agonist CL trigger white adipocyte adiponectin exocytosis via Epac-dependent mechanisms. *Left:* Example capacitance traces of 3T3-L1 adipocytes infused with a non-stimulatory pipette solution (lacking cAMP) with extracellular addition of CL or ADR as indicated by the arrows. Light blue trace represents cells pre-treated for 30 min with Epac antagonist ESI-09 prior to addition of extracellular ADR. *Right:* Adrenergic/β3AR-stimulation of adiponectin secretion in IWAT adipocytes during 30 min as well as effects of pretreatment with ESI-09 prior to addition of ADR or CL. Data are mean values ± SEM of 9 experiments. *P<0.05 vs control.

Adiponectin release stimulated by CL remained unchanged in adipocytes pretreated with the protein synthesis inhibitor cycloheximide (10 μM). Also, short-term incubation of cultured adipocytes with CL was without effect on gene expression level of adiponectin. These results support our hypothesis that short-term adrenergic stimulation triggers the release of prestored adiponectin-containing vesicles with little contribution to increased adiponectin synthesis or expression.

Measurements of cAMP content in cells exposed to ADR or CL for 30 min revealed that ADR elevated cAMP ~7.5-fold, while levels in CL-treated cells were only 3-fold increased. This finding is not surprising since ADR activates all cAMP increasing βARs. The observation that the 3-fold elevation of cAMP via β3AR signalling stimulates adiponectin exocytosis/secretion of an equal magnitude as ADR (which increases cAMP much more and via all βARs), further proposes little or no contribution of β1- and β2ARs in adrenergically triggered adiponectin release. Together our findings suggest that activation of β3ARs and Epac1 triggers release of pre-existing adiponectin-containing vesicles with no observed contribution to neither adiponectin gene expression nor protein synthesis.

The role of Ca^{2+} in adrenergically stimulated adiponectin secretion

Next we studied the role of $[Ca^{2+}]_i$ on adrenergically stimulated adiponectin secretion as ADR is known to elevate concentrations of the ion via actions on α1ARs. ADR -stimulated adiponectin release remained intact upon Ca^{2+} chelation with BAPTA (50 μM; 30 min pretreatment), in both cultured and in primary mouse IWAT adipocytes. Ratiometric Ca^{2+} measurements demonstrated a heterogeneous response of ADR on Ca^{2+} elevation. The effects on Ca^{2+} were overall small and thus in agreement with obtained low gene expression of α1AR. Our results are similar to previously reported heterogeneous Ca^{2+} elevations in human adipocytes exposed to NA, an effect reported to depend on an uneven intercellular distribution of AR subtypes (Seydoux *et al.*, 1996). We conclude that the Ca^{2+} elevation resulting from α1AR activation is of little importance for adrenergically stimulated adiponectin release.

Blunted adiponectin secretion in adipocytes isolated from high-fat diet fed mice

To study the association between obesity/T2D and catecholamine-stimulated adiponectin release, IWAT adipocytes were isolated from mice fed chow (chow adipocytes) or HFD (HFD adipocytes) for 8 weeks. HFD-fed mice had elevated serum glucose and insulin levels, representing a diabetic condition. Additionally the HFD-fed mice had increased bodyweight (46.3 ±0.8 g. compared with 31.8 ± 0.6 g, for chow-fed mice). There was no change in circulating adiponectin levels in HFD-fed mice, in contrast to previous reports in obese/diabetic subject (Panidis *et al.*, 2003; Saltevo *et al.*, 2008). However, we observed a significant reduction in levels of circulating HMW adiponectin (~50%), corresponding to what has been reported in obese/type 2 diabetic individuals (Hara *et al.*, 2006; Tabara *et al.*, 2008; Zhu *et al.*, 2010).

A combination of the cAMP-elevating agents forskolin (FSK; 10 µM) and IBMX (200 µM; FSK/IBMX), CL or ADR increased the release of adiponectin ~2-fold in IWAT adipocytes isolated from chow-fed mice. In adipocytes from HFD-fed mice, the stimulatory effect of FSK/IBMX was significantly reduced (~30%) compared to chow adipocytes (Fig. 10: *left*). Strikingly, adiponectin secretion triggered by ADR or CL was essentially abolished in adipocytes from obese/diabetic mice coupled with a 2-fold increase of basal release (unstimulated conditions; Fig. 10: *right*).

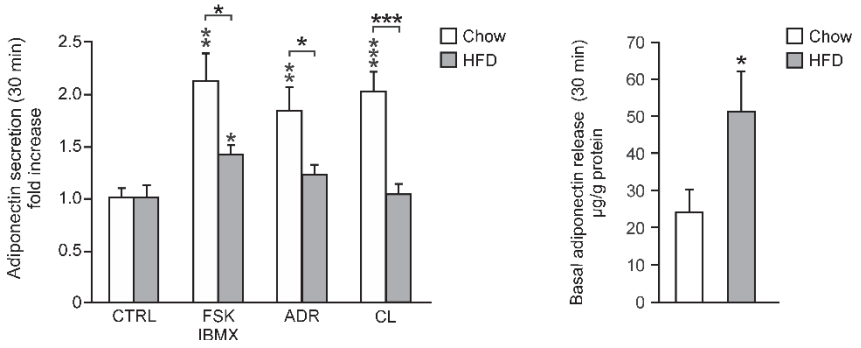

Fig. 10: Adrenergically/cAMP-stimulated adiponectin secretion from lean and obese/diabetic mice. Stimulated adiponectin secretion (l*eft*) and basal release (*right*) in IWAT adipocytes isolated from chow- or HFD-fed mice during 30 min of incubation. Data are from 8 chow-fed and 8 HFD-fed animals. Data are presented as mean values ± SEM. *P<0.05; **P<0.01; ***P<0.001.

We evaluated whether reduced adiponectin content could explain the blunted secretion in HFD adipocytes. Several studies have proposed that the obese/diabetic state is associated with reduced adipocyte adiponectin expression, both at the gene and protein level (Hu *et al.*, 1996). IWAT adipocyte adiponectin content was unaltered in HFD-fed mice. Moreover, the fraction secreted upon extracellular stimulation with ADR or CL was much smaller in HFD adipocytes. All things considered, our data suggests that the adrenergically regulated adiponectin release is impaired in IWAT adipocytes from HFD-fed mice.

Gene expression analysis of key players involved in adrenergically stimulated adiponectin secretion supported the existence of a secretory defect. Gene expression data showed that IWAT adipocytes from HFD-fed mice, displayed reduced mRNA levels of β3AR (~5-fold) and Epac1 (40 %). The reduced abundance of β3AR and Epac1 in adipocytes from HFD-fed mice was also confirmed at the protein level. Measurements showed that Epac1 was decreased by ~30%, similar in magnitude to the reduced effect of FSK/IBMX-stimulated adiponectin secretion observed in HFD adipocytes. The protein level of β3AR was 35% lower in IWAT adipocytes from HFD-fed mice.

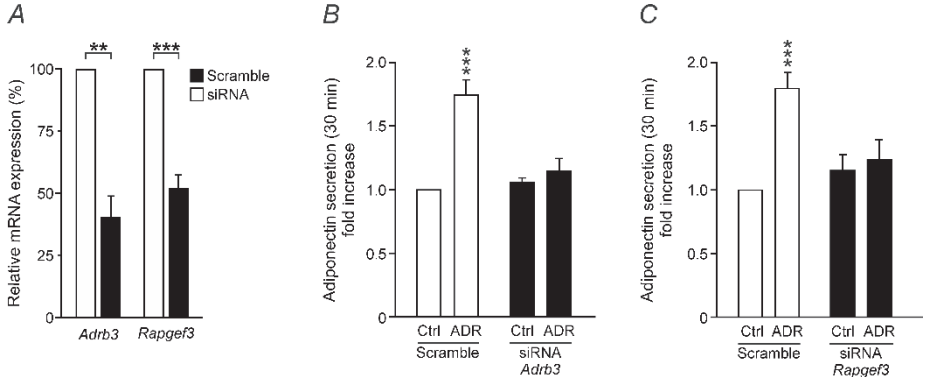

Fig. 11: Blunted adrenaline-stimulated adiponectin secretion upon β3AR and Epac1 siRNA knockdown. *A:* Relative mRNA expression in 3T3-L1 adipocytes transfected with β3AR (*Adrb3*) or Epac1 (*Rapgef3*) siRNA for 60 hours. The siRNA gene expression for each gene of interest is normalised against its expression in concurrently scramble transfected cells (negative control). *B-C*: Adrenaline-stimulated adiponectin secretion (30 min) in β3AR or Epac1 silenced 3T3-adipocytes as well as scramble transfected cells. Data in A-C are from five separate experiments. **P<0.01; ***P<0.001.

To further verify the significance of β3AR and Epac1 in catecholamine-stimulated adiponectin secretion, we carried out siRNA knockdown in 3T3-L1 adipocytes (Fig. 11A). In adipocytes with silenced β3AR expression (~60% knockdown), ADR was not able to induce adiponectin release (Fig. 11B). Likewise, silencing of Epac1 (~50%) resulted in an abrogated ADR effect on adiponectin secretion (Fig. 11C). Adipocytes transfected with scramble siRNA (negative control) remained responsive. We postulate that the reduced levels of β3AR together with lower abundance of Epac1 might provide an explanation for the blunted CL/ADR-stimulated release of adiponectin.

Summary of findings in paper I

We here move our knowledge forward and show that the physiological regulation of adiponectin exocytosis in subcutaneous adipocytes involves catecholaminergic signalling, mainly via β3AR and further downstream activation of Epac1 (Fig. 12: *left*). We also provide evidence for a defect adrenergically stimulated adiponectin release in subcutaneous adipocytes isolated from diet-induced obese/diabetic mice due to reduced abundance of key proteins β3AR and Epac1, in a condition we refer to as "*catecholamine resistance*" (Fig. 12: *right*).

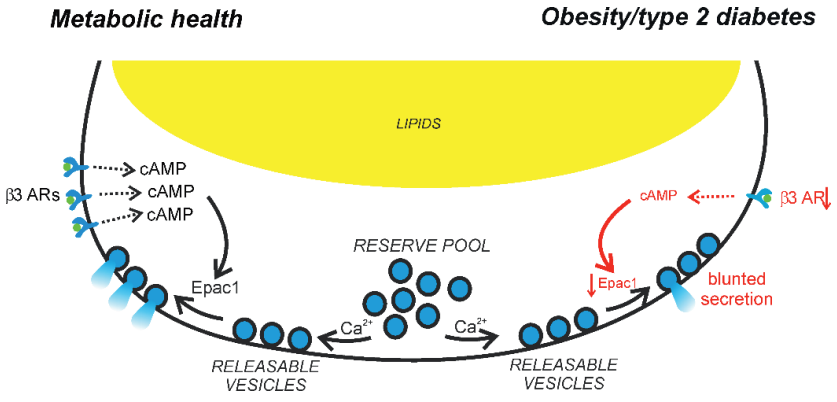

Fig. 12: Graphical conclusion based on our findings in paper I. See text for further details.

Paper II

Several studies report that insulin induces adiponectin release from both cultured and primary mouse adipocytes. However, in paper I, we show an alternative pathway of catecholamine-stimulated adiponectin secretion. The cAMP/catecholamine-triggered adiponectin exocytosis is difficult to reconcile with a stimulatory effect of insulin, which decreases intracellular cAMP levels via activation of PDE3B (Degerman *et al.*, 2011). In paper II, we investigate adiponectin exocytosis/secretion in response to insulin and ADR, using cultured 3T3-L1 adipocytes and primary subcutaneous adipocytes isolated from mice and humans.

Insulin and adrenaline/CL-stimulated adiponectin secretion involves different signalling pathways and show dissimilar time-kinetics

3T3-L1 adipocytes were exposed to the β3AR agonist CL (1 μM) or insulin (200 nM) for 30 min. In agreement with findings in paper I, CL stimulated adiponectin secretion (~1.7-fold). Insulin-stimulated adiponectin release was of similar magnitude. The combination of CL and insulin resulted in released adiponectin in amounts comparable to stimulation with each substance separately. Reports of insulin-stimulated adiponectin release suggest the involvement of PI3K (Blumer *et al.*, 2008; Cong *et al.*, 2007; Lim *et al.*, 2015; Pereira & Draznin, 2005). To investigate this, we measured adiponectin release from 3T3-L1 adipocytes pretreated with PI3K-inhibitor Wortmannin (100 nM) for 30 min prior to incubation with insulin or ADR. Inhibition of PI3K action abolished the insulin-induced adiponectin release whereas ADR-stimulated adiponectin release remained intact. Furthermore, inhibition of Epac had no effect on insulin-stimulated adiponectin release while, in agreement with paper I, ADR-triggered adiponectin secretion was diminished.

To further study the similarities and differences in more detail, we performed time-course experiments of adiponectin release (Fig. 13). CL stimulated adiponectin release at early time points (measurable amounts collected over 15 min in static incubations). However, the insulin effect was first observed at 30 minutes. The observed effect of insulin-stimulation on adiponectin release is similar to results from a previous study (Lim *et al.*, 2015), demonstrating that insulin was without effect on adiponectin release

at early time points with a more prominent effect observed at later time points (60-120 min). Our data propose that insulin acts via mechanisms acting downstream of the exocytotic machinery whereas catecholamines trigger release at the level of the exocytotic machinery via a direct actions on readily releasable adiponectin vesicles.

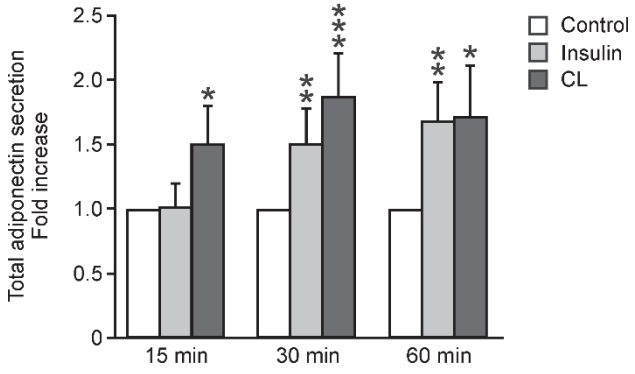

Fig. 13: Insulin-stimulated adiponectin release has slower time-kinetics than β3AR-triggered adiponectin secretion Time-course series in 3T3-L1 adipocytes representing the effects of insulin and CL on adiponectin release at 15, 30 and 60 minutes. Results are from 5 experiments and are expressed as mean ± SEM *P<0.05; **P<0.01; ***P<0.001 vs control (unstimulated conditions).

The role of Ca^{2+} and cAMP in insulin-induced adiponectin release

Insulin-induced adiponectin release was unaffected by Ca^{2+}-depletion, and ratiometric Ca^{2+}-imaging further demonstrated that changes in intracellular Ca^{2+} do not underlie insulin-stimulated adiponectin secretion. In agreement with findings in paper I, ADR stimulated short-term (30 min incubation) adiponectin release in IWAT adipocytes isolated from mice. Consistent with previous reports (Cong *et al.*, 2007), insulin (20 or 200 nM) also induced adiponectin release in isolated mouse IWAT adipocytes. Measurements of cAMP levels in cells exposed to insulin for 30 min confirmed that cAMP was elevated and thus not involved in the stimulatory mechanisms. The observation that adrenergic stimulation and insulin exposure both stimulate the release of adiponectin may appear contradictory due to their opposing physiological roles.

Both adrenaline- and insulin-stimulated adiponectin secretion is blunted in adipocytes isolated from obese/diabetic mice

In paper I of this thesis, we demonstrate that adrenergically stimulated adiponectin exocytosis is blunted in IWAT adipocytes isolated from mice with diet-induced obesity/T2D, due to reduced abundance of β3ARs and Epac1 (Komai *et al.*, 2016). To study the effect of obesity/T2D on insulin-stimulated adiponectin release, we exposed IWAT adipocytes isolated from chow- or HFD-fed mice (8 weeks) to insulin (20 nM) or ADR (5 µM). Consistent with previous experiments in this study, insulin and ADR increased the amount of released adiponectin in chow adipocytes. In adipocytes isolated from HFD-fed mice, neither ADR nor insulin could stimulate adiponectin release (Fig. 14: *left*). The latter is likely due to insulin resistance in the HFD-fed mice, displayed by elevated glucose and insulin levels as well as reduced insulin receptor expression. Similar to findings in paper I, basal (unstimulated) adiponectin release tended to be elevated although this did not reach statistical significance (P=0.067). Measurements of adipocyte adiponectin content (Fig. 14: *right*) showed that the blunted secretion was not due to a reduction of the hormone content.

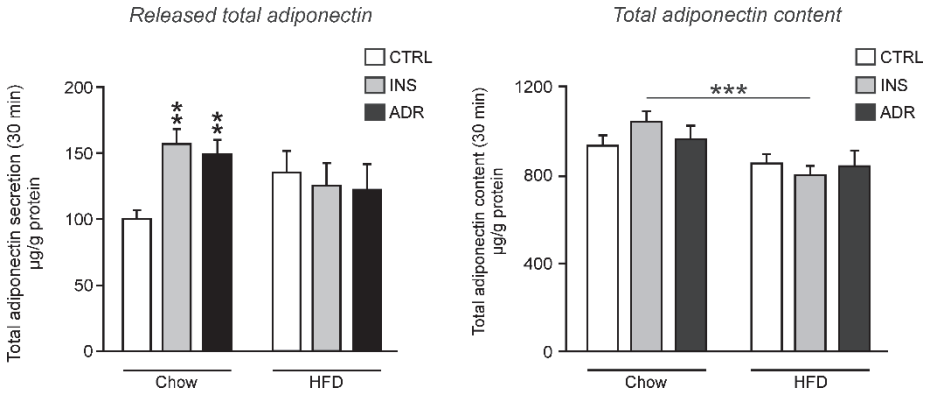

Fig. 14: Insulin- and ADR-stimulated adiponectin secretion is blunted in obese/diabetic mice. Adiponectin secretion (*left)* and adiponectin content (*right*) in IWAT adipocytes isolated from chow- or HFD-fed mice (8 weeks). Results are from 5-6 chow- and 5-6 HFD-fed mice. **P<0.01; ***P<0.001 vs control.

There was a tendency to a small increase of adiponectin content in chow adipocytes exposed to insulin (P=0.075 vs control), indicating that extracellular insulin upregulates adiponectin synthesis. This slightly enhanced content observed in chow adipocyte yielded a significant difference when compared to insulin-treated HFD adipocytes. Adiponectin content was neither altered in cells stimulated with ADR nor in control cells, in adipocytes from chow- or HFD-fed mice.

Adrenaline triggers exocytosis of high-molecular weight adiponectin whereas insulin induces release of smaller adiponectin forms

The physiological meaning of adiponectin release stimulated via both a catabolic (adrenergic) and an anabolic (insulin) signalling pathway is puzzling. However adiponectin is a complex hormone existing in diverse circulating molecular form, reported to have different effects on target tissues (Yamauchi *et al.*, 2002). We hypothesised that adiponectin secretion induced by adrenergic or insulin stimulation reflects release of different adiponectin forms. To investigate this, we measured HMW levels in medium samples from mouse IWAT adipocytes treated with ADR or insulin for 30 min (Fig. 15). Interestingly, release of HMW adiponectin was unaffected by insulin treatment but potently triggered by ADR.

The stimulated secretion of HMW adiponectin is abrogated in adipocytes from obese/ diabetic mice

Next we measured released HMW adiponectin from HFD adipocytes (Fig. 15). Notable, ADR-stimulated HMW adiponectin secretion was entirely obliterated in HFD adipocytes whereas the basal release of HMW adiponectin was unaffected. To confirm that the blunted HMW adiponectin release was not due to HFD-induced diminished adiponectin synthesis, we measured levels of the molecular form in homogenates from chow and HFD adipocytes. The content of HMW adiponectin was unaltered in adipocytes from fat mice. Thus, the obesity/T2D-associated blunted release is likely the result of a secretory defect and not due to reduced intracellular levels.

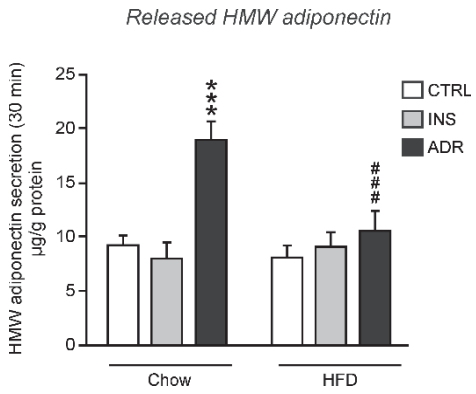

Fig. 15: ADR triggers release of HMW adiponectin whereas insulin induces release of smaller adiponectin forms only. HMW adiponectin release upon stimulation with insulin or ADR (in same samples as in fig.14). Data are from adipocytes isolated from 5-6 chow- and 5-6 HFD-fed mice. ***P<0.001 vs control. ###P<0.001 vs ADR; chow.

In conclusion, we propose that the blunted insulin-induced release of smaller forms is due to insulin resistance. We further speculate that the reduced stimulated release of smaller forms is compensated for by elevated basal (unstimulated) secretion. This would provide an explanation for the observed unaltered serum total adiponectin levels in obese/diabetic mice. However, the adipocytes isolated from HFD-fed mice seem unable to compensate for the diminished stimulated secretion of HMW adiponectin, as shown by the reduced levels of serum HMW adiponectin in obese/diabetic mice.

HMW adiponectin secretion is triggered by catecholamines also in human adipocytes

To validate our findings using mouse adipocytes for human physiology, we isolated subcutaneous abdominal adipocytes from healthy individuals undergoing elective surgery. In agreement with experiments in cultured and subcutaneous mouse adipocytes; insulin and ADR both stimulated adiponectin secretion in human adipocytes during 30 min incubations. Interestingly, CL readily stimulated adiponectin secretion in human adipocytes (Fig. 16: *left*). The physiological role of β3ARs in human adipocyte function has, as mentioned, been widely debated although β3ARs expression has been observed in human adipocytes (Berkowitz *et al.,* 1995; Krief *et al.*, 1993; Revelli *et al.*, 1993) Our secretion measurements with CL propose a new role for β3ARs in regulation of human adipocyte metabolism. Pretreatment of human adipocytes with Epac inhibitor ESI-09 confirmed the involvement of Epac in adrenergically stimulated adiponectin release, which is in agreement with earlier

observations in mouse adipocytes. Next we measured released HMW adiponectin in our human medium samples exposed to insulin, ADR or CL for 30 min. In line with our experiments in mouse adipocytes- both ADR and CL triggered the release of HMW adiponectin while insulin was not able to induce secretion of this molecular form (Fig. 16: *right*).

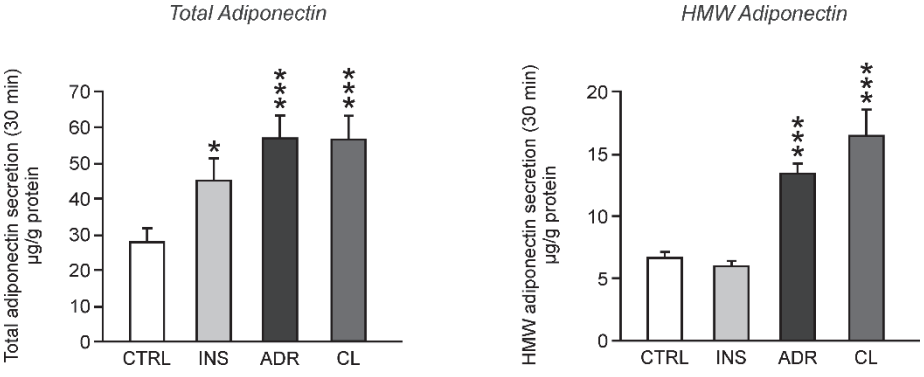

Fig. 16: β3AR-activation stimulates release of HMW adiponectin in human subcutaneous primary adipocytes Effect of stimulatory agents insulin, ADR and CL on total adiponectin release (*left*) and HMW adiponectin release in the same samples (*right*). Results are from 8 patients. *P<0.05; ***P<0.001 vs control.

Summary of findings in Paper II

In this study we show that insulin- and catecholamine stimulation have entirely diverse roles in the regulation of adiponectin release (Fig. 17: *top*). Catecholamine-triggered exocytosis involving β3ARs and Epac1 signalling results in rapid release of HMW adiponectin containing vesicles whereas insulin induces the release of smaller forms via PI3K-dependent mechanisms at later time-points. Additionally, we show that in obese/diabetic conditions, short-term stimulated secretion of both total and HMW adiponectin was blunted with a tendency to an upregulated basal release of smaller adiponectin forms (confirmed in paper; Fig. 17:*bottom*). Our findings further suggest that the inability of metabolically deprived adipocytes to respond to catecholamine stimulation (catecholamine resistance) causes the reduction of circulatory HMW adiponectin observed in metabolic disease.

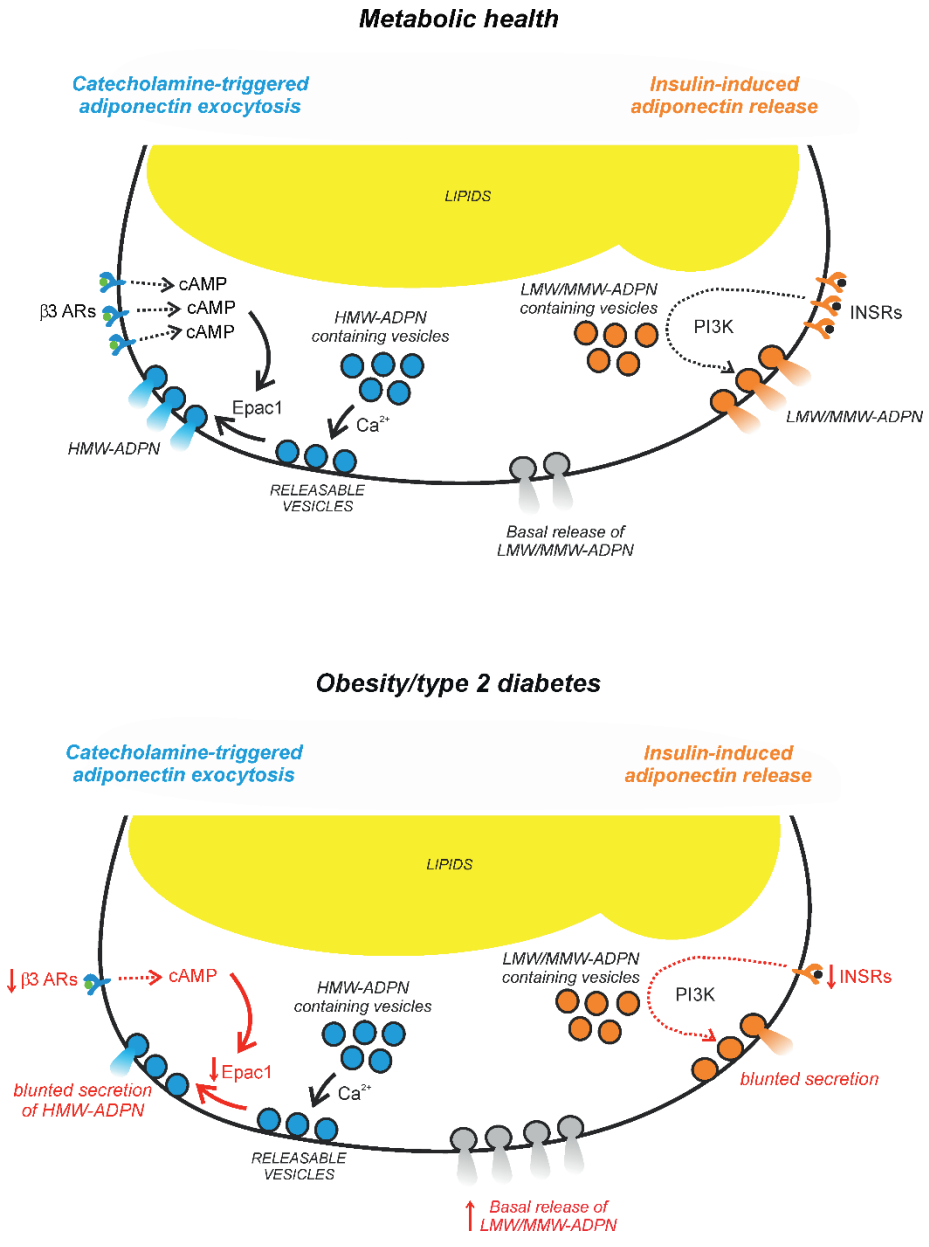

Fig. 17: Graphical conclusion based on our findings in paper II. See text for further details.

Paper III

The finding in paper I that adiponectin exocytosis is triggered by catecholamines proposes the involvement of the SNS in regulation of adiponectin secretion and suggests a possible regulatory role for simultaneously released ATP. In paper III of this thesis we investigate the involvement of sympathetic innervation in the regulation of white adipocyte adiponectin release.

Extracellularly applied noradrenaline stimulates white adipocyte adiponectin exocytosis/secretion

Short-term incubation of mouse IWAT adipocytes with NA (100 nM) for 30 min stimulated adiponectin release of a similar magnitude as demonstrated in paper I with ADR or the β3-agonist CL (Komai *et al.*, 2016). Further, NA-stimulated adiponectin release was unaffected in Ca^{2+}-depleted cells. Exocytotic recordings of vesicle fusion revealed that NA, as expected, triggered adipocyte exocytosis via Epac-dependent mechanisms (Fig. 18). Our data propose that NA triggers adiponectin exocytosis/secretion through β3AR-activation, elevating cAMP levels and finally resulting in a further downstream activation of Epac1.

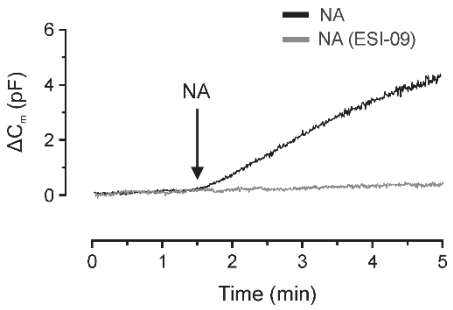

Fig. 18: Effects of NA on white adipocyte exocytosis. Example capacitance traces of 3T3-L1 cells infused with a non-stimulatory pipette solution (lacking cAMP) with extracellular addition of NA as indicated by the arrow. Light grey trace represents cells pre-treated for 30 min with Epac antagonist ESI-09 prior to addition of extracellular NA.

The role of extracellular ATP in the regulation of adiponectin release

Extracellular ATP can bind to ionotropic P2X or metabotropic P2Y receptors and activate purinergic signalling, leading to diverse biological effects depending on receptor subtype (Burnstock, 2007). In order to study the effects of external ATP, 3T3-L1 adipocytes were incubated with ATP for 30 minutes. Measurements of released adiponectin revealed that ATP stimulated adiponectin secretion ~2-fold over basal. We have recently demonstrated that extracellular application of ATP increases

3T3-L1 adipocyte Ca^{2+} levels (confirmed in the current study) via activation of P2Y2Rs (G_q-coupled and increases intracellular Ca^{2+}; El Hachmane *et al.*, 2018). The ability of external ATP to elevate adipocyte Ca^{2+} was also confirmed in the current study. ATP-stimulated adiponectin secretion was less potent in BAPTA-pretreated cells during 30 min incubations and was blunted upon stimulation during 60 min (in agreement with that Ca^{2+} has an important role for adiponectin vesicle replenishment and for secretion over longer periods).

The significance of extracellular ATP

ATP is a complex molecule involved in many cellular processes. In the cell, ATP provides energy to several biochemical reactions as well as activates various kinases, important for the signal transduction pathway. In addition to its intracellular functions, ATP can also be released extracellularly from cells. ATP has been suggested to be secreted via different mechanisms, such as cellular diffusion, channel proteins or vesicle fusion. Release of ATP from sympathetic nerve terminals has already been mentioned (Burnstock, 2007), and co-release via vesicle fusion in other cell types has been previously shown in chromaffin cells (Rojas *et al.*, 1985) and pancreatic β-cells (Detimary *et al.*, 1996). It is feasible to suggest that ATP is also released from the white adipocyte, perhaps in adipokine-containing vesicles, but this has never been shown. Regardless of origin (sympathetic nerve endings or the adipocyte itself), ATP release occurs in close proximately to adipocytes and thus may act locally in high concentration. Due to the biochemical properties of ATP, it is a very instable molecule that is rapidly hydrolysed. Although difficult to measure, reports demonstrate a range of local ATP concentration ranging from 10-100 μM (thus a concentration similar to the one used in this study; Detimary *et al.*, 1996). In white adipocytes, purinergic signalling has been suggested to regulate adipogenesis via P2Y2. The possible role of P2Y2 in lipid metabolism has been debated; some report that extracellular ATP stimulates lipolysis while other show no lipolytic effect of ATP. The receptor subtype P2Y1 has been proposed to be involved in leptin secretion (Laplante *et al.*, 2010), however our own gene expression data show very low levels.

Effects of noradrenaline and ATP on adiponectin release in primary adipocytes isolated from lean and obese/diabetic mice

In view of our previously demonstrated roles of cAMP (triggers adiponectin exocytosis) and Ca^{2+} (potentiates the cAMP-stimulated secretion), we hypothesised that sympathetic innervation of adipose tissue, via co-secretion of NA and ATP, perhaps compose the physiological pathway for stimulation of adiponectin secretion. We moreover considered that this regulation may be perturbed in obesity/metabolic disease. To investigate this, we incubated IWAT adipocytes isolated from lean and obese/diabetic mice with NA, ATP or a combination of NA and ATP (mimicking the co-release from sympathetic nerves; Fig. 19). As previously shown with ADR in paper I, short-term incubation of IWAT adipocytes from healthy mice stimulated adiponectin release. In agreement with results above using 3T3-L1 adipocytes, ATP increased secretion of adiponectin. The combination of NA and ATP potently stimulated adiponectin release (P<0.01 vs NA alone).

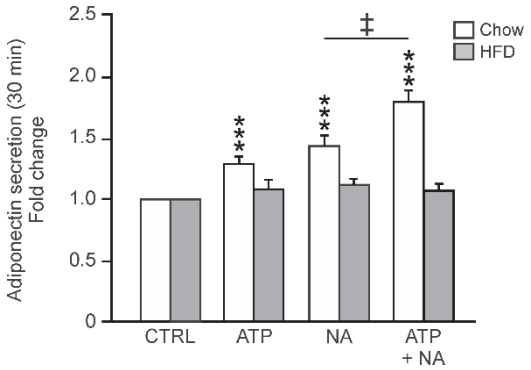

Fig. 19: Purinergic and adrenergic stimulation of adiponectin secretion in adipocytes isolated from lean and obese/diabetic mice Adiponectin secretion (30 min) in primary IWAT adipocytes from chow- or HFD-fed mice. Cells were incubated with ATP, NA or a combination of ATP and NA. Results are from 10-13 chow 10 chow- and 11 HFD-fed mice. ***P<0.001, ‡ P<0.01 ATP+NA vs NA; chow.

We confirmed that catecholamine-stimulated adiponectin secretion was blunted in obese/diabetic conditions, due to reduced levels of β3ARs and Epac1, and thus the previously shown catecholamine resistance. An indication for disrupted purinergic signalling was observed as 60% lower P2Y2R mRNA levels in HFD adipocytes.

Furthermore, measured adiponectin cell content was only marginally decreased in HFD compared to chow adipocytes. However, analysis of the released fraction of the total adiponectin revealed that HFD adipocytes released a smaller proportion, indicating defect secretory capacity. Our results suggest that ATP augments the NA-stimulated adiponectin release in a Ca^{2+}-dependent manner. To validate the involvement of P2Y2Rs (elevates Ca^{2+}) in the current study, we performed secretion measurements with the P2Y2R antagonist AR-C 118925XX (AR-C, 1 μM). In agreement with previous findings, the combination of NA and ATP potently stimulated adiponectin release. However, the secretory response was significantly reduced in adipocytes treated with the antagonist.

Obesity-induced reduction of sympathetic sensitivity in white adipose tissue has been reported (Lonnqvist *et al.*, 1992; Reynisdottir *et al.*, 1994). To examine the SNS innervation in adipose tissue, we performed immunohistochemical staining of tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis and a commonly used marker for sympathetic nerves. Additionally we measured adipose tissue NA-levels. Indeed, quantification of TH and NA levels revealed reduced SNS activity in IWAT from HFD-fed mice compared to chow. Our findings propose that diabetes/obesity is associated with decreased SNS innervation of white adipose tissue, resulting in blunted catecholamine-stimulated release of adiponectin and that defect adipocyte purinergic signalling further reduces the adiponectin secretion.

Effects of noradrenaline and ATP on secretion of HMW adiponectin

In order to study the effect of purinergic signalling in adiponectin release in more detail, we measured released HMW adiponectin from IWAT adipocytes exposed to NA or ATP for 30 minutes (Fig. 20). Preliminary results show that NA, in agreement with findings with ADR in paper II, stimulated secretion of this molecular form. ATP on the other hand only induced release of smaller adiponectin forms. This demonstrates that ATP, in accordance with the finding that the nucleotide is without effect on cytoplasmic cAMP, has no effect on release of HMW adiponectin.

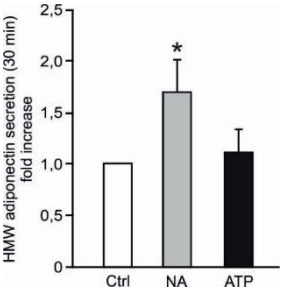

Fig. 20: Effects of NA and purinergic signalling on released HMW adiponectin. Secreted HMW adiponectin in IWAT adipocytes incubated with NA or ATP for 30 min. Results are presented in fold increase. Data are from 4 chow mice. *P<0.05.

Summary of findings Paper III

In paper III of this thesis, we show that, as expected, NA stimulates adiponectin release of a similar magnitude as that obtained with ADR. Further we show that external ATP induces adiponectin release via Ca^{2+}-dependent pathways. Interestingly, ATP augments the NA-triggered adiponectin secretion and this augmentation is diminished in adipocytes isolated from obese/diabetic mice. The blunted ATP potentiation is paralleled by a downregulation of purinergic P2Y2R gene expression and decreased adipose tissue NA levels (Fig. 21). Our studies propose that adiponectin exocytosis is physiologically regulated by NA and ATP, co-released from sympathetic nerves innervating the white adipose tissue. The combination of the disruptions of purinergic signalling and decreased NA levels within the adipose tissue and the documented catecholamine resistance shown in this thesis, likely jointly contributes to the obesity/T2D-induced reduced adiponectin release and to the lower adiponectin levels observed in metabolic disorders.

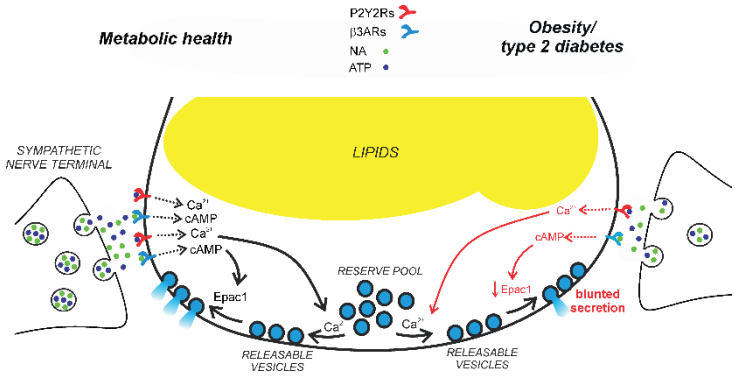

Fig. 21: Graphical conclusion based on our findings in paper III. See text for further details.

Paper IV

In paper IV we studied if the pathophysiological regulation of adiponectin release in visceral adipocytes was similar to that demonstrated in subcutaneous adipocytes (Paper I-III). Visceral adipocytes have been shown to be more sensitive to catecholamine stimulation due to high expression of ARs (Arner *et al.*, 1990; Bolinder *et al.*, 1983). This suggest a role of adrenergic signalling in stimulation of visceral adipocyte adiponectin release.

Visceral adipocyte adiponectin release is stimulated via β3ARs and Epac1

Incubation of visceral GWAT mouse adipocytes with FSK (10 μM) together with IBMX (200 μM), ADR (5 μM) or β3-agonist CL (1 μM) stimulated short-term adiponectin secretion. Measurements of released product revealed that a combination of FSK/IBMX (elevates cAMP) increased adiponectin secretion >2-fold over basal whereas the effect of ADR or CL was slightly lower. To study the role of Epac in visceral adiponectin release, GWAT adipocytes were pretreated with the Epac antagonist ESI-09 (10 μM for 30 minutes) prior to incubation with ADR (Fig. 22). Adiponectin release caused by ADR was abolished in cells preincubated with ESI-09. We propose that visceral adipocyte short-term adiponectin release is stimulated by the same mechanism as shown in subcutaneous adipocytes (Paper I), involving activation of β3ARs and Epac1.

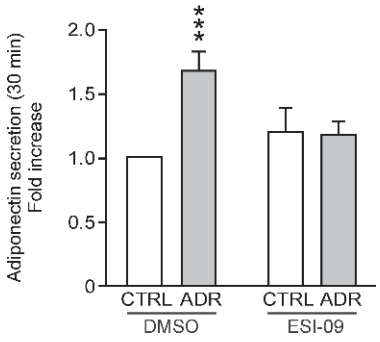

Fig. 22: Adrenergic stimulation of adiponectin secretion in GWAT adipocytes is Epac-dependent. Adrenaline-stimulated adiponectin release in isolated primary GWAT adipocytes pretreated with Epac-antagonist ESI-09 for 30 min (10 μM). Data is expressed in fold change and from 7 mice. ***P<0.001.

Measurement of total adiponectin content in cells treated with either stimulatory agent, revealed no difference in cellular levels of the hormone. This confirmed that the stimulatory effect on adiponectin release was due to effects on the secretory machinery, and not as a result of increased adiponectin synthesis. Further we showed that only a small fraction (~1.5 %) of total adiponectin content was release upon stimulation for 30 min.

Reduced stimulated adiponectin secretion in visceral adipocytes isolated from obese/diabetic mice

We next investigated how diet-induced obesity/diabetes affected adrenergically stimulated adiponectin release using adipocytes isolated from mice fed HFD (Fig. 23). In GWAT adipocytes isolated from chow-fed mice, ADR or CL could stimulate adiponectin release. Similar to finding in subcutaneous adipocytes, GWAT adipocytes isolated from diet induced obese/diabetic mice had reduced levels of secreted adiponektin upon stimulation with ADR or CL. Unstimulated adiponectin release (basal) from GWAT was unaffected by HFD-feeding. Total adiponectin content analysis, showed a small reduction (~20%) of the adipokine in HFD adipocytes compared to chow controls.

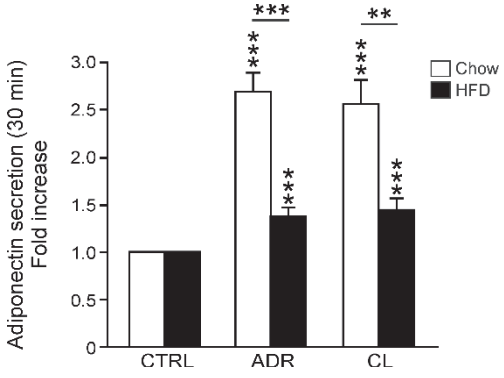

Fig. 23: Adrenergic stimulation of adiponectin secretion in GWAT cells from lean and obese/diabetic mice. Adrenergically stimulated adiponectin secretion from GWAT adipocytes isolated from chow- and HFD-fed mice. Data are from 10 chow- and 11 HFD-fed mice. **P<0.01; *** P<0.001.

In Paper I we demonstrated that in IWAT adipocytes, HFD results in a complete abolishment of adrenergically stimulated adiponectin release largely due to reduced levels of β3ARs and Epac 1. To investigate if similar molecular defects could explain the reduced stimulated adiponectin release in GWAT adipocytes from obese/diabetic mice, we performed gene expression analysis of ARs and Epac isoforms.

Similar to findings in IWAT adipocytes, β3AR was highly expressed in chow GWAT adipocytes (Fig. 24: *left*). The β1 and β2 adrenergic receptors were expressed at ~100-fold lower levels than β3AR (*not shown*). Also α1DAR was detected in GWAT adipocytes. Contrary to findings using IWAT adipocytes, there was no change in mRNA levels of β3AR in HFD adipocytes. Comparable to findings in IWAT adipocytes, Epac1 was expressed in GWAT adipocytes with no presence of the other isoform (Fig. 24: *right*). Interestingly gene expression of Epac1 was doubled in HFD adipocytes compared to adipocytes isolated from chow-fed mice.

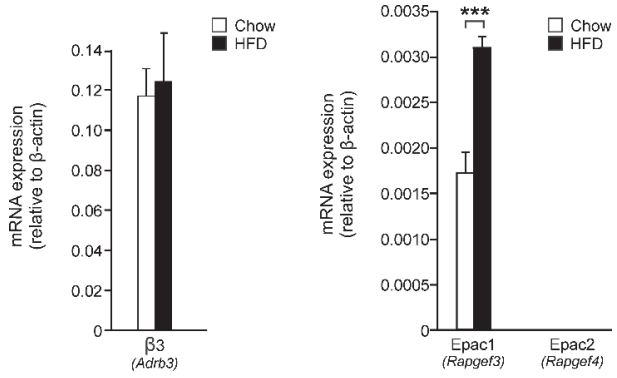

Fig. 24: Relative gene expression of β3AR and Epac isoforms in GWAT adipocytes from lean and obese/diabetic mice. Gene expression of ARs (*left*) and Epac isoforms (*right*). Data are from 5 chow- and 5 HFD-fed mice. ***P<0.001.

We speculated that perhaps the upregulation of Epac1 was due to lower levels of intracellular cAMP in the obese/diabetic adipocytes. We measured intracellular cAMP levels in chow and HFD GWAT adipocytes treated with the stimulatory agents. Rises of intracellular cAMP triggered by ADR or CL were similar in cells isolated from chow- and HFD-fed mice. Unaltered levels of cAMP in HFD adipocytes are in agreement with the detected maintained mRNA level of β3ARs. This is supported by that in IWAT adipocytes isolated from HFD-fed mice, the cAMP increase in response to CL was ~60% lower. Our results propose that diet-induced blunted adiponectin release in visceral (GWAT) mouse adipocytes are caused by other secretory defects than those described in subcutaneous (IWAT) mouse adipocytes.

Role of exocytotic proteins in blunted adiponectin release from visceral HFD-adipocytes

We hypothesised that the reduced stimulated adiponectin release in visceral HFD adipocytes is perhaps due to defects of the exocytotic process itself. Hormone-containing vesicle fusion is typically mediated by interaction of SNARE proteins. Guided by previous publications (Kawanishi *et al.*, 2000; Olson *et al.*, 1997; St-Denis *et al.*, 1999) and adipocyte micro array data, we decided to measure the expression of a selection of proteins known to be involved in regulated exocytosis. The SNARE SNAP-23 is present in adipocytes and has important roles in the translocation of GLUT4 to the plasma membrane (St-Denis *et al.*, 1999). SNAREs need to interact with other SNAREs such as syntaxins and vesicle-associated membrane proteins (VAMPs) in order to complete vesicle fusion (Han *et al.*, 2017). Gene expression analysis revealed a small reduction of SNAP-23 in HFD adipocytes ($P=0.06$) whereas levels of VAMP2 and VAMP4 were more reduced ($P<0.05$). Also, the small GTPase Rab3a was expressed at a slightly lower level ($P<0.05$) whereas Rap1 (isoforms a and b) remained unaltered. Based on our gene expression data comparing normal and obese/diabetic conditions, we propose that the defected stimulated adiponectin release may be due to defects in expression of proteins important for the exocytotic process. However, several exocytotic proteins are present in white adipocytes and their roles are incompletely understood.

Summary of findings in paper IV

In paper IV, we demonstrate that short-term adiponectin secretion in GWAT adipocytes is stimulated via β3AR and activation of Epac1 as shown in subcutaneous adipocytes (Paper I-III). Moreover, we show that stimulated adiponectin release in GWAT adipocytes isolated from obese/diabetic mice is reduced. It seems that the underlying mechanism for the disturbed adiponectin release clearly differs between depots since the gene expression level of β3AR and Epac1 remain unaltered.

FINAL DISCUSSION

The metabolic health improving effects of adiponectin is widely recognised and suggest adiponectin replacement therapy as promising pharmacological treatment of obesity-related diseases. Eukaryotic cells are a successful (although expensive) model system for synthesis of all molecular forms of adiponectin. Nonetheless, development of exogenous adiponectin has experienced many difficulties. First, adiponectin cannot be administrated orally due to its polypeptide structure. Secondly, intravenous administration would be challenging to maintain since adiponectin has a short biological half-life (Halberg *et al.*, 2009). Thus, the prospect to instead increase levels of endogenous adiponectin is attractive. Studies demonstrate that pharmacological elevation of circulating adiponectin improves metabolic status (Pajvani *et al.*, 2004) and can be a promising therapeutic strategy for treatment of metabolic diseases. Still little is known about the regulation of adiponectin secretion. In order to pave the way for future treatments aimed at elevating secretion of the body's own adiponectin, we must increase our knowledge about the cellular and molecular regulation of the adiponectin exocytosis process.

Catecholamine resistance

As a natural continuation of the findings in (Komai *et al.*, 2014), we show in paper I of this thesis that adiponectin exocytosis is stimulated via catecholamine-activation of β3ARs and Epac1 in subcutaneous adipocytes. We further demonstrate that adrenergically stimulated adiponectin secretion is blunted in obesity/diabetes, in a state we refer to as *catecholamine resistance*. Professor Arner with colleagues was first to describe catecholamine resistance in subcutaneous adipocytes isolated from obese individuals (Lonnqvist *et al.*, 1992). The observed disturbed lipolytic activity was attributed to decreased β2AR abundance (~70%) as well as defected downstream signalling (reduced PKA levels). Similar to those findings, we have now demonstrated that the inability of subcutaneous adipocytes isolated from obese/diabetic mice to respond to catecholamine-stimulation with elevated adiponectin release is caused by lowered density of β3ARs and Epac1.

Due to its wide distribution in the body, SAT has been proposed to be more likely to affect circulating levels of secretory products. Likewise, studies show that subcutaneous adipocytes secrete more adiponectin compared to visceral fat cells, thus contributing more to circulating levels of the adipokine (Fisher *et al.*, 2002; Meyer *et al.*, 2013). In paper I, we observed that catecholamine resistance in the subcutaneous adipocytes is associated with a reduction of circulating HMW adiponectin (~50%). Interestingly total adiponectin levels were unaltered in obese/diabetic mice compared to healthy control mice. We speculated that the increased basal adiponectin release observed in obese/diabetic adipocytes acts as a compensatory mechanism for reduced stimulated secretory response, hence maintaining high circulating adiponectin levels.

Adrenergic signalling stimulates release of HMW adiponectin

Results from paper I contributed to our hypothesis in paper II that adrenergic signalling is explicitly involved in the regulation of HMW adiponectin secretion. Short-term secretion measurements revealed that both ADR and β3-agonist CL triggered the release of HMW adiponectin. Our findings from cultured and primary mouse subcutaneous adipocytes were confirmed in isolated primary human subcutaneous adipocytes. Although the expression of β3AR was dramatically lower in human adipocytes compared to observations in mouse adipocytes, specific β3AR-activation resulted in increased release of HMW adiponectin. The coeval finding that insulin also induces adiponectin secretion lead us to further investigate the similarities and differences between adiponectin release stimulated via the two signalling pathways. Our studies demonstrated that insulin acts via different signalling mechanisms and induces adiponectin release with slower time-kinetics.

The observation that adiponectin secretion was induced by two opposing signalling molecules may appear controversial from a physiological perspective. In the body, levels of circulating insulin are high as glucose levels rise following a meal (fed state). Endocrine functions of insulin include increased glucose uptake in peripheral tissues. On a cellular level, insulin maintains expression of several transcriptional factors, essential for lipid and glucose metabolism (Furtado *et al.*, 2002; Stahl *et al.*, 2002). A small increase of adiponectin content was observed in adipocytes exposed to insulin

for 30 minutes, proposing that insulin increases synthesis of adiponectin. However, results from Professor Han and colleagues demonstrated that inhibition of protein synthesis did not affect insulin-stimulated adiponectin secretion (Lim *et al.*, 2015) and the effect of insulin on adiponectin expression need to be further investigated.

Under the opposite physiological condition, serum insulin drops and circulating catecholamines increases (fasted state). In addition, adipocyte lipolysis is increased in the fasted state to satisfy the higher FFA demand of peripheral tissues for energy consumptions. Considering the complexity of circulating adiponectin molecular structures, it is feasible that different mechanisms can regulate release of diverse adiponectin forms. We considered the possibility that insulin stimulates the release of smaller adiponectin forms. Measurements of secreted product confirmed that short-term insulin exposure did not stimulate HMW adiponectin secretion but only the release of smaller adiponectin forms. We found that in adipocytes from obese/diabetic mice, the stimulated release of both HMW adiponectin and simpler forms were disturbed due to catecholamine resistance coupled with insulin resistance. The maintained total adiponectin levels appear to instead be achieved by the diabesity-associated increase of unregulated release of smaller adiponectin forms.

Role of sympathetic innervation in regulation of adiponectin release

Due to the demonstrated rich innervation of white adipose tissue by the sympathetic nervous system (Youngstrom & Bartness, 1995), we in paper III determine the role of NA and ATP that are co-released from sympathetic nerves, in the regulation of adiponectin exocytosis. We speculated that β3ARs are more likely to be activated by high NA concentrations released from the close vicinity of the sympathetic nerve terminal than by catecholamines released from the adrenal medulla into the circulation. This suggestion was supported by quantification of catecholamine content in rat IWAT, revealing 80-fold higher levels of NA compared to ADR (Vargovic *et al.*, 2011), thus implying NA as the main catecholamine regulating adiponectin exocytosis. Our findings in paper III indeed propose that adiponectin secretion is physiologically triggered by innervation of sympathetic nerves, NA elevates cAMP levels to trigger

adiponectin exocytosis and ATP acts to increase Ca^{2+} via P2Y2R, thus potentiating the cAMP-stimulated secretion. This regulation is in agreement with the mechanistic molecular control shown for cAMP and Ca^{2+} in our previous publication (Komai *et al.*, 2014).

The regulation of HMW adiponectin secretion by sympathetic innervation offers a very precise and rapid means of controlling adiponectin secretion, perhaps in an adipose depot specific manner. Our preliminary data indicate that only catecholamines/cAMP trigger the release of HMW adiponectin whereas ATP, like insulin, triggers release of smaller forms of the adipokine. However, we also demonstrate that the stimulatory effect of ATP is largely dependent on Ca^{2+} while insulin-stimulated adiponectin release occurs without alterations of intracellular Ca^{2+}. In addition, we have previously shown that Ca^{2+} can not trigger adipocyte exocytosis in the absence of cytosolic cAMP (Komai *et al.*, 2014). It seems unlikely that ATP affects the same adiponectin pool as insulin, as we show that insulin-stimulated adiponectin release is Ca^{2+}-independent. The results thus indicate that ATP, via Ca^{2+} elevations (and likely by initiation of additional signalling cascades), act on a third adiponectin pool. Clearly, the involvement of diverse signalling pathways in secretion of different adiponectin forms is perplex and further studies are required in order to resolve the details. .

Adipose-depot specific adiponectin release in health and metabolic disease

In the final work of this thesis (paper IV), we show that catecholamines trigger adiponectin release also in visceral rodent (gonadal) adipocytes. Similar to results in subcutaneous adipocytes, regulation of adiponectin secretion involves β3AR and Epac1 signalling. Interestingly, our results demonstrate a blunted adiponectin release in gonadal adipocytes from obese/diabetic mice. However, the underlying mechanism for the disturbed stimulated release in visceral HFD adipocytes differ from the catecholamine resistance defined in subcutaneous adipocytes as shown by maintained gene expression of β3ARs and Epac1 as well as largely sustained cAMP levels; the defects appear to lie further down the exocytotic pathway, perhaps involving proteins essential for the adiponectin exocytosis process. It is worthy of note that local

altercations of cAMP concentrations, in regions near the plasma membrane where adiponectin exocytosis occurs, may exist. Compartmentalisation of cAMP signalling molecules such as ARs, ACs and PDEs has been reported. Furthermore, formation of cellular cAMP gradients have been observed (Houslay & Adams, 2003) as well as a restricted distribution of Epac1 (Schmidt *et al.*, 2013). It is thus possible that the signalling involved in regulation of adiponectin exocytosis/secretion is localised to specific cellular micro-domains containing the key regulatory molecular players (β3AR and Epac1) and that the local signalling at these sites is disturbed. Interestingly, in cardiomyocytes, β2ARs have been suggested to be located in cholesterol rich structures called caveolae. These lipid rich structures are enriched in white adipocytes and are proposed to be responsible for heterogeneous distribution of signalling proteins in different micro-domains. Unpublished findings from our group suggest that caveolae are important for adiponectin secretion (Brännmark et al., in preparation).

The data in this thesis propose that similar mechanisms regulate adiponectin secretion in subcutaneous and visceral adiponectin but that different release disturbances arise in association to obesity and diabetes. Subcutaneous and visceral adipose tissues have different origin (Chau *et al.*, 2014) and also differ in metabolic functions (Wajchenberg *et al.*, 2002). Adipocytes in visceral fat are more metabolically active as demonstrated by increased lipolytic activity and thus higher expression of βARs together with lower insulin-sensitivity (Arner *et al.*, 1990; Bolinder *et al.*, 1983). Furthermore, the size of visceral fat mass correlates positively to insulin resistance (Kloting *et al.*, 2010). Individuals with enlarged VAT are at a higher risk for developing obesity-related diseases. The anatomical position of VAT, with direct portal drainage to the liver (Rytka *et al.*, 2011), enables hormones such as adiponectin released from VAT to have a direct effect on glucose metabolism though paracrine effects. Considering the above, the similarities with regard to adiponectin release mechanisms between subcutaneous and visceral adipocytes may actually appear more surprising than the differences.

Pathophysiological relevance

The importance of regulated secretion of a hormone as abundant in the circulation as adiponectin may be questioned. Also, even if adrenergic stimulation specifically regulates release of HMW adiponectin, this form also exists at rather constantly high serum levels (30-50% of the total adiponectin; Aso *et al.*, 2006; Bluher *et al.*, 2007; Hamilton *et al.*, 2011). Other endocrine hormones typically circulate at low levels and are rapidly and transiently increased upon physiological demand (such as insulin that systemically increases in response to food intake). We propose that the answer lies within the distinct characteristics of adipose tissue in combination with where the released adiponectin is to exert its effects.

White adipose tissue differs from other hormone releasing organs in that it is located in diverse regional depots and contribute to as much as a fifth of the whole body mass. The wide distribution of white adipose tissue expectedly maintains the incessantly high systemic levels of adiponectin. It is worth speculating that the regulated secretion of adiponectin, in particular the HMW form, is perhaps more important for local autocrine/paracrine actions and not so much for endocrine effects. Thus, the regulated exocytotic pathway of HMW adiponectin may exist in order to locally increase the hormone to very high levels, acting at nearby cells, tissues and organs. The close anatomical location of visceral adipose tissue to several metabolically active organs such as pancreas and liver suggests an important role for visceral adiponectin release. An alternative/complementing explanation is that the regulated secretion of HMW adiponectin does not necessarily exist with the aim to induce acute effects on the target tissues but instead to preserve the high circulating levels. Our data in paper I support this hypothesis, as we demonstrate that serum levels of HMW adiponectin are decreased by ~50% in obese and catecholamine resistant mice. Our data defining the regulation of adiponectin secretion (HMW and smaller forms) is so far largely limited to results from subcutaneous adipocytes. Other adipose tissue depots, such as VAT that has been shown to differ from SAT need to be more thoroughly investigated with respect to the regulated adiponectin release.

The finding presented here, showing that secretion of HMW and smaller forms of adiponectin are stimulated under opposite metabolic conditions propose that the regulation of adiponectin is a very complex process. More detailed future research defining the release of specific adiponectin forms and its molecular and cellular regulation is required to fully explain the role of adiponectin in metabolic disease. However, we are of the opinion that our studies jointly propose that adiponectin secretory defects underlie the reduced circulating HMW adiponectin levels reported in metabolic disease.

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