The role of beta 3 adrenergic receptor in white adipocyte adiponectin exocytosis

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Cover illustration by Marina Kalds Said: Beta 3 adrenergic receptor activation triggers the release of different molecular forms of adiponectin in the white adipocyte.

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Simplicity is the ultimate sophistication.

Leonardo da Vinci

To my parents and sisters.

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ABSTRACT

In this thesis we define the physiological and underlying mechanisms involved in the white adipocyte adiponectin exocytosis, with a special focus on the beta 3 adrenergic receptor (β_3AR) in health and metabolic disease. In **paper I,** we define the role of sympathetic innervation and purinergic signaling in the regulation of white adipocyte adiponectin release**.** We hypothesized that adiponectin exocytosis is regulated by sympathetic nerves, co-releasing noradrenaline and ATP within the white adipose tissue, acting on adrenergic and purinergic receptors, elevating cytosolic cAMP and Ca^{2+}, respectively. A combination of electrophysiological recordings and secretion measurements confirmed that NA stimulate the release of adiponectin, whereas ATP augments it. This regulation is abrogated in adipocytes isolated from obese and diabetic mice, due to a reduction of protein expression of both receptors, and that this is associated with reduced circulating high molecular weight adiponectin. In **paper II,** we investigated the role of β_3AR in white adipocyte adiponectin release and in metabolic health in more detail, using mice that are genetically ablated for β_3AR (β_3AR-KO). Our data show that the disturbed signaling mechanism in the KO mice results in abrogated adiponectin release and reduced serum levels of total and HMW adiponectin. Furthermore, fasting insulin and glucose indicate that KO mice have an improved insulin sensitivity and are metabolically healthy, despite the lower adiponectin levels. However, β_3AR-KO mice become insulin resistant when challenged with high fat diet. Finally, in **paper III**, we show that an activation of β_3AR rapidly triggers the release of HMW adiponectin, while insulin induces release of only smaller molecular forms of adiponectin. Adiponectin secretion is diminished in adipocyte isolated from obese individuals, connected to ~50% reduced abundance of β_3ARs in adipocytes and lower circulating HMW adiponectin. Treatment with a β_3AR agonist increased serum HMW adiponectin in both lean and obese mice.

Keywords: White adipocyte, adiponectin exocytosis/secretion, beta 3 adrenergic receptor

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SAMMANFATTNING PÅ SVENSKA

Vit fettvävnad, som finns både subkutant (under hud) och visceralt (runt organen i bukhålan), är kroppens främsta energireserver (lagra energi i form av fett) och består till största del av fettceller (adipocyter). Efter några års forskning har man upptäckt att den vita fettväven även fungerar som ett endokrint organ, med förmågan att kommunicera med kroppens andra organ genom att frisätta aktiva signalmolekyler/hormoner direkt till blodet. Ett utav dessa hormoner är *adiponektin*, som har fått stor uppmärksamhet för dess gynnsamma effekter. Adiponektin hjälper framförallt till med att sänka inflammation, öka fettförbränning och förbättra insulinkänslighet, som i sin tur hjälper till med att öka upptaget av socker (glukos) från blodet efter en måltid. Det är alltså ett anti-diabetiskt hormon som minskar risken att utveckla typ 2 diabetes. Visst låter det som ett sund organ? Det finns dock en liten hake. Vid överskott av fettlagring under en längre tidsperiod, kan dess funktioner försämras och leda till hälsoproblem, i form av övervikt och fetma.

Övervikt/fetma är idag ett av våra allvarligaste globala folkhälsoproblem, som växer snabbt bland vuxna såväl som barn och unga. Övervikt är ingen sjukdom i sig, men kan öka risken till att utveckla fetma, vilket i sin tur ökar risken att utveckla andra allvarliga sjukdomar som till exempel typ 2 diabetes och hjärt- och kärlsjukdomar. Vid fetma expanderar den vita fettväven; fett lagras mer och mer i fettcellerna samtidigt som adiponektin frisätts mindre till blodet. För att motverka fetma är det önskvärt att öka mängden adiponektin i kroppen. Adiponektin är relativt stort och komplex i sin storlek, har kort livslängd i blodet och frisätts i olika former. Framställningen av adiponektin i form av ett läkemedel är därmed komplicerat. Därför vore det med fördel om man istället kunde hitta sätt/medel att reglera kroppens egen frisättning av adiponektin.

Vår grupp har tidigare visat att vid aktivering av beta 3 adrenerga receptorer (β_3AR), ökar innehållet av den sekundära signalbudbärare cAMP, som i sin tur ökar frisättningen av adiponektin via signaleringsproteinet Epac1, framförallt den hög-molekylära formen av adiponektin (aktiva formen). Vi har därför, i denna avhandling valt att studera regleringen av adiponektinfrisättningen från vita fettceller med avseende på β_3AR roll.

Vi börjar avhandlingen med att undersöka hur regleringen av adiponektin kan stimuleras från närliggande källor till vita fettvävnaden, som sympatiska nerver, som frisätter noradrenalin (aktiverar β_3AR) och ATP. Vi går därefter djupare in i avhandlingen, där vi utvärderar β_3AR roll och funktion i frisättningen av adiponektin. Vi avslutar med att studera andra signaleringsvägar av adiponektinfrisättning, där vi inkluderar stimulering av adiponektinfrisättningen med hormonet insulin (utsöndras från bukspottskörteln). Vi kommer även att undersöka hur regleringen av adiponektin (speciellt den hög molekylära formen av adiponektin) påverkas under ohälsosamma tillstånd såsom fetma och typ 2 diabetes. Våra fynd kommer med förhoppning ge oss en bättre förståelse om de bakomliggande processerna bakom regleringen av adiponektinfrisättningen. Om vi kan lyckas styra regleringen, kan vi på så sätt ta oss ett steg närmre till hur vi kan behandla fetma på ett effektivt sätt. Detta kan således bidra till en minskning på ett av världens växande sjukdomsproblem i framtiden.

LIST OF PAPERS

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

I. Musovic, S., Komai, A. M., **Said, M. K.**, Shrestha, M. M., Wu, Y., Wernstedt Asterholm, I., & Olofsson, C. S. (2022). Noradrenaline and ATP regulate adiponectin exocytosis in white adipocytes: Disturbed adrenergic and purinergic signalling in obese and insulin-resistant mice. *Molecular and cellular endocrinology*, *549*, 111619.

II. **Kalds Said, M.,** Mohan Shrestha, M., Musovic, S., Begum Samad, M., Dou, H., Wernstedt Asterholm, I., & Olofsson, C. S. Mice genetically ablated for β_3 adrenergic receptor display improved metabolic health despite reduced circulating adiponectin but are prone to develop insulin resistance when challenged with a high-fat diet. *Manuscript*

III. Musovic, S., Shrestha, M. M., Komai, A. M., **Kalds Said, M.,** Maimaiti, S., Banke, E., Skibicka, K., Wernstedt Asterholm, I., Barg, S., Rorsman, P., Blüher, M., & Olofsson, C. S. Beta 3 adrenergic receptor agonists stimulate white adipocyte exocytosis of high molecular weight (HMW) adiponectin in mice and humans and prevent a decrease of circulating HMW adiponectin levels in obese mice. *Manuscript*

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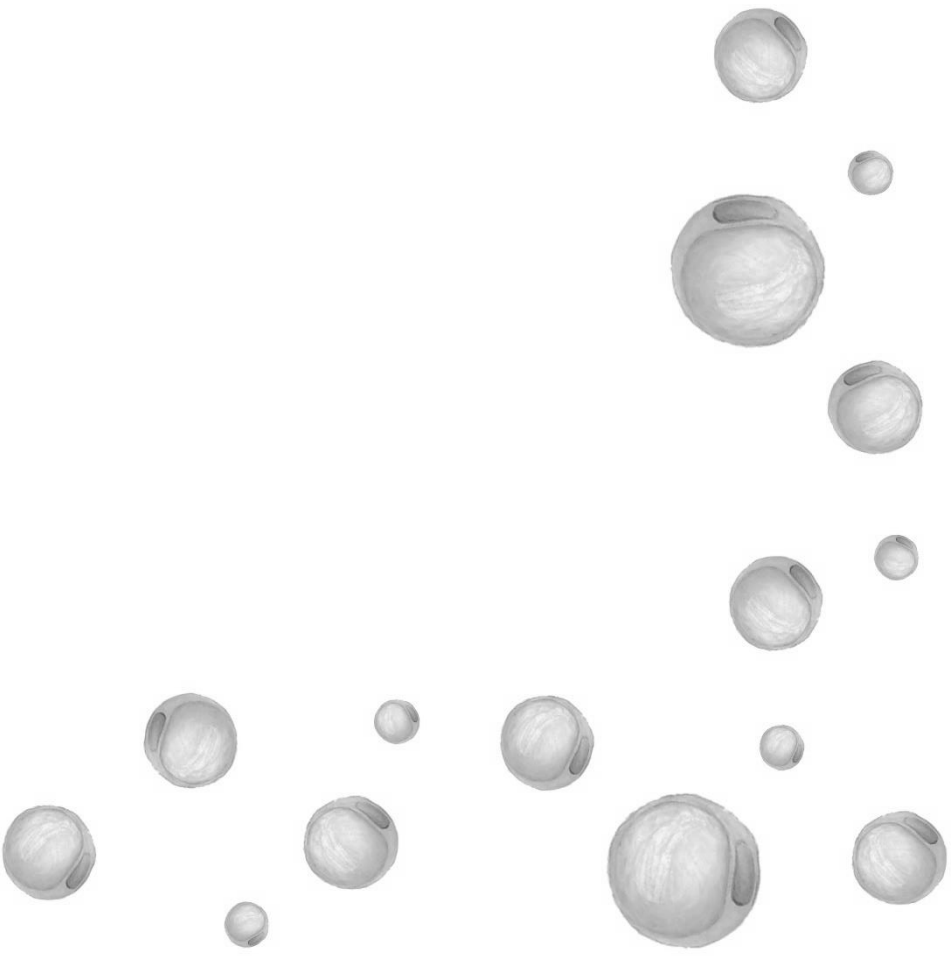
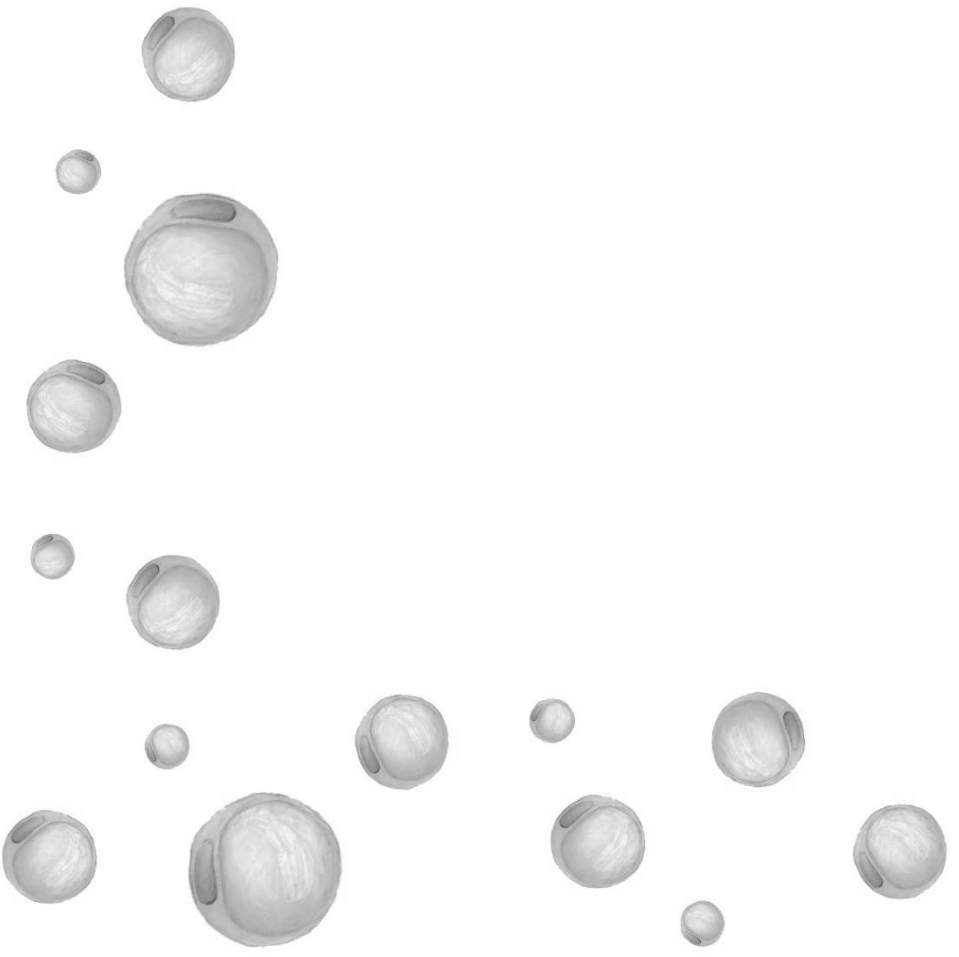
ABBREVIATIONS

$[Ca^{2+}]_i$	*Intracellular calcium ions*
AC	*Adenylyl cyclase*
AdipoR1	*Adiponectin receptor 1*
AdipoR2	*Adiponectin receptor 2*
ADR	*Adrenaline*
AR	*adrenergic receptor*
AT	*Adipose tissue*
ATP	*adenosine triphosphate*
BAPTA	*membrane-permeable Ca^{2+} chelator (1,2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)*
BAT	*Brown adipose tissue*
cAMP	*cyclic Adenosine Monophosphate*
CL	*β_3AR agonist, CL316,243*
DAG	*diacylglycerol*
Dexa	*Dexamethasone*
DINP	*diisononyl phthalate oil*
Dio2	*Iodothyronine Deiodinase 2*
DMSO	*dimethyl sulfoxide*
DsbA-L	*disulfide bond A oxidoreductase-like protein*
Epac	*Exchange Protein directly Activated by cAMP*
ER	*endoplasmic reticulum*
Ero1-Lα	*ER membrane-associated oxidoreductase-1-Lα*
ERp44	*Endoplasmic reticulum resident protein 44*
FBS	*Fetal Bovine Serum*
FFA	*Free fatty acids*
Fsk	*Forskolin*
FVB	*Friend leukemia virus B*
GPCR	*G-protein coupled receptor*
GWAT	*Gonadal white adipose tissue*
HFD	*High fat diet*
HMW	*high molecular weight*
i.p.	*intraperitoneal*
IBMX	*3-Isobutyl-1-methylxanthine*
IP3	*inositol trisphosphate*
IWAT	*Inguinal white adipose tissue*
LMW	*low molecular weight*
MMW	*middle molecular weight*
mRNA	*messenger RNA*
NA	*Noradrenaline*
NBCS	*New born Calf Serum*
OGTT	*Oral glucose tolerance test*
P/S	*Penicillin-streptomycin*

P2Y2R	*Purinergic P2Y2 receptor*
PDE3B	*phosphodiesterase 3*
PI3K	*phosphoinositide 3 kinase*
PKA	*Protein kinase A*
PKB/Akt	*Protein kinas B*
PLC	*phospholipase C*
SEM	*Standard Error of the Mean*
SNS	*sympathetic nerve system*
SVF	*Stromal vascular fraction*
T2D	*Type 2 diabetes*
TH	*tyrosine hydroxylase*
TIRF	*Total internal reflection fluorescence*
Ucp1	*uncoupling protein 1*
WAT	*White adipose tissue*
WHO	*World Health organisation*
WT	*Wild type*
β_3AR	*beta 3 adrenergic receptor*
β_3AR-KO	*beta 3 adrenergic receptor knockout*





INTRODUCTION

Obesity and overweight, defined as an increase of lipid accumulation in white adipocytes (fat cells), results from an imbalance between energy intake and energy expenditure. This growing epidemic have globally become a public health problem among adults and children, causing more deaths than underweight. World Health Organisation (WHO) has reported (in 2016) that more than 1.9 billion adults (age $\geq$ 18) were classified as overweight of whom 650 million were obese. Obesity is associated with several health problems summarized as metabolic syndrome, such as hypertension (high blood pressure), hyperglycemia (high blood glucose) and hyperlipidemia (high levels of lipids), increasing the risk of developing metabolic diseases, such as, type 2 diabetes (T2D) and cardiovascular diseases (Lavie et al., 2009).

Unlike other organs, adipose tissue (AT) depots are distributed throughout the body in a variety of locations, which vary in function as well as morphology (Cinti, 2012; Ibrahim, 2010; Trujillo and Scherer, 2006). There are two main types of AT, brown adipose tissue (BAT) and white adipose tissue (WAT). BAT has an ability to transfer energy from food into heat and plays an essential role in regulation of temperature in the body through non-shivering thermogenesis (an increase in metabolic heat production). The brown adipocyte contains several small lipid droplets and is rich in number of mitochondria, which exclusively express uncoupling protein 1 (Ucp1), mediating energy expenditure and heat production (Cannon and Nedergaard, 2004).

WAT is an organ that functions as a reservoir for energy-storage (in form of triacylglycerols), with important roles in the control of energy homeostasis and modulation of lipid metabolism. In human, WAT can be divided into two main depots; the visceral AT (surrounding the internal organs in abdominal cavity) and the subcutaneous AT (situated under the skin). The WAT in rodent is similar to human, but have some dissimilarities (figures 2A and 2B). For instance, the visceral gonadal WAT (GWAT) is located in the perigonadal region in rodent, but is absent in human (Chusyd et al., 2016). The white adipocyte contain only one large lipid droplet, which

covers approximately 95% of the total cell volume, pushing the nucleus and cytosolic components to the edge of the cell plasma membrane (figure 1). Due to the high lipid-storage properties, the white adipocytes are very heterogeneous in size and can vary between 25 to 200 µm in diameter (Meyer et al., 2013). The enlargement of AT can either be as a result of hypertrophy (adipocyte size increase due to increased lipid accumulation) or hyperplasia (cell number increase due to differentiation of preadipocytes to mature adipocytes; Jo et al., 2009).

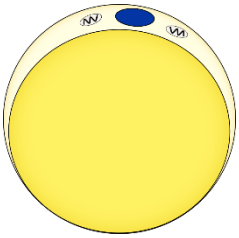


Figure 1| The white adipocyte (*Created in Affinity Designer).*

This unique organ consists of not only lipid-filled adipocytes, but is highly vascularized and contains also of a heterogeneous collection of other cells known as stromal vascular fraction (SVF). SVF includes, for instance, preadipocytes, endothelial cells, macrophages, pericytes, monocytes as well as other cell types, which jointly plays an important role in maintaining the AT homeostasis (Lee et al., 2013).

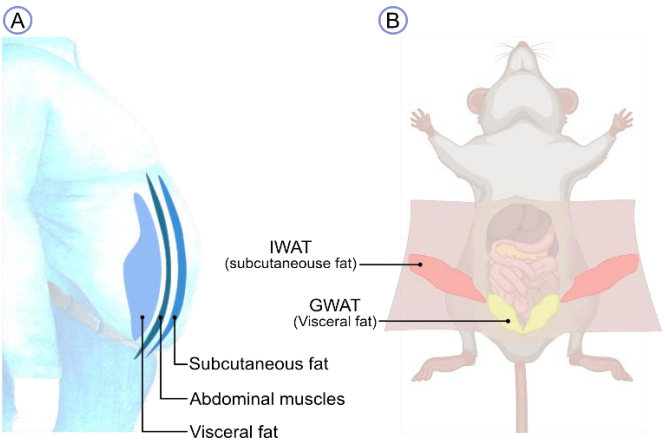


Figure 2| WAT depots **(A)** in human, and **(B)** in rodent. Inguinal WAT tissue (IWAT; subcutaneous fat under the skin) and GWAT (visceral fat surrounding the organs; *Created with BioRender.com and Affinity Designer).*

WAT is not only a reservoir for energy-storage, but has over the last decades been shown to also function as an active endocrine organ with the ability to secrete several metabolically active molecules, known as *adipokines*, into the blood circulation (Kershaw and Flier, 2004; Trujillo and Scherer, 2006). These signaling molecules have important effects and impact both locally (autocrine and paracrine) and systematically

(endocrine) throughout the body. Among the numerous secreted adipokines, *adiponectin* has gained considerable attention due to its beneficial metabolic properties.

Adiponectin was discovered in 1995 by Scherer and colleagues' as a novel protein hormone highly specific to fully differentiated adipocytes in culture (Scherer et al., 1995). Additionally, adiponectin started getting recognized for its insulin sensitizing (Hotta et al., 2000), fat-burning (Yamauchi et al., 2002), anti-inflammatory (Ouchi et al., 2003) and anti-oxidant (Fruebis et al., 2001) properties. Adiponectin plays an important roles in the regulation of glucose homeostasis, by reducing glucose production (gluconeogenesis) by the liver (Combs et al., 2001) and enhancing lipid metabolism by fatty acid oxidation in skeletal muscle (Fruebis et al., 2001). Moreover, adiponectin appears to have a protective role, reducing the risk of developing obesity-associated metabolic disease. Numerous studies have shown decreased levels of circulating adiponectin in relation to obesity, T2D, and cardiovascular disease (Arita et al., 1999; Cnop et al., 2003; Hotta et al., 2000; Spranger et al., 2003; Weyer et al., 2001; Yamauchi et al., 2001).

Adiponectin is a highly abundant plasma protein (circulates at concentrations of μg/ml) and amounts to approximately 0.01% of total plasma protein. This can be compared to other secretory hormones such as insulin and leptin with concentrations in the range of ng/ml in human (Arita et al., 1999; Pajvani et al., 2003). Adiponectin is an adipocyte-secreted hormone of 30 kDa, containing 244-247 amino acid long polypeptide represented as a full-length adiponectin. A full-length adiponectin consists of four different regions (figure 3A): a signaling peptide domain at the N-terminal, a non-conserved variable region, followed by a collagenous domain and lastly, a globular domain at the C-terminal (Scherer et al., 1995). Adiponectin is present in the circulation as different molecular forms (figure 3B); low molecular weight (LMW) trimer, middle molecular weight (MMW) hexamer and high molecular weight (HMW) multimer (consists of 12-18 hexamers forming a bouquet-like structure), and are linked together with covalent disulfide bonds (Pajvani et al., 2003; Scherer et al., 1995; Tsao et al., 2003; Wang et al., 2006). It has been proposed that HMW adiponectin is the

most biologically active form of adiponectin, and that specifically this higher order form of adiponectin is decreased in obese and T2D individuals (Hara et al., 2006) and is tightly connected to insulin sensitivity (Pajvani et al., 2004).

Full-length adiponectin undergoes several post-translational modifications within the white adipocyte endoplasmic reticulum (ER), where it is synthesized, folded and thereafter passed to the Golgi complex before being released into the blood circulation (Wang et al., 2002). The disulfide bond formation occurs in the ER through oxidation, and the assembly of higher molecular forms of adiponectin is tightly regulated and dependent on the ER chaperones; Endoplasmic reticulum resident protein 44 (ERp44) and ER membrane-associated oxidoreductase-1-Lα (Ero1-Lα; Hampe et al., 2015a; Wang et al., 2007). Disulfide bond A oxidoreductase-like protein (DsbA-L), which is another ER chaperon protein, has also been shown to be involved in the regulation of adiponectin multimerisation of the higher molecular form of adiponectin, due to its ability to form disulfide bonds (Liu et al., 2008; Qiang et al., 2007; Wang et al., 2007). Once the different molecular forms of adiponectin are secreted to the blood circulation, the forms stay stable and do not interconvert (Pajvani et al., 2004; Schraw et al., 2008).

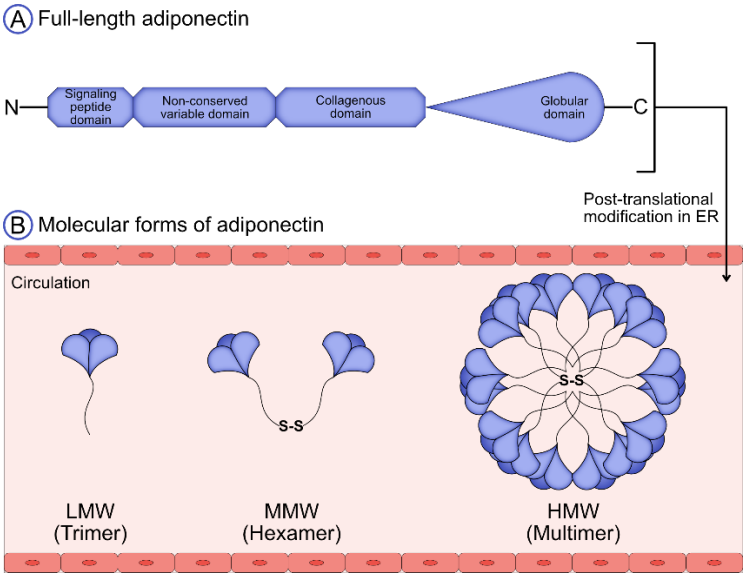
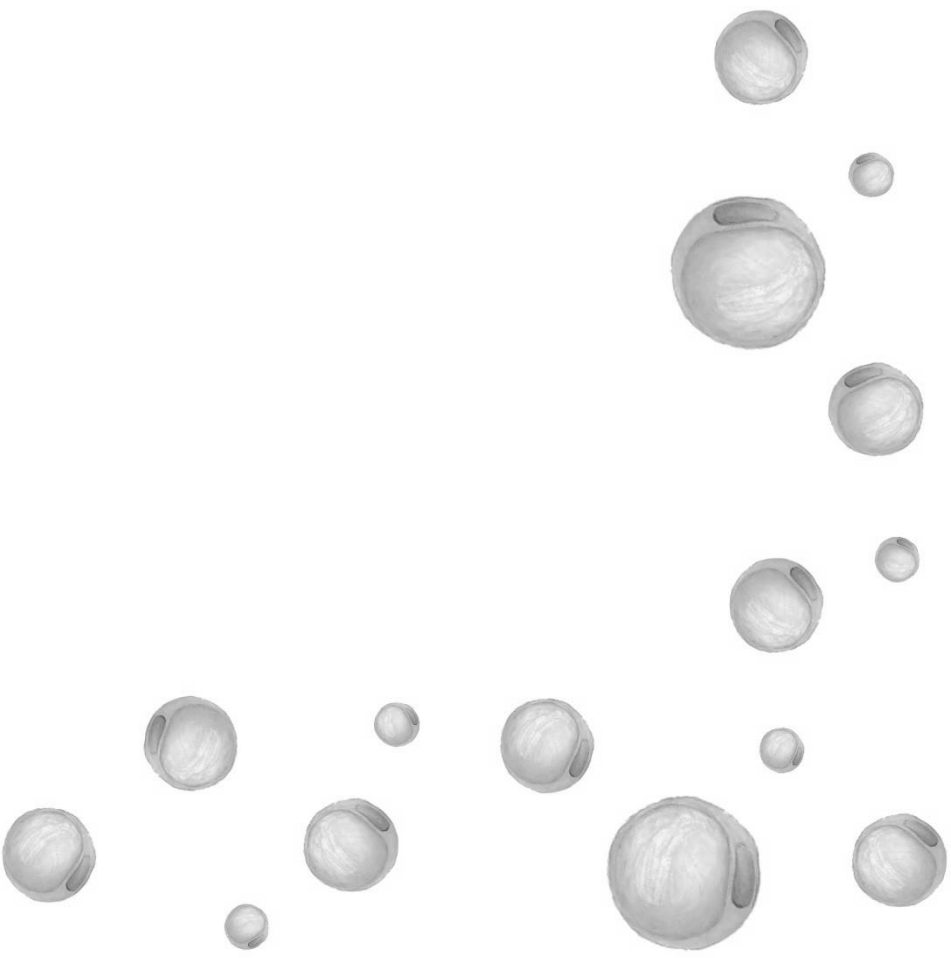


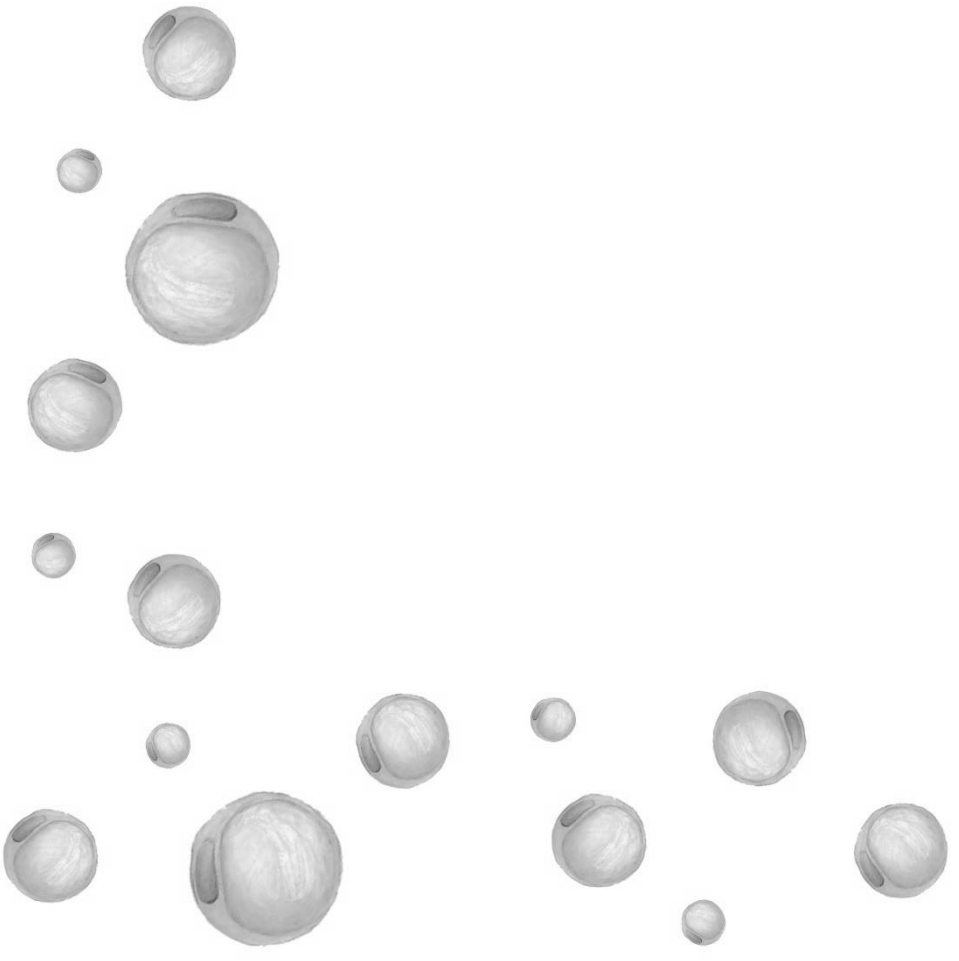
Figure 3| *A:* The full-length adiponectin, containing four different regions, undergoes post-translational modifications in ER into different molecular forms of adiponectin before being released into the blood circulation. ***B:*** The different forms of adiponectin (LWM, MMW and HMW), linked together with covalent disulfide bonds in the blood circulation (*created in Affinity Designer*).

As mentioned above, adiponectin has beneficial effects on the metabolic health, and can improve insulin sensitivity and the regulation of glucose and lipid metabolism. Adiponectin mediates its endocrine effects through two identified seven transmembrane receptors; adiponectin receptor 1 and 2 (AdipoR1 respective AdipoR2). These two receptors, structurally and functionally differ distinct from the normal G-protein coupled receptors (GPCR), since the N-terminal is faced intracellular and the C-terminal faced extracellular, which is the opposite of the classic GPCRs. AdipoR1 is mainly expressed in skeletal muscle, where it activates/simulates fatty acid oxidation, and has higher affinity for the globular domain of adiponectin. AdipoR2 is mainly present in the liver, where it together with insulin inhibits hepatic glucose production, and display a high affinity for the full-length adiponectin (Berg et al., 2001; Yamauchi et al., 2003). The two adiponectin receptors are also distributed in several other tissues, such as AT, pancreas, and heart. Therefore, adiponectin has extensive physiological effects throughout the body (Ding et al., 2007; Kharroubi et al., 2003; Rasmussen et al., 2006). T-cadherin is a third adiponectin receptor that can be found in endothelial and smooth muscle cells, playing an important role in cell growth and vasculature development. T-cadherin has been suggested to bind to the higher order forms of adiponectin and not the smaller forms of adiponectin (Hug et al., 2004).

In the first work by Scherer et. al, it was suggested that adiponectin is secreted via regulated exocytosis (Scherer et al., 1995). In other words, adiponectin, like several other peptide and protein hormones, is released when vesicles containing adiponectin fuse with the plasma membrane in response to a triggering signal. Almost two decades later, the Olofsson laboratory demonstrated that adiponectin exocytosis is triggered by an intracellular elevation of cyclic adenosine monophosphate (cAMP) and activation of *E*xchange *P*rotein directly *A*ctivated by *c*AMP (Epac) in a protein kinase A (PKA)- and calcium (Ca^{2+})-independent manner. However, a combination of elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) and adenosine triphosphate (ATP) is necessary for recruiting new readily releasable adiponectin vesicles from the functional reserve pool to the releasable pool, and thus to maintain adiponectin secretion over longer time periods (Komai et al., 2014).

Later, the physiological regulation of white adipocyte adiponectin release and how this regulation is disturbed in obesity and T2D was further investigated (Komai&Musovic. et al., 2016). The study demonstrated that adiponectin exocytosis is stimulated via adrenergic signaling pathway, chiefly involving the beta 3 adrenergic receptor (β_3AR). Interestingly, adiponectin secretion could not be stimulated by neither catecholamine or by a highly selective β_3AR agonist (CL; CL316,243) in inguinal (Komai&Musovic. et al., 2016) or gonodal (Musovic and Olofsson, 2019) adipocytes isolated from obese and T2D mice. Remarkably, HMW adiponectin was markedly decreased and the protein level of β_3AR and Epac isoform 1 (Epac1) was ~30% lower in the obese and diabetic mice, compared to lean mice (Komai&Musovic. et al., 2016). Collectively, the findings suggest that adiponectin release is stimulated via an adrenergic signaling pathway involving β_3AR, and that this receptor plays a key role for secretion and circulating levels of the higher molecular form of adiponectin.





AIM

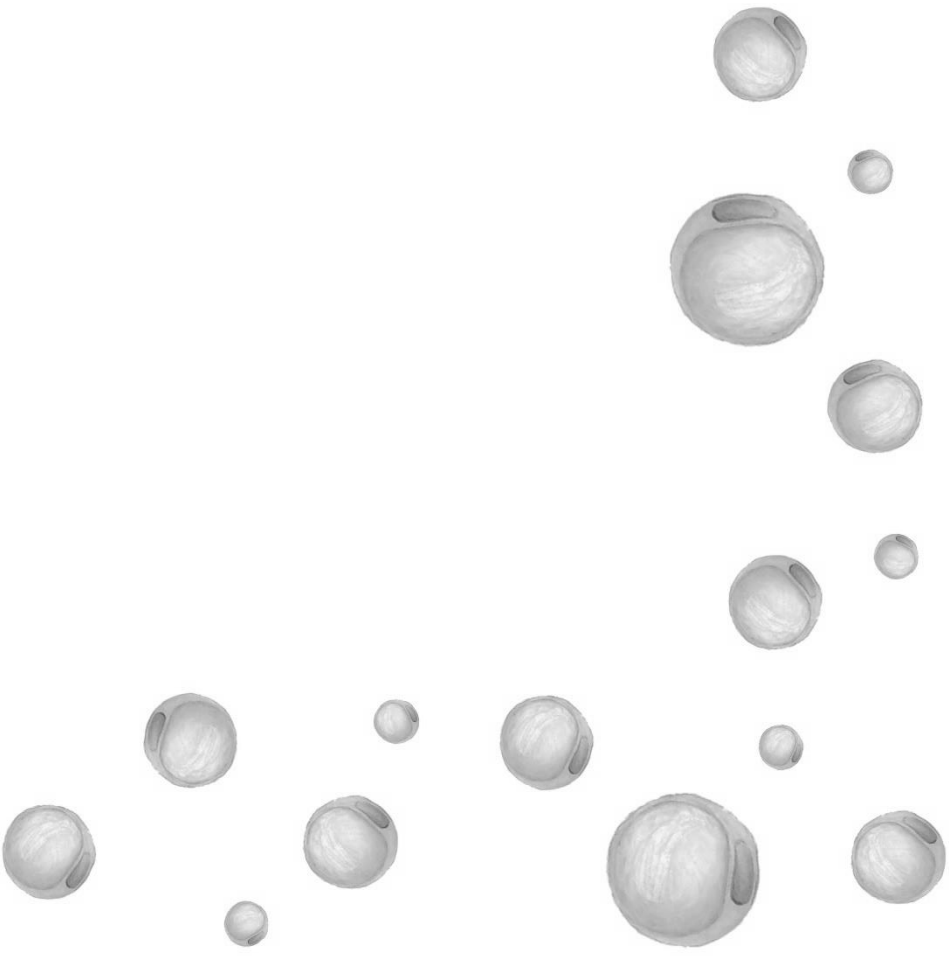
The overall goal of this thesis was to define the physiological regulation and underlying mechanism of white adipocyte adiponectin exocytosis. The specific aims were to:

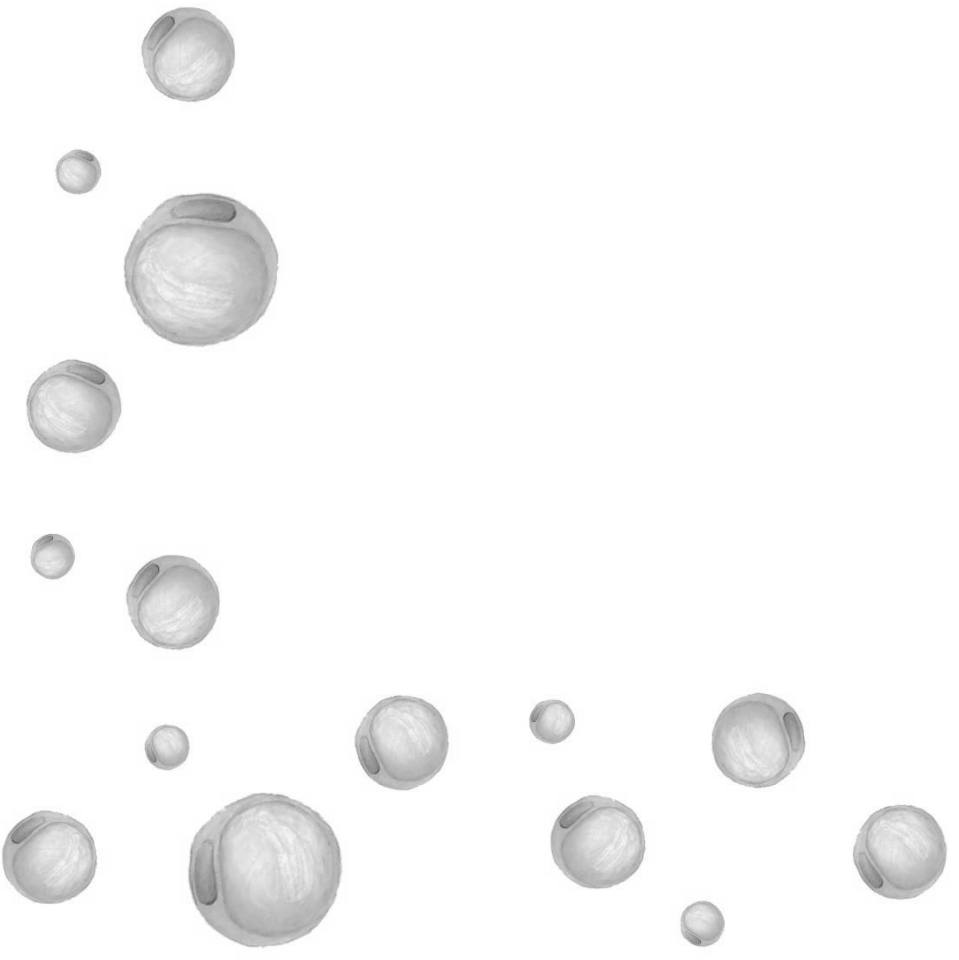
Specific aims

I. Define the role of sympathetic innervation and purinergic signaling in the regulation of white adipocyte adiponectin exocytosis/secretion.

II. Detail the role of β_3AR in white adipocyte adiponectin exocytosis/secretion and in metabolic health using mice that are genetically ablated for β_3AR.

III. Determine the role of β_3AR specifically for secretion/exocytosis of HMW adiponectin, in metabolic health and in obesity-associated disease.





METHODS

All animal studies were approved by the Regional Ethical Review Board at the University of Gothenburg. Human adipocytes were obtained from subcutaneous WAT biopsies from surgery under general anesthesia at Sahlgrenska University Hospital, Gothenburg, Sweden. The procedures were approved by the Regional Ethical Review Board, University of Gothenburg and were carried out in compliance with the Declaration of Helsinki. Only a short and simplified description of the main methods will be mentioned in this method section. The reader is referred to paper I-III for more detailed description of materials and methods used for each paper.

MOUSE MODELS

Male C57BL/6J (paper I and III), Friend leukemia virus B (FVB) wild type (WT) and FVB β_3AR knockout (β_3AR-KO) mice (paper II) were used and housed in a 12 hours light/dark cycle (21-22°C) with unlimited access to food and water if nothing else is stated. Mice in paper II were bred and maintained under standard housing conditions, and routinely genotyped at the animal facility, to produce homozygote β_3AR-KO and WT littermates.

CELL CULTURE OF 3T3-L1 ADIPOCYTES

Cultured clonal 3T3-L1 preadipocytes are a mouse embryonic fibroblast cell line that can be differentiated into mature adipocytes and used for experiments at day 8-9. The cells are commonly used in studies of adipocyte biology and mechanism *in vitro*. The fibroblast-like 3T3-L1 preadipocytes were seeded and proliferated in high glucose medium containing 10% New Born Calf Serum (NBCS) and 1% penicillin-streptomycin (P/S) in a T75 flask, and incubated in a suitable environment of 5% CO_2 at 37°C. Subsequently, when cells reached a confluency of ~70% within 2-3 days, cells were removed using trypsin and seeded into either new flasks, plates or petri dishes of desire. Before initiating the differentiation, cells need to proliferate into ~80-90% confluence to be able to obtain good differentiation into adipocytes. Differentiation (day 0) was initiated by an addition of a "cocktail" mixture containing 3-Isobutyl-1-methylxanthine (IBMX, 0.5 mM), dexamethasone (Dexa, 1 μM) and

insulin (850 nM) mixed in a high glucose medium (differentiation medium) containing 10% Fetal Bovine Serum (FBS) and 1% P/S. This "cocktail" is essential to stop the proliferation and initiate the transition to mature adipocyte. After 2 days, medium was replaced with fresh differentiation medium containing only insulin (day 2) to help the adipocyte to accumulate more lipid droplets through glucose uptake and triglyceride synthesis. The medium was thereafter replaced with only fresh medium every second day up until the differentiated cells were used for experiments (figure 4).

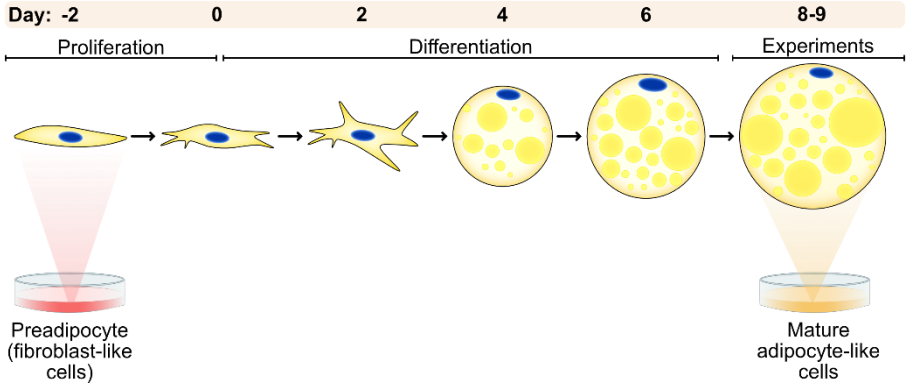


Figure 4| Differentiation of 3T3-L1 fibroblast-like preadipocyte into mature adipocyte (*Created in Affinity Designer*)**.**

ISOLATION OF PRIMARY WHITE ADIPOCYTES

Mouse primary white adipocytes (figure 5) were isolated from different adipose tissue depots (IWAT and GWAT). Human adipocytes were isolated from obtained subcutaneous WAT biopsies. WAT depots were collected and minced, followed by incubation in collagenase type II (1 mg/mL) for 45-60 min at 37°C under gentle shaking conditions in order to activate the enzyme. The suspension was poured through a nylon mesh filter into a tube to ensure that non-degraded tissue pieces and lymph nodes remains in the filter. The adipocytes were allowed to float up to the top while the SVF was removed with a syringe. Isolated mouse adipocytes were then washed with buffer, and thereafter either used directly for adipocyte hormone secretion experiments or stored at -80°C for future investigations. Human adipocytes isolation was performed as described in (Komai et al., 2014), thereafter incubated overnight in 5% CO_2 at 37°C before starting with adipocyte hormone secretion the day after.

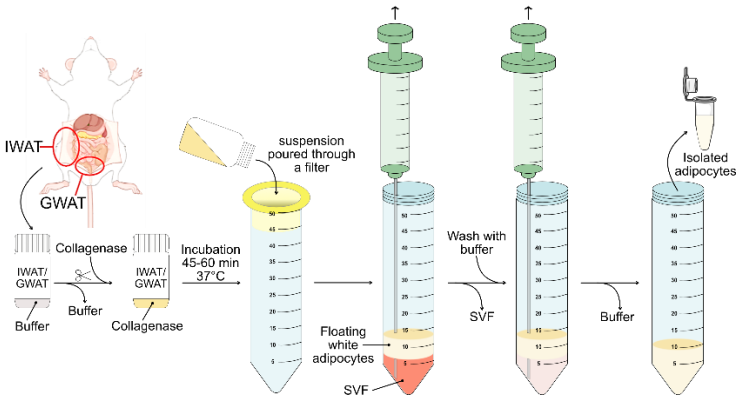


Figure 5| An illustration of the procedure for isolation of primary mouse adipocytes (*created in Affinity Designer*)**.**

MEASUREMENTS OF WHITE ADIPOCYTE ADIPONECTIN SECRETION

To investigate short-term adipocyte adiponectin secretion, differentiated 3T3-L1 adipocytes as well as primary isolated adipocytes (10-15% volume/volume; figure 6) were incubated with an extracellular solution containing 5 mM glucose, together with test substances of interest for 15-60 min at 32°C under gentle shaking condition. At termination of incubation of 3T3-L1 adipocytes, the medium (containing secreted product) and the adipocytes attached at the bottom of each well was collected and further investigated. At termination of isolated primary adipocytes, cells were separated from media by centrifuging through small tubes filled with diisononyl phthalate oil and instantly snap frozen in dry ice. The oil-tubes were cut at two different points to separate adipocytes from media and removing the middle layer containing the oil. Cells were lysed with a cell lysis buffer and thereafter stored at -80°C for future investigation.

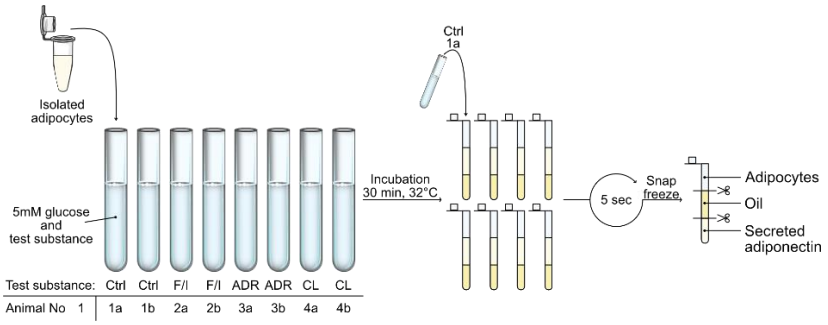


Figure 6| The procedure for stimulation of adipocyte adiponectin secretion in isolated primary adipocytes in paper II (*created in Affinity Designer*).

IN VIVO EXPERIMENTAL DESIGN

Paper II

To be able to understand and investigate the role of β_3AR in obesity and metabolic disturbance as well as functionality of the white adipocyte and adiponectin secretion, mice were kept on a chow or high fat diet (HFD; 60% kcal from fat) for 8 weeks. The metabolic status of mice were observed by oral glucose tolerance test (OGTT) before and after 8 weeks of diet conditions, to investigate the insulin release and how fast exogenous glucose was cleared from the circulation (figure 7). Mice (9-11 weeks old) were fasted for 4 hours and weighed before initiating the OGTT. Blood glucose levels were measured at time point 0 (fasting glucose) before glucose solution (2.5 g/kg of body weight) was administered orally through a gavage tube. Thereafter, blood glucose levels were measured at 15, 30, 60 and 120 min, and mice were afterwards left for 1 week of recovery. Subsequently, mice were randomly divided into groups.

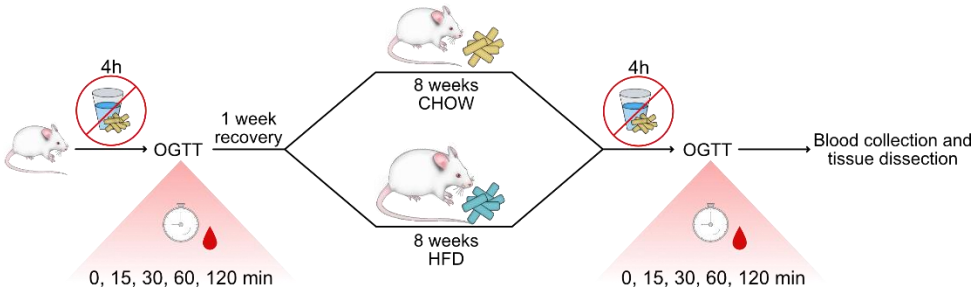


Figure 7| The experimental setup of the in vivo experimental setup in paper II (*created in Affinity Designer*).

Paper III

To determine the effect of β_3AR agonist on the regulation of circulating adiponectin and under catecholamine resistance condition, intraperitoneal (i.p) injection was performed. Before initiating with the treatment and to avoid induction of an interfering stress response that could affect the experiment, mice were habituated during one-two weeks. This was necessary to do because the body releases catecholamines in response to stress, which can trigger adiponectin release through adrenergic stimulation.

2 days treatment with CL:

Habituated mice (10-20 weeks old) were weighed and afterwards injected with saline or CL (1 mg/kg) daily. Fasting blood glucose was measure and blood samples collected at day 0, prior to treatment, and on day 2 (figure 8A).

2 weeks treatment with CL:

Habituated mice (5 weeks old) were put on a chow or HFD for 8 weeks. After diet challenge, mice were weighed and injected daily with saline or CL (1 mg/kg) at 8 am. The mice were fasted for 4 hours before blood glucose and blood sample collection every second day. Mice remained on their respective diet throughout the treatment and until the day of termination (figure 8B).

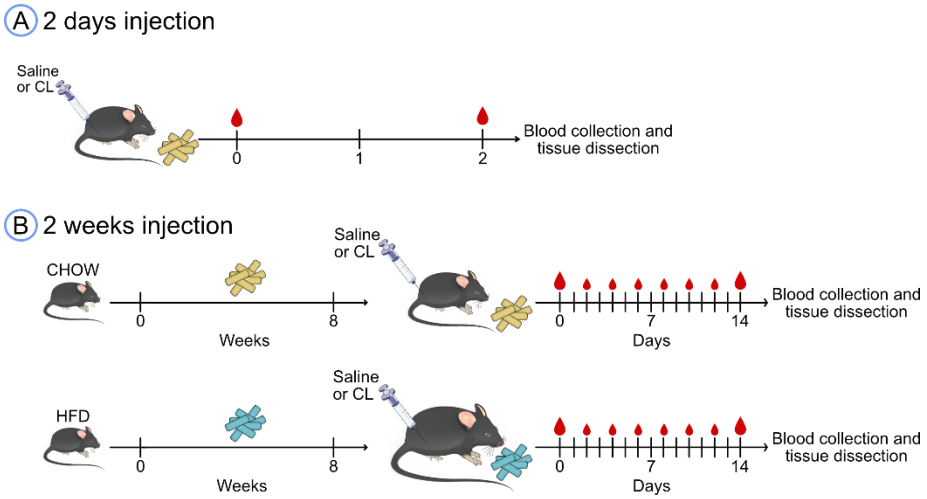


Figure 8| The experimental setups of the treatment with CL for ***A:*** 2 days and ***B:*** 2 weeks (*created in Affinity Designer*).

CAPACITANCE MEASUREMENT

Regulated exocytosis is an essential biological process in the cell, and controls the release of secretory components from cells to the surrounding environment. Regulated exocytosis can be measured as membrane capacitance change over time using the patch-clamp technique, since the increase in membrane capacitance occurs when vesicles fuse with the plasma membrane and the cell surface area increases. The membrane capacitance can be calculated from the equation $C_m = A \times \mathcal{E}/d$,

where C_m (membrane capacitance) is proportional to A (plasma membrane area). $\mathcal{E}$ (specific membrane capacitance) and d (thickness of plasma membrane) is constant. The patch-clamp technique can measure the electrical properties with the help of an electrode connected to an amplifier, either over a small part of the membrane or over the entire cell membrane (the whole-cell configuration). In order to attach the glass pipette to the cell, a gentle suction need to be applied to the cell membrane surface to form a tight so called a "giga-ohm seal. To subsequently gain full access to the inside of the cell, gentle suction is again applied to rupture the enclosed membrane and allows the pipette solution containing substance of interest to wash into the patched cell (figure 9). In paper I and III, the increase in cell capacitance of differentiated 3T3-L1 single adipocytes were measured using this so called *standard whole-cell configuration of the patch-clamp technique*. This configuration allows us to control the intracellular environment with the pipette filling solution. Thus, intracellular levels of mediators that are important for adiponectin vesicle exocytosis, such as Ca^{2+}, cAMP and ATP, can be controlled within the recorded cell.

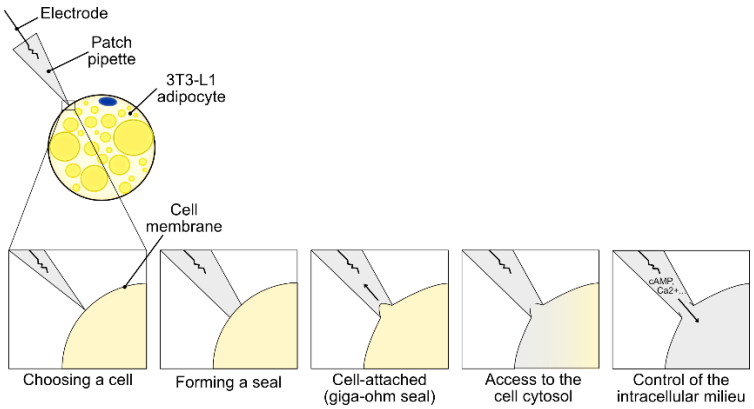


Figure 9| A simplified illustration of a standard whole-cell patch-clamp technique (*created in Affinity Designer*).

TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY

Although, membrane capacitance recording provide detailed information about vesicle exocytosis in live single cell, it does not yield information about what is actually secreted. To gain this information, the membrane capacitance patch-clamp recording need to be frequently compared to biochemical measurements of hormone secreted from cells that have been exposed to similar condition and incubation time as in

capacitance recordings. It is certainly desirable to be able to investigate the adiponectin exocytosis in detail in real-time. To do this, in paper III, total internal reflection fluorescence (TIRF) microscopy was applied. TIRF visualizes the process of adipocyte vesicle dynamic and exocytosis in a high temporal and spatial resolution, allowing us to observe cell events that occur near the plasma membrane within ~100-200 nm in the "TIRF zone" (evanescent field). In order to investigate adiponectin vesicle exocytosis, we used 3T3-L1 adipocytes that stably express mCherry fused to human adiponectin (adiponectin-mCherry). In these cells, adiponectin vesicles are visualised by TIRF as a punctate pattern of fluorescence. When the correct extracellular stimulation is added to the cell dish containing the fluorescently labelled adipocytes, some adiponectin containing vesicles are released (visible as the rapid disappearance of fluorescence puncta; *black dots*) whereas other vesicles stay stable (*red dots*). Once stimulated and the fluorescence puncta disappears, can that be interpreted as an exocytosis event (figure 8).

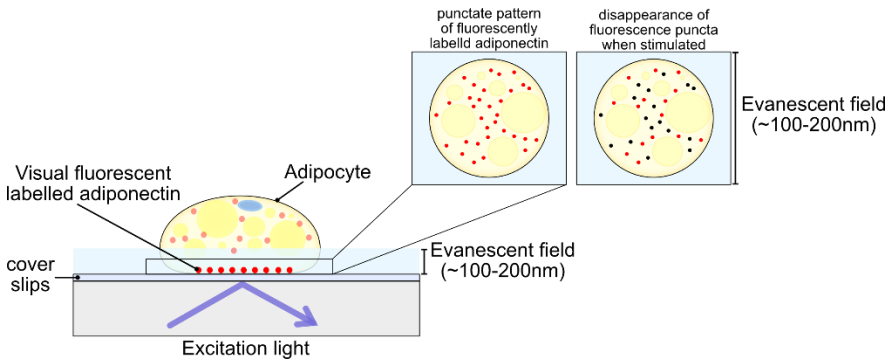
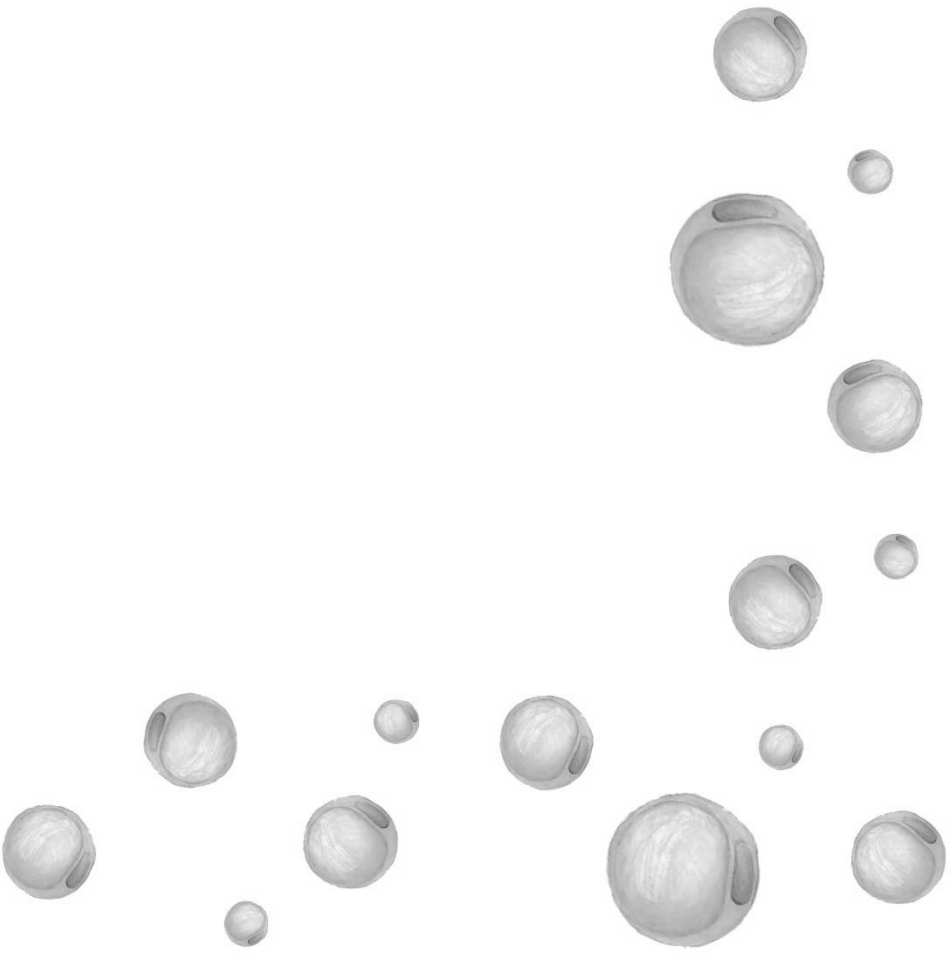
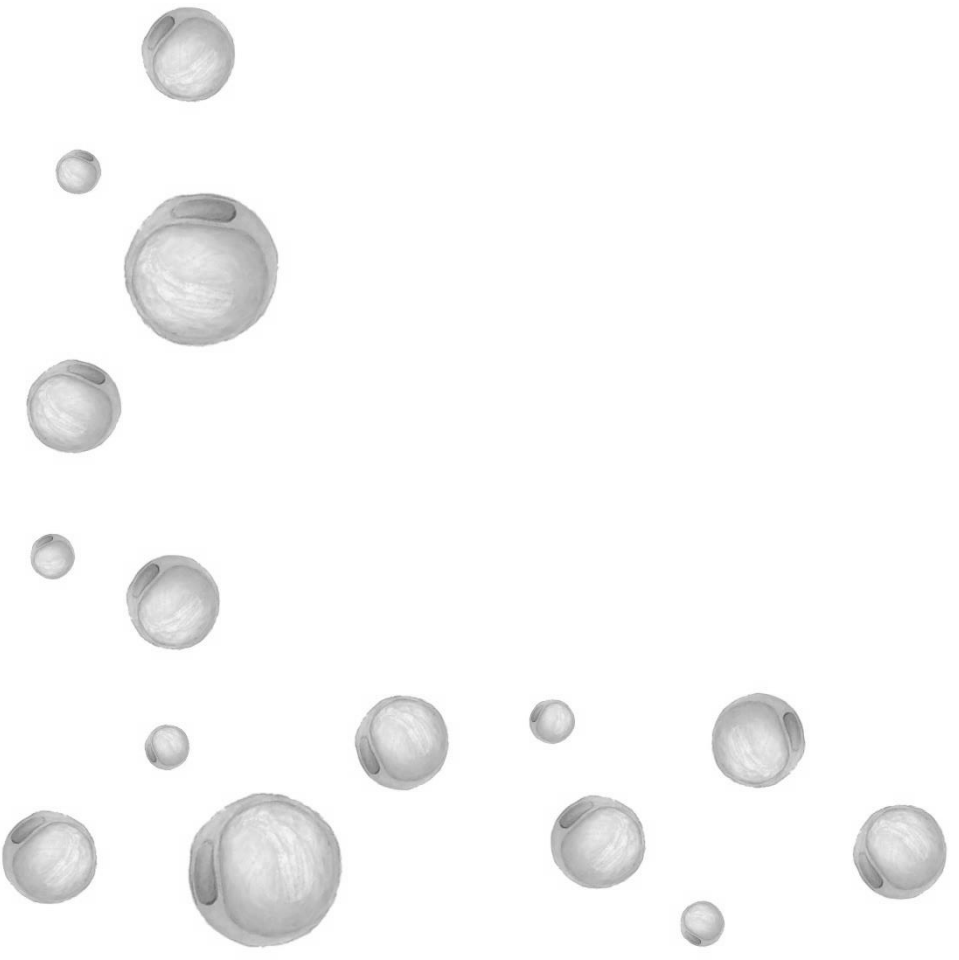


Figure 8| A simplified illustration of the TIRF technique (*created in Affinity Designer*).

DATA ANALYSIS

All data in this thesis are represented as mean values ± Standard Error of the Mean (SEM), expressed as either in absolute concentrations or in fold change over basal (unstimulated condition). Statistical significance was calculated with GraphPad Prism 8. Student's t-test (unpaired or paired as appropriate) was used to determine significance between two experimental groups. One- or two-way analysis of variance (ANOVA) was applied to determine statistical significance between two or more independent groups and/or conditions.





BACK TO HOW IT ALL STARTED

Normal cellular function, cell-cell communication and interaction is essential for functional mammalian multicellular systems. Due to the size and complexity of protein molecules, they can not be transported via normal diffusion mechanisms over the plasma membrane and need therefore some other way to be released. One way of doing that is through *regulated exocytosis*, the process in which secretory vesicles fuse with the plasma membrane in response to a triggering stimuli. Secretory vesicles are typically divided into two functional pools; the *reserve pool* (immature vesicles) and the *readily releasable pool* (mature and release-ready vesicles; Denker and Rizzoli, 2010). Vesicles that are to be released via regulated exocytosis is transported from the *trans* Golgi and accumulated in the cytoplasm as readily releasable vesicles close to the plasma membrane, and undergo fusion with the plasma membrane in response to the correct stimulatory signal. When the readily releasable pool of vesicles are depleted, vesicles from the reserve pool need to be chemically modified into mature vesicle and sometimes be physically translocated to the plasma membrane before being released (Burgoyne and Morgan, 2003).

Regulated exocytosis is an essential physiological process in secretory cells such as neuroendocrine and endocrine cells and this process is determined by many factors (Kits and Mansvelder, 2000). The regulated exocytosis of hormone- or neuropeptide-containing vesicles are, in the majority of cases, triggered by an elevation of $[Ca^{2+}]_i$ due to an influx through membrane-bound calcium channels and/or release of Ca^{2+} from intracellular stores. The membrane-bound Ca^{2+} channels are typically localized near the readily releasable vesicles, which upon activation increases the local $[Ca^{2+}]$ close to the vesicles leading to a rapid vesicle release (Barg et al., 2001; Becherer et al., 2003). Although, Ca^{2+} is the exocytosis trigger, other intracellular molecules such as cAMP and ATP are essential for vesicle exocytosis to occur (Parsons et al., 1995). The readily releasable pool of vesicles can be depleted and need to be refilled by vesicles from reserve pool. These vesicles need to undergo a series of maturation steps in order to become mature and discharge their cargo upon fusion with the plasma membrane. Besides the triggering vesicle fusion, Ca^{2+} helps with the physical

translocation and *docking* (stable attachment to the plasma membrane) of vesicles. In order to gain enough competence to release and refill the readily releasable pool, docked vesicles need to undergo *priming* (chemical modifications that make docked vesicles releasable), which involves Ca^{2+}-, ATP-, cAMP-dependent modifications (Becherer et al., 2003; Burgoyne and Morgan, 2003; Seino and Shibasaki, 2005). The regulated exocytosis of several hormones are rather well understood (Burgoyne and Morgan, 2003), but the cellular and molecular mechanisms and mediators that control white adipocyte adiponectin vesicle release has until recently remained unclear. However, during the last decade, work from our group has begun to elucidate the detailed control of white adipocyte adiponectin exocytosis.

The underlying mechanisms and mediators controlling adiponectin vesicle release

A number of studies have demonstrated the regulation of adiponectin secretion under long-term/chronic conditions (>4 hours to days), concerning the changes in gene expression, synthesis and release when stimulated by insulin (Blümer et al., 2008; Bogan and Lodish, 1999; Cong et al., 2007; Lim et al., 2015; Scherer et al., 1995; Xie et al., 2008). It has been reported that insulin can affect the gene expression of adiponectin by inducing it (Cong et al., 2007) or reducing it (Fasshauer et al., 2002). These dissimilarities can perhaps be explained by the use of different adipocyte sources (cultured or rodent adipocytes), the variety of AT depots or experimental conditions (the use of agonist or antagonist, incubation time and temperature). The long-term exposure to insulin has shown to induce adiponectin release via phosphoinositide 3 kinase- (PI3K) and phosphodiesterase 3B (PDE3B)-dependent pathway (Cong et al., 2007). The adiponectin secretion during short-term exposure to insulin (30-120 min) has been less studied. Few papers have shown that insulin increases adiponectin release in a PI3K-dependent manner, with no effects on gene expression nor protein synthesis (Bogan and Lodish, 1999; Lim et al., 2015). In the same works, they show that the highest adiponectin content was localized nearby the plasma membrane proposing that insulin may be involved in the release of already synthesized and modified adiponectin vesicles (Bogan and Lodish, 1999; Cong et al., 2007; Lim et al., 2015).

In 2014, our group published the first study that elucidated the intracellular mediators and mechanisms involved in regulation of short-term white adipocyte adiponectin exocytosis, by combining membrane capacitance patch-clamp recordings and biochemical measurements of adiponectin secretion in both cultured 3T3-L1 adipocyte and human adipocytes. The study showed that an elevation of cAMP triggers the release of readily releasable adiponectin-containing vesicles via activation of Epac (Komai et al., 2014). PKA was initially thought to be the primarily target of cAMP in cAMP-regulated exocytosis, but can as well activate Epac (Cheng et al., 2008; Holz et al., 2006). The Epac protein exists in two isoforms, Epac1 and Epac2, which vary in tissue distribution. Epac1 is present ubiquitously in all tissues, while Epac2 has a limited tissue distribution, predominantly present in the brain, pancreas and adrenal gland (de Rooij et al., 1998; Kawasaki et al., 1998). Although the PKA signaling pathway is well recognized in the regulation of lipolysis (breakdown of triglyceride into fatty acids and glycerol) in the white adipocyte, the Epac signaling in the white adipocyte is less studied.

Proposed model of the regulation of white adipocyte adiponectin exocytosis is shown in figure 9. The readily releasable pool of adiponectin-containing vesicle is refilled by new vesicles from the reserve pool in a Ca^{2+}-dependent manner. In the absence of Ca^{2+}, the readily releasable pool is depleted and exocytosis cannot last for longer time-periods, even when cAMP is elevated (reaching a plateau in the capacitance measurements; Komai et al., 2014). Additionally, a combination of Ca^{2+} and ATP augments adiponectin-containing vesicle release. Thus, it is clear that Ca^{2+} plays an important role for maintenance of

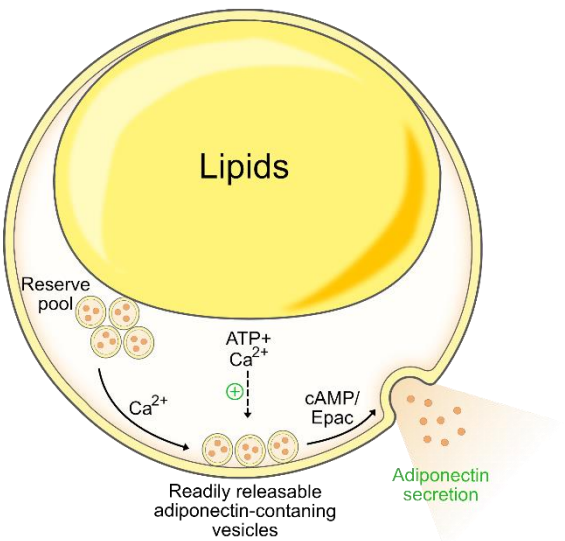


Figure 9| Proposed model of the regulation of white adipocyte exocytosis in a cAMP/Epac-dependent pathway including the involvement of cytosolic Ca^{2+} and ATP (*created in Affinity Designer*).

adiponectin exocytosis over prolonged time-periods, but how is $[Ca^{2+}]_i$ physiologically elevated in the white adipocytes?

ATP is the main intracellular source of energy for most cells, but has also important role as an extracellular signaling molecule. Extracellular ATP acts on plasma membrane-located purinergic receptors, such as P2Y (metabotropic GPCR) and P2X (ionotropic ligand-gated ion channel receptors), resulting in different downstream intracellular signaling cascades depending on which receptor is stimulated (Burnstock, 2007). Investigations have shown that extracellular ATP elevates cytosolic Ca^{2+} via phospholipase C (PLC) followed by an activation of the second messenger inositol trisphosphate (IP3) and diacylglycerol (DAG), by binding to P2Y2 receptor (P2Y2R). P2Y2Rs are abundantly expressed in differentiated adipocytes, and has been reported to regulate adipogenesis and lipid metabolism (El Hachmane et al., 2018; Lee et al., 2005; Taylor and Tovey, 2010; Tozzi and Novak, 2017). It is proposed that the sympathetic nerve system (SNS) has an impact on AT physiology through actions on for instance lipolysis, by mediating the lipid metabolism via direct innervation of sympathetic postganglionic nerves, co-releasing ATP and NA (Bartness et al., 2010; Youngstrom and Bartness, 1995) and mediates part of its affect via P2Y2R. The SNS may therefore be a key regulator of Ca^{2+}-dependent processes, such as adiponectin vesicle exocytosis in the white adipocyte. Hence, we aimed to define the role of sympathetic innervation and purinergic signaling in the regulation of white adipocyte adiponectin secretion (paper I).

The important role of catecholamines for the regulation of adiponectin exocytosis

As already mentioned, previous study from our group have demonstrated that adrenergic signaling is involved in the control of white adipocyte adiponectin release. Catecholamines such as NA and adrenaline (ADR), binds to adrenergic receptors (AR) alfa (α) and beta (β) at the plasma membrane resulting in activation of intracellular signaling cascades. The α_1AR elevates cytosolic Ca^{2+} and $\beta_{1\text{-}3}$AR elevates cAMP upon activation, while α_2AR decreases cAMP production. To determine which receptor subtype influences adiponectin release, our group investigated the effect of ADR and CL on adiponectin release using a combination of electrophysiological and

biochemical measurements. The results showed that white adipocyte adiponectin exocytosis is stimulated via β_3AR and downstream activation of Epac1 and that this adrenergic signaling pathway was disrupted in IWAT adipocytes isolated from obese and diabetic mice. Interestingly, further investigation showed that obese and diabetic mice had reduced gene and protein expression of β_3AR and Epac1, in a state we defined as *catecholamine resistance* (figure 10). The disturbance was associated with blunted adiponectin secretion, and a ~50% reduction of HMW adiponectin concentrations in serum of obese and diabetic mice (Komai&Musovic. et al., 2016). This proposes a central role of β_3AR for secretion and circulating levels of specifically the higher molecular form of adiponectin. In order to better understand the connection between β_3AR and adiponectin, in paper II we investigate the role of β_3AR in white adipocyte adiponectin release, circulating level and whole-body metabolic health using mice that are genetically ablated for β_3ARs. In paper III we next determine the role of β_3AR specifically for release of HMW adiponectin in both metabolic health and in obesity-associated metabolic disease.

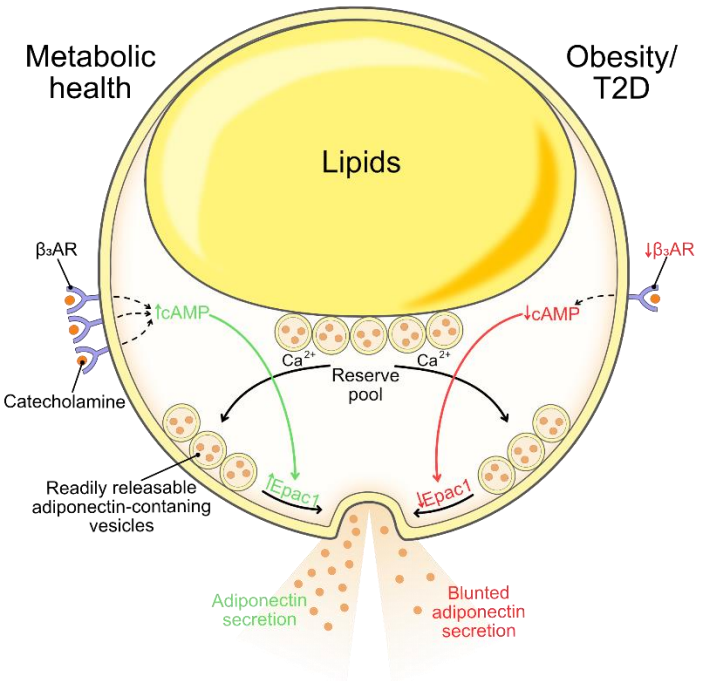
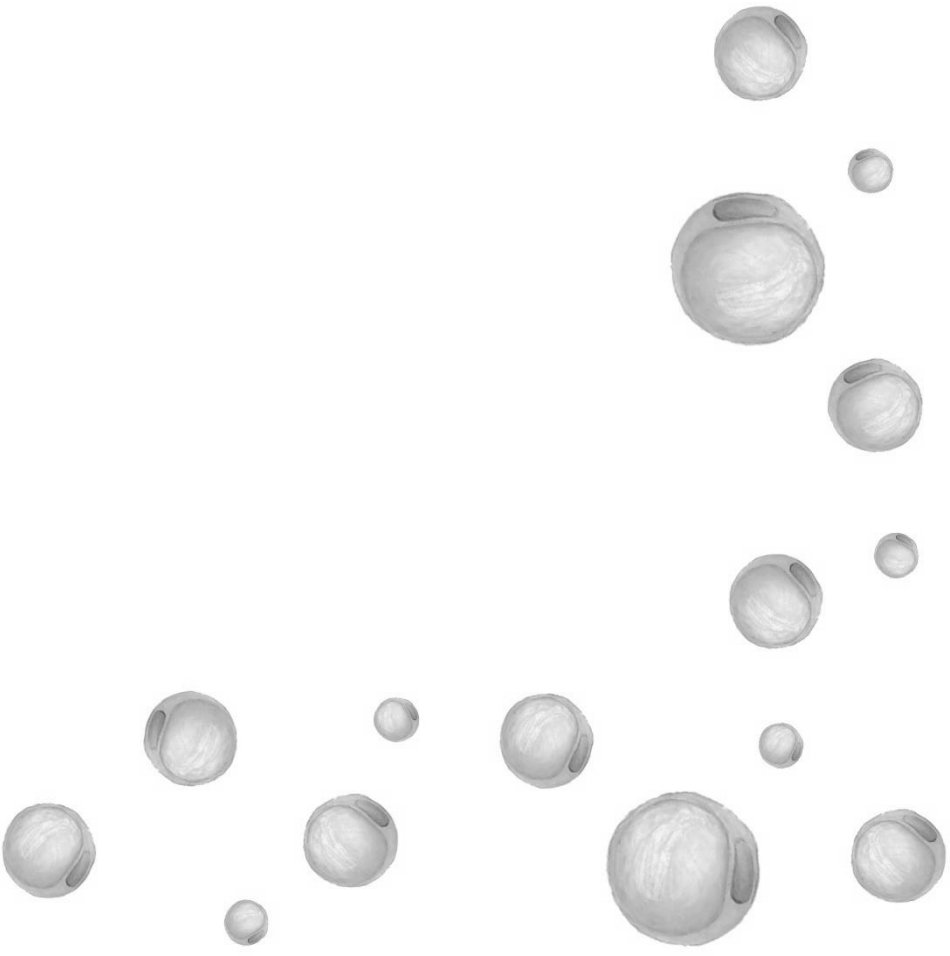
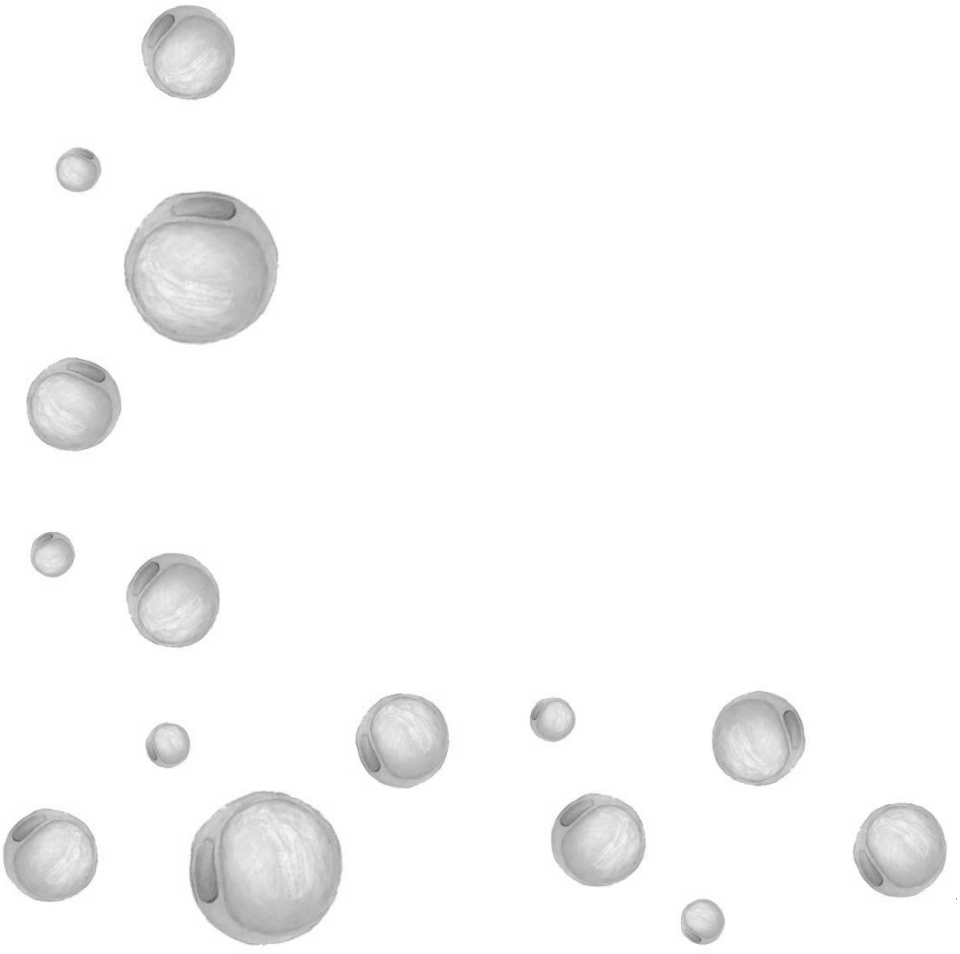


Figure 10| Graphical model of the regulation of white adipocyte adiponectin exocytosis in metabolically healthy and obese/T2D condition (*created in Affinity Designer*)**.**





WHERE ARE WE NOW?

The role of sympathetic innervation and ATP signaling in white adipocyte adiponectin exocytosis (paper I)

The WAT is known to be richly innervated by the SNS (Bartness et al., 2010). It has been suggested that SNS is primarily involved in lipolysis by co-releasing NA and ATP through a direct innervation of sympathetic postganglionic nerves in AT (Bartness et al., 2014; Bartness et al., 2010; Burnstock, 2007; Duncan et al., 2007; Tozzi et al., 2020). Evidence has shown that NA has a higher affinity for the β_3AR compared to ADR (Lohse et al., 2003), and when it binds to the receptor at the plasma membrane, intracellular cAMP increases. ATP acts on P2Y2Rs that are located at the plasma membrane in white adipocytes, increasing cytosolic Ca^{2+} (Burnstock, 2014; El Hachmane et al., 2018; Kelly et al., 1989; Laplante et al., 2010; Lee et al., 2005). The fact that sympathetic nerves co-releases NA and ATP lead us to hypothesize that sympathetic innervation is central for the physiological regulation of adipocyte adiponectin release.

Extracellularly applied NA and ATP stimulates adiponectin release

In order to investigate the role of sympathetic innervation and ATP on white adipocyte adiponectin secretion, we first examined the effects of NA and extracellular ATP on white adipocyte adiponectin release in primary mouse IWAT adipocytes. The cells were incubated with different concentrations of ATP (0.5, 10 and 100 μM), either alone or in combination with NA (100 nM) for 30 min (figure 11 and *figure 2 in paper I*). As expected, and in agreement with previous results using ADR or CL (Komai&Musovic. et al., 2016), NA stimulated adiponectin release. Low concentrations of ATP (0.5 and 10 μM) alone or in a combination with NA did not affect nor augment the release of adiponectin. However, a high concentration of ATP (100 μM) stimulated adiponectin release compared to control. Adiponectin release in response to a combination of NA and ATP was significantly higher than that induced by ATP alone. Following experiments in this work was done using 100 μM concentration of ATP.

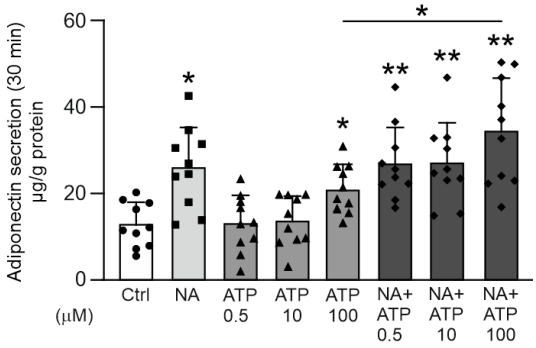


Figure 11| The effect of extracellularly applied NA and/or different concentrations of ATP on IWAT mouse adipocyte adiponectin release during 30 min. Results are expressed as µg/g protein vs. control (unstimulated condition) in each group. *P<0.05, **P<0.01, vs control.

To define the role of NA and ATP on adiponectin release at the single cell level, whole-cell patch-clamp capacitance measurement of exocytosis was performed using differentiated 3T3-L1 adipocytes. In agreement with previous results (Komai et al., 2014), infusion of matured 3T3-L1 adipocytes (day 8-9 of differentiation) with a non-stimulatory pipette-filled solution (lacking both cAMP and Ca^{2+}) did not affect the adipocyte exocytosis. However, the subsequent addition of extracellularly NA into the cell culture dish with adipocytes triggered adipocyte exocytosis; the stimulatory effect of NA was absent in adipocytes pretreated with the Epac inhibitor ESI-09 (10 µM) for 30 min (*figure 2B-C in paper I*). This finding is in agreement with previous work, confirming that adiponectin release is stimulated via cAMP/Epac1 signaling pathway (Komai&Musovic. et al., 2016).

Next, we investigated the involvement of Ca^{2+} in adipocyte adiponectin exocytosis stimulated by ATP. Differentiated 3T3-L1 adipocytes were incubated with ATP or NA for 30 and 60 min, with or without BAPTA-AM (membrane-permeable Ca^{2+} chelator). ATP stimulated adiponectin release ~2-fold over basal during 30 min exposure. The adiponectin release tended to still be elevated by ATP during 30 min (P=0.1), but was diminished in 60 min incubation in BAPTA-pretreated adipocytes (*figure 3A-C in paper I).* This suggests that an ATP-induced $[Ca^{2+}]_i$ elevation has an important role for adiponectin vesicle replenishment and adiponectin release over longer time periods.

Collectively, our results suggest that NA triggers white adipocyte adiponectin release via an elevation of cAMP, whereas ATP potentiates the cAMP-triggered adiponectin release by an increase of $[Ca^{2+}]_i$. However, ATP can as well stimulate adiponectin release in the absence of an accompanying elevation of cAMP.

Reduced HMW/total adiponectin is associated with low SNS innervation in obese/diabetic mice

To determine the importance of SNS innervation of WAT for adiponectin secretion in health and in obesity-associated metabolic disease, mice were put on either chow or HFD for 8 weeks. It has been proposed that the sympathetic sensitivity is reduced in WAT in obesity, suggesting that there is a dysregulation of production and secretion of AT secretory products, such as adiponectin (Reynisdottir et al., 1994). In order to understand how SNS innervation in WAT is affected by obesity, immunohistochemical staining of tyrosine hydroxylase (TH) was performed. TH is a marker used for staining of catecholamine-containing neurons and endocrine cells. The enzyme is mainly present in the cytosol, but also to some extent at the plasma membrane and is involved in catecholamine synthesis. Interestingly, we found that TH was significantly reduced in primary IWAT adipocytes isolated from HFD-fed mice compared to mice on chow-diet (figure 13A, *figure 1B in paper I*). Additionally, adipose tissue NA levels were reduced in mice with diet-induced obesity (figure 13B, *figure 1D in paper I*). Measurements of serum adiponectin level displayed >40% reduction of HMW to total adiponectin ratio in obese compared to lean mice (figure 13C, *figure 1E in paper I).*

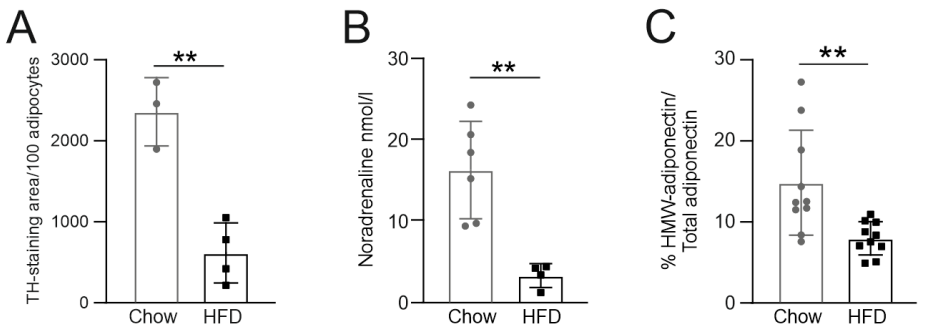


Figure 13| Quantification of TH and catecholamine levels show reduced SNS activity in WAT, associated with reduced HMW to total adiponectin ratio in obese mice. **P<0.01 vs chow

Our group has previously demonstrated that adrenergically triggered adiponectin release is diminished in adipocyte isolated from obese/T2D mice (Komai&Musovic. et al., 2016). To determine whether ATP-stimulated adiponectin release is also disturbed under similar conditions, primary IWAT adipocytes from lean and diet-induced obese mice were incubated with NA, ATP or a combination of both NA and ATP for 30 min (*figure 5D-E in paper* I). NA or ATP alone stimulated adiponectin release in adipocytes isolated from lean mice, and the combination of both NA and ATP triggered adiponectin secretion more potent than ATP alone. However, adipocyte adiponectin release could not be stimulated in obese/T2D mice when incubated with NA or ATP alone or in combination of the catecholamine and nucleotide. The adiponectin content was unaltered in adipocytes isolated from diet-induced obese mice, showing that the blunted adiponectin release in obese/T2D mice are due to a secretion defect rather than synthesis of the hormone.

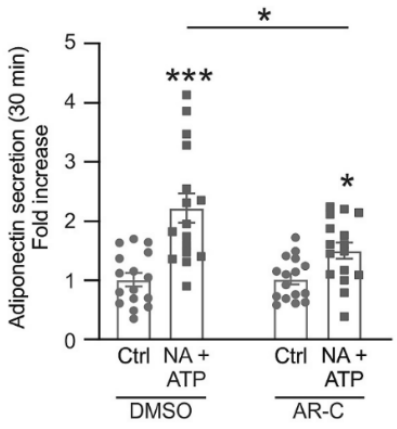


Figure 14| The effect of NA combined with ATP on adiponectin secretion using lean IWAT adipocytes pretreated with the P2Y2R antagonist AR-C for 30 min. Results are expressed as fold increase vs. control (unstimulated conditions) in each group. *P<0.05, ***P<0.001.

We next investigated the molecular details of the ATP signaling pathway involved in the control of adiponectin release. P2Y2Rs are known to be highly expressed in mouse adipocytes and we confirmed the presence of disrupted purinergic signaling pathway observing a reduction of both mRNA and protein levels of P2Y2Rs in adipocytes isolated from obese mice (*figure 6A-B in paper I*). To understand the ATP signaling pathway and validate the involvement of P2Y2R, adiponectin release was measured in lean IWAT adipocytes pretreated with the P2Y2R antagonist 118925XX (AR-C, 1 μM) for 30 min. Those adipocytes were thereafter exposed to a combination of NA and ATP for further 30 min. Adiponectin secretion was triggered >2-fold when NA and ATP were combined under untreated conditions (control/dimethyl sulfoxide; Ctrl/DMSO).

However, adiponectin release was still triggered in the presence of the P2Y2R antagonist, but in a significantly lower magnitude than untreated cells (figure 14).

To summaries, we suggest that adiponectin exocytosis may be physiologically regulated by SNS innervation in WAT, by co-releasing NA and ATP. When NA binds to β_3AR, intracellular cAMP increases followed by an increased adiponectin release. In addition, ATP acts on P2Y2R increasing cytosolic Ca^{2+} and potentiating adiponectin exocytosis. Thus, NA can stimulate adiponectin release alone, but the secretion is augmented by ATP. The adiponectin secretion is reduced in obese and diabetic mice, and this was associated with lower abundance of P2Y2Rs. Our results propose that diet-induced obesity is associated with decreased SNS innervation of WAT, and that this is linked to blunted catecholamine-stimulated white adipocyte adiponectin release and reduced circulating levels of the adipocyte hormone, as represented in the graphical model in figure 15.

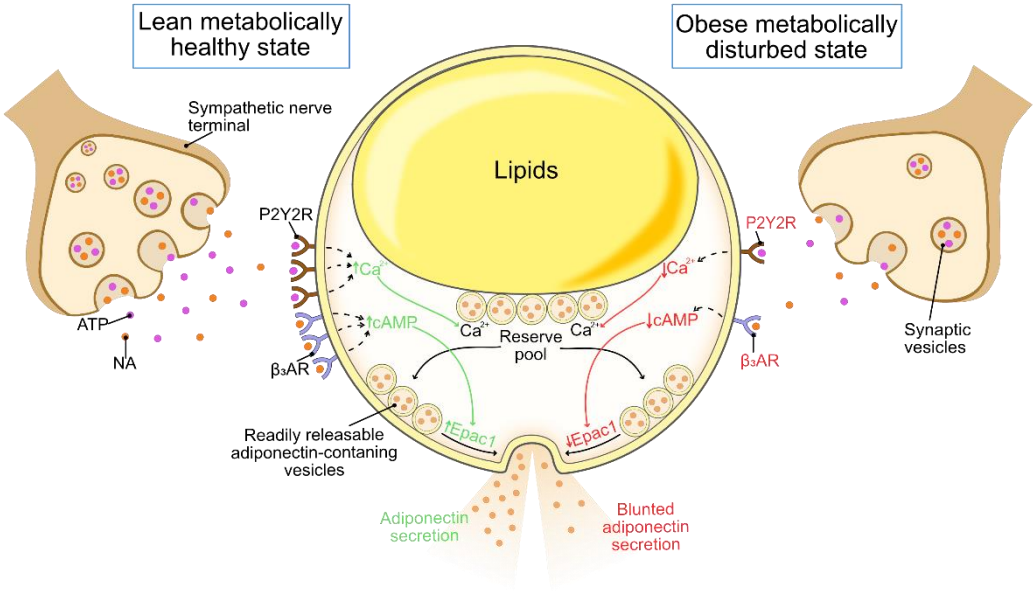


Figure 15| Graphical model showing a conclusion based on the findings in paper I (*created in Affinity Designer*)**.**

The effect of β_3AR ablation on adiponectin and metabolic health (paper II)

As a natural continuation of paper I, and based on our previous work (Komai&Musovic. et al., 2016), in paper II we wanted to further detail the role of β_3AR in white adipocyte adiponectin release and in overall metabolic health using mice that are genetically ablated for β_3AR. In order to confirm the absence of β_3AR, as well as to examine if there are any compensatory upregulation of other beta adrenergic receptors, we measured gene expression of all beta adrenergic receptors ($\beta_{1\text{-}3}$) in primary white adipocytes isolated from both WT and KO mice. As expected, β_3ARs were not expressed in adipocytes isolated from β_3AR-KO mice and no compensatory upregulation of either β_1- or β_2ARs was observed (*figure 3F-H in paper II*).

B_3AR-KO mice have reduced circulating adiponectin levels but are yet metabolically healthy

In previous work from our group, we defined that the catecholamine-induced adiponectin release depend on activation of β_3AR and that this adrenergic stimulation is abolished in white adipocytes isolated from obese and diabetic mice (Komai et al., 2016; Musovic and Olofsson, 2019). To study the effect of the absence of β_3AR on adiponectin release, we measured serum total and HMW adiponectin. Interestingly, KO mice had ~50% reduced serum total, as well as HMW adiponectin (*figure 2G-H in paper II*). We hypothesized that the reduction in circulating adiponectin could be a consequence of abolished white adipocyte stimulated adiponectin release. To test this, primary white adipocytes isolated from both KO and WT chow-fed mice, were incubated with cAMP-elevating agents (figure 16, *right*); forskolin (10 μM) together with IBMX (200 μM; Fsk/IBMX), ADR or CL for 30 min (figure 16, *left*). As shown in figure 3A and 3B in paper II, Fsk/IBMX triggered adiponectin release in both genotypes in IWAT adipocytes. However, adiponectin secretion was stimulated by ADR in a significantly lower amount in GWAT (P=0.003) β_3AR-KO adipocytes compared to WT littermates, while CL could not stimulate adiponectin secretion over basal in KO mice in either AT depot. We propose that the defect of adrenergically stimulated adiponectin release underlies the reduced serum adiponectin levels found in β_3AR null mice.

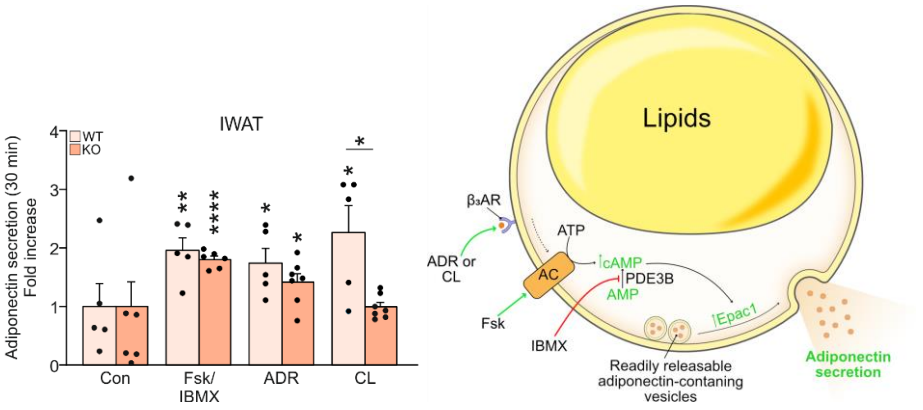


Figure 16| To the *left*; the cAMP-stimulated adiponectin release from IWAT adipocytes isolated from chow-fed mice. To the *right*; Primary white adipocyte incubated with cAMP-elevating agents. Results are expressed as fold increase vs. control (unstimulated conditions). *P<0.05, **P<0.01, ****P<0.0001 vs control.

Interestingly, serum insulin levels were reduced by almost 50% in fasted KO mice, while blood glucose concentrations were similar between the genotypes (*figure 2E-F in paper II*). The fasting insulin and glucose data propose that β_3AR null mice have improved insulin sensitivity compared to WT littermates and that they are metabolically healthy, despite the lower serum adiponectin levels. To investigate this, in *in vivo* and in diet-induced obesity and metabolic disturbances mice were put on HFD for 8 weeks. To determine how ablation of β_3AR affects whole-body metabolic health of the mice, OGTT was carried out before and after diet challenge. Before diet challenge, blood glucose levels peaked after oral glucose administration but rapidly decreased at later time points, despite the unchanged insulin levels in young mice. After 8 weeks on chow diet, KO mice displayed lower fasting glucose during all time points, concomitant with reduced fasting serum insulin. However, after 8 weeks on HFD diet, the insulin sensitivity of β_3AR null mice were decreased, as shown by blood glucose levels and serum insulin that remained high after glucose oral administration, (figure 17 and *figure 5 in paper II for more detailed information*). This suggests that β_3AR-KO mice are metabolically healthy and are more sensitive to insulin compared to WT. However, when challenged with HFD, KO mice display to be less sensitive to insulin as demonstrated by the remaining high blood glucose levels throughout the OGTT.

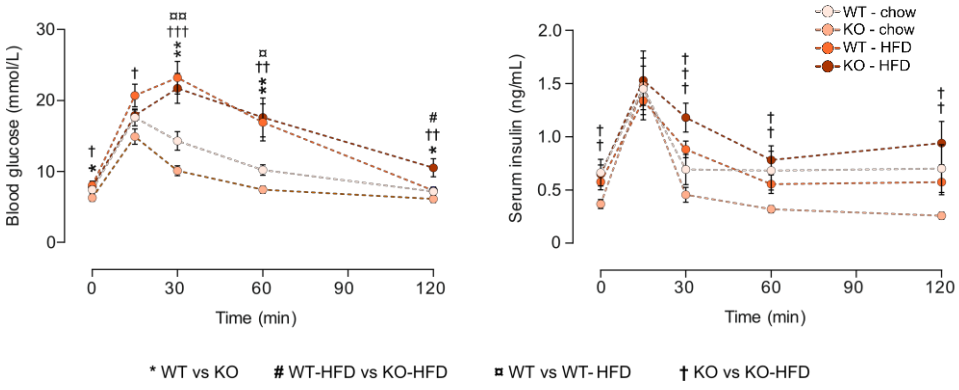


Figure 17| Measurements of blood glucose level and serum insulin after 8 weeks on chow- or HFD at different time points after glucose administration. *For more detailed information, see figure 5 in paper II.*

Subcutaneous WAT of β3AR-KO show signs of browning, increased adipogenesis and enhanced angiogenesis

Upon observation during dissection, IWAT appeared to be more brown in color in KO compared to WT mice. To understand this visual observation in more detail, histological sections of IWAT was investigated. IWAT from β_3AR null mice appeared to have a larger number of smaller sized adipocytes compared to littermates (figure 18). We suspected that the morphological changes observed in IWAT KO mice can be typical WAT browning (Bartelt and Heeren, 2014). We thus measured the gene expression of different browning markers. The mRNA levels of Ucp1 were significantly increased and the thyroid hormone iodothyronine deiodinase 2 (dio2; induces browning of WAT) tended to be higher (P=0.09), whereas the mRNA levels of the other browning markers were unchanged in IWAT from KO mice. We next considered that the increased number of adipocytes and IWAT appearance can be due to enhanced adipogenesis or angiogenesis (Ghaben and Scherer, 2019). This was indeed supported by gene expression data, showing an increase of adipogenesis (formation of new adipocytes from preadipocytes) and angiogenesis (the growth of new blood vessels from existing vasculature) genetic markers in IWAT tissue from KO mice (*figure 6 and 7 in paper II*).

We suggest that the growth of WAT via adipogenesis and concomitant angiogenesis is a way for the KO mice to balance the negative metabolic effects observed in obesity, where the size of AT mass correlates positively to insulin resistance (Meyer et al., 2013; Stenkula and Erlanson-Albertsson, 2018).

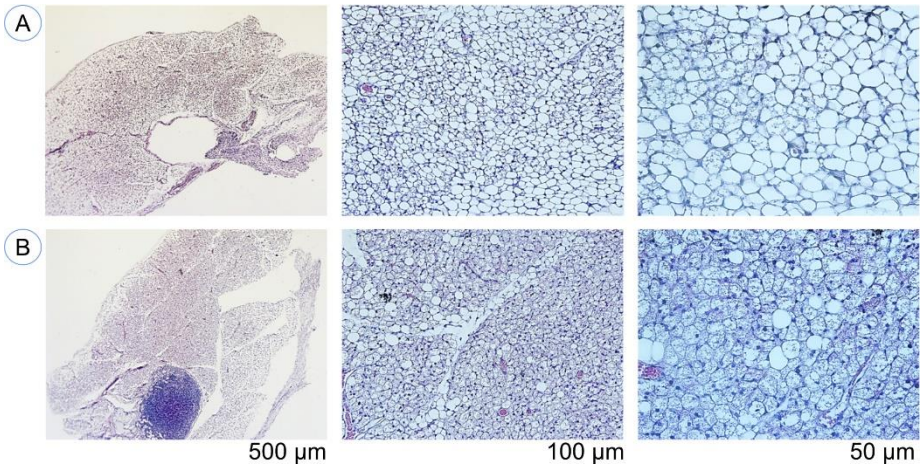


Figure 18| Representative Hematoxylin and Eosin Y solution staining in IWAT from (**A**) WT and (**B**) KO chow-fed mice. Scale bar: 500 – 50 µm.

To summaries, findings from paper II confirm that β_3AR signaling is central for the regulation of white adipocyte adiponectin release. The KO mice have ~50% reduced circulating levels of total as well as HMW adiponectin, and this is associated with disturbed adiponectin release in white adipocyte. The β_3AR agonist did not have any effect on white adipocyte adiponectin release in KO mice. Moreover, we propose that lean KO mice are metabolically healthy due to reduced serum insulin levels, concomitant with unaltered blood glucose compared to WT. However, obese KO mice become less insulin sensitive when challenged with HFD, to the same degree as WT HFD-fed mice.

The link between β_3AR and HMW adiponectin in diabetic individuals (paper III)

Our current understanding of how adiponectin release is regulated is still not completely clear. In addition to the catecholamine-induced adiponectin release via cAMP/Epac signaling pathway (Komai et al., 2014; Komai&Musovic. et al., 2016), insulin, secreted by pancreatic β cells, is another adiponectin-inducing secretagogue (Blümer et al., 2008; Bogan and Lodish, 1999; Lim et al., 2015; Szkudelski et al., 2011). Insulin, increases when glucose and free fatty acid (FFA) levels rise in the circulation after food intake. Actions of white adipocyte insulin-signaling (figure 19) stimulate FFA uptake and inhibits lipolysis, by activating PDE3B via PI3K/Protein kinase B (PKB) signaling pathway, breaking down cAMP (Degerman et al., 2011; Stahl et al., 2002). It is difficult to reconcile between adrenergic-stimulated and insulin-stimulated adiponectin release, due to the fact that catecholamines increase intracellular cAMP, while insulin decreases it. In paper III, we investigate how adiponectin release of different molecular forms are induced by insulin or adrenergic stimulation in more detail. We also determine the role of β_3AR specifically for the release of HMW adiponectin in white adipocytes isolated from primary rodent and human adipocytes, under metabolic healthy and obesity-associated metabolic disease condition.

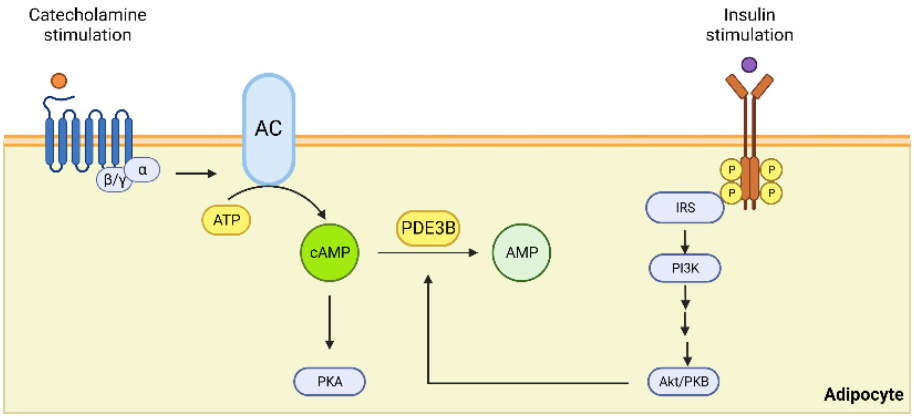


Figure 19| Different signaling pathways involved in white adipocyte lipid metabolism *(created with BioRender.com).*

Adrenergic-signaling triggers release of HMW adiponectin while insulin induces only the release of smaller adiponectin forms

Reduced levels of HMW adiponectin has been associated with development of metabolic disease in regard to the regulation of glucose homeostasis and insulin sensitivity (Wang et al., 2006; Yamauchi et al., 2001). We know from previous own work (Komai&Musovic. et al., 2016), that adrenergically stimulated adiponectin release is blunted in primary white adipocytes isolated from obese/T2D mice, due to a lower abundance of β_3ARs and Epac1. Here, we wanted to determine how insulin affect secretion of smaller and larger forms of adiponectin under similar condition. In order to do this, adiponectin release was measured using primary IWAT adipocytes isolated from 8 weeks chow- or HFD-fed mice. IWAT adipocytes were incubated with either insulin (20 nM) or ADR (5 μM) for 30 min. In agreement with previous study, as can be seen in the left part of figure 20A, both insulin and ADR stimulated secretion of adiponectin in adipocytes isolated from lean mice. In contrast, the effect of the two secretagouges on adiponectin release was abolished in adipocytes isolated from obese/T2D mice (figure 20A, *right*). This could be due to the reduced insulin sensitivity and elevated blood glucose level observed in obese/T2D mice. The basal adiponectin secretion tended to be elevated (P=0.07) in adipocytes isolated from HFD-fed mice, which is in agreement with previous findings (Komai&Musovic. et al., 2016).

Next, we measured HMW adiponectin release (figure 20B) in same samples as in figure 20A. Interestingly, the effect of ADR on HMW adiponectin secretion was completely abolished in white adipocyte isolated from HFD-fed mice, whereas the basal HMW adiponectin release was not changed. However, total content of HMW adiponectin, confirmed that the abolished release of HMW adiponectin is not due to a reduction of the HMW adiponectin content, displaying similar content in adipocyte isolated from chow- and HFD-fed mice *(figure 1G in paper III).*

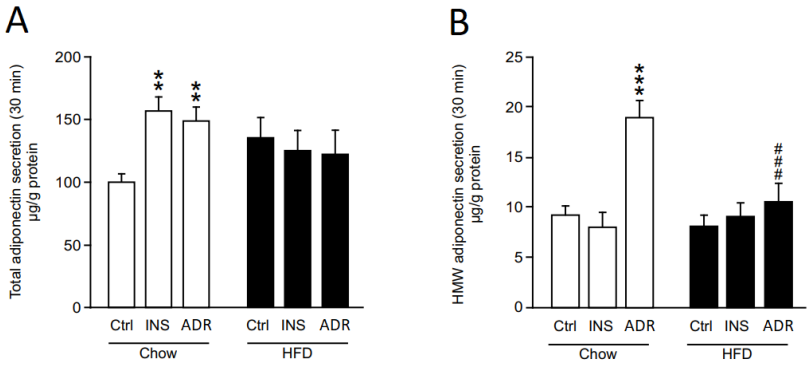


Figure 20| The effect of insulin and ADR on total and HMW adiponectin release in lean and obese/T2D mice. Results are expressed as ug/g protein vs. control (unstimulated conditions) in each group. **P<0.01, ***P<0.001 vs control, ###P<0.001 vs ADR chow.

The signaling pathways involved in insulin- and catecholamine-stimulated adiponectin secretion

To gain a detailed understanding of the signaling pathways involved in adiponectin secretion stimulated via insulin or adrenergic signaling, differentiated 3T3-L1 adipocytes were preincubated with or without the PI3K inhibitor (Wortmannin; 100 nM) for 30 min, followed by an incubation with insulin or ADR for further 30 min *(figure 2B in paper III*). The insulin-induced adiponectin secretion was abolished in cells treated with the PI3K-inhibitor, while ADR-stimulated adiponectin secretion remained unaffected in same conditions. On the other hand, pretreatment with the Epac inhibitor ESI-09 (10 µM) had no effect on insulin-stimulated adiponectin secretion, whereas ADR-stimulated adiponectin release was diminished (*figure 2D in paper III).*

Additionally, to further study the similarities and dissimilarities between the two signaling pathways, a time-course experiment was performed in differentiated 3T3-L1 adipocytes, at 15, 30 and 60 min, in response to insulin (200 nM) or CL (1 uM; figure 21 and *figure 2E in paper III*). CL stimulated adiponectin release already at early time-point as well as at

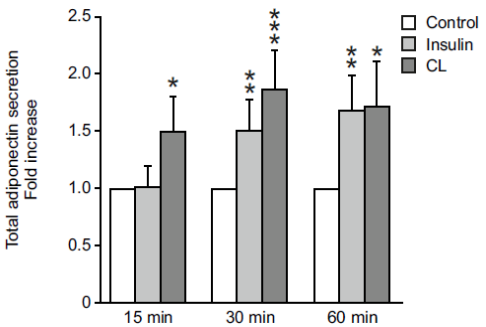


Figure 21| The time course series shows the effect of insulin and CL on adiponectin release at 15, 30 and 60 min. Results are expressed as fold increase vs. control. *P<0.05, **P<0.01, ***P<0.001 vs control.

later time points. However, insulin-stimulated adiponectin release was first observed after 30 min and later. This insulin stimulatory effect of adiponectin release is in agreement with similar results observed in other studies, where insulin was not able to stimulate adiponectin secretion at early time points, but only at later time points (Cong et al., 2007; Lim et al., 2015). Our results suggest that activation of β_3AR trigger the rapid secretion of HMW form of adiponectin via downstream activation of Epac1, while insulin induces the release of the smaller forms of adiponectin involving PI3K- and PDE3B-dependent mechanisms and with delayed time kinetics.

TIRF demonstrates rapid exocytosis of adiponectin-containing vesicles via adrenergic signaling pathway

To investigate the effect of insulin and ADR at the level of single adiponectin vesicle release, live TIRF microscope imaging was performed on 3T3-L1 adipocytes stably expressing human adiponectin fused to mCherry (Shrestha et al., 2022). Extracellular addition of insulin (200 nM), during TIRF recordings displayed a disappearance of adiponectin vesicles in form of vanished fluorescence puncta pattern starting from 10 min after adding insulin. We interpreted this vanished fluorescence puncta as fusion of adiponectin vesicles with the plasma membrane and release of their cargo. However, only a fraction of the adiponectin vesicles had been released while the majority of adiponectin vesicles remained stable throughout the recording (30 min). In contrast, the ADR (5 μM) effect was first observed after ~2 min, displaying a rapid and potent adiponectin exocytosis. Significantly fewer ADR-triggered adiponectin containing vesicles remained in the stimulated cells after 15 minutes recordings (figure 22 and *figure 3 in paper III*).

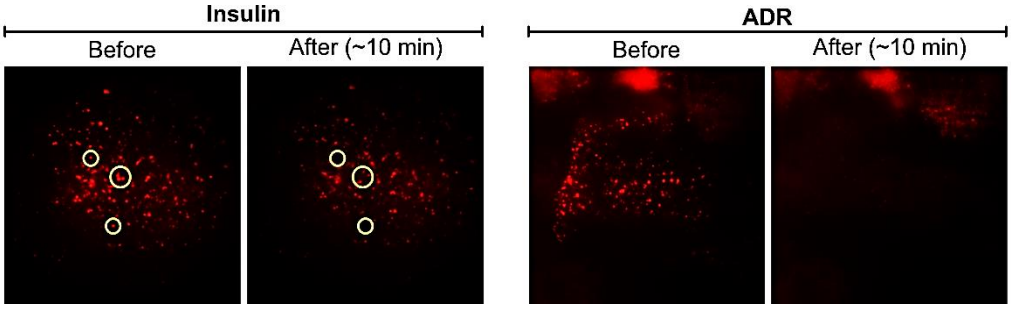


Figure 22| TIRF imaging of the mCherry-expressing adiponectin containing vesicles visualized as punctate pattern of fluorescence. Scale bar = 2 μm.

To further study the similarities and dissimilarities of the time-kinetics between insulin- and ADR-stimulated adiponectin releases, patch-clamp technique was applied to 3T3-L1 adipocytes and change in membrane capacitance (representative of exocytosis) was recorded. An addition of insulin induced slow exocytosis, whereas an addition extracellular ADR to insulin-stimulated cells triggered further exocytosis in a rapid manner. To further confirm the ability of ADR to stimulated adiponectin secretion, 3T3-L1 adipocytes were first pretreated with insulin for 30 min, followed by an incubation with ADR (or insulin again) for further 30 min. As expected, ADR was able to stimulate adiponectin secretion in cells that had already been exposed to insulin as shown in figure 4D in paper III. Our data suggest that the different time-kinetics between ADR-stimulated and insulin-induced adiponectin release, show that the two hormones secrete different size of adiponectin. This is supported by the ADR-stimulated release of adiponectin in cells pre-exposed to insulin.

Collectively, our data suggest that HMW adiponectin is released via adrenergic-signaling pathway, while insulin induces secretion of the smaller forms of adiponectin only. However, the stimulated release of both HMW and smaller forms of adiponectin is abrogated in obese/T2D mice. TIRF and patch-clamp data, together with biochemical measurements, propose that ADR trigger fast exocytosis of both smaller and larger adiponectin forms, while insulin trigger release of smaller forms of adiponectin, with slower time-kinetics.

Adrenergic signaling triggers the secretion of HMW adiponectin also in human primary adipocyte

To extend our findings in cultured and mouse-derived adipocytes to human pathophysiology, isolated adipocytes from healthy and obese/diabetic individuals were incubated with insulin, ADR or CL for 30 min. In agreement with findings of experiment in cultured and mouse adipocytes, all stimulatory agents triggered adiponectin release in human adipocytes (figure 23A and *figure 5A in paper III*). Moreover, pretreatment with Epac inhibitor for 30 min (*figure 5B in paper III*) confirmed previous results with mouse adipocytes that there is an involvement of Epac signaling pathway in adrenergically triggered adiponectin release also in human

adipocytes. Furthermore, the release of HMW adiponectin was measured from same samples in figure 23A. As expected, both ADR and CL stimulated the release of HMW adiponectin, whereas insulin had no effect on the HMW adiponectin release (figure 23B and *figure 5C in paper III*). In the view of metabolic disturbed condition, human adipocytes isolated from obese individuals showed that none of the stimulatory agents could induced adiponectin release (figure 23C and *figure 5E in paper III*). This was associated with ~40% reduced serum HMW adiponectin in obese adipocytes compared to lean control group. In agreement with our previous findings in obese/diabetic mice (Komai&Musovic. et al., 2016), the protein level of β_3AR was ~50% reduced in adipocytes from obese/T2D mice.

Collectively, the results demonstrate that HMW adiponectin release is triggered via β_3AR in mouse and human adipocytes. Insulin induces the release of only smaller molecular forms of adiponectin in mouse as well as in human adipocytes. Adiponectin release stimulated by the two secretagouges are blunted in adipocytes isolated from obese/T2D individuals.

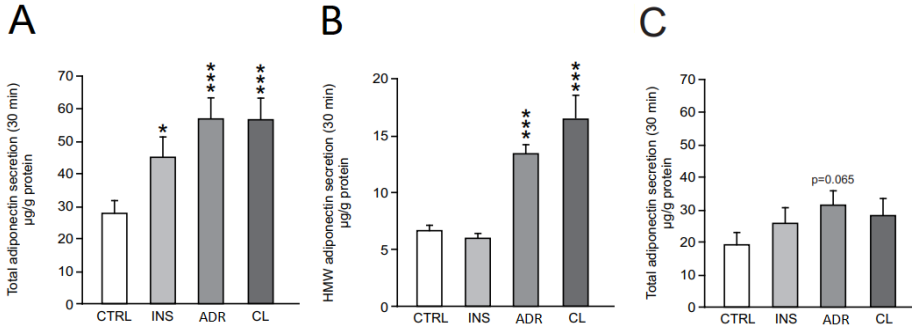


Figure 23| The effect of insulin, ADR and CL on ***A:*** Total adiponectin release, ***B:*** HMW adiponectin release (in the same samples in ***A***) in adipocytes isolated from lean individuals. ***C:*** Total adiponectin release in obese individuals. Results are expressed as µg/g protein vs. control in each group. *P<0.05, ***P<0.001 vs control.

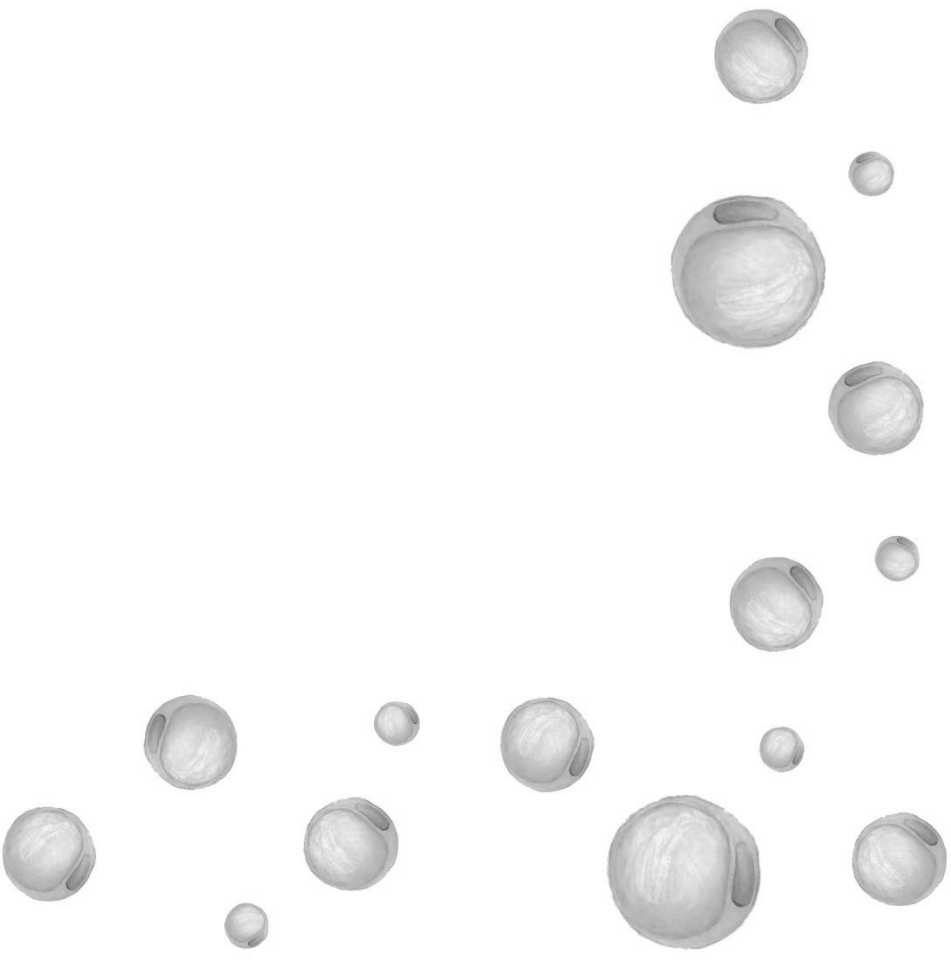
Daily injection with CL elevates HMW adiponectin levels in lean and obese mice

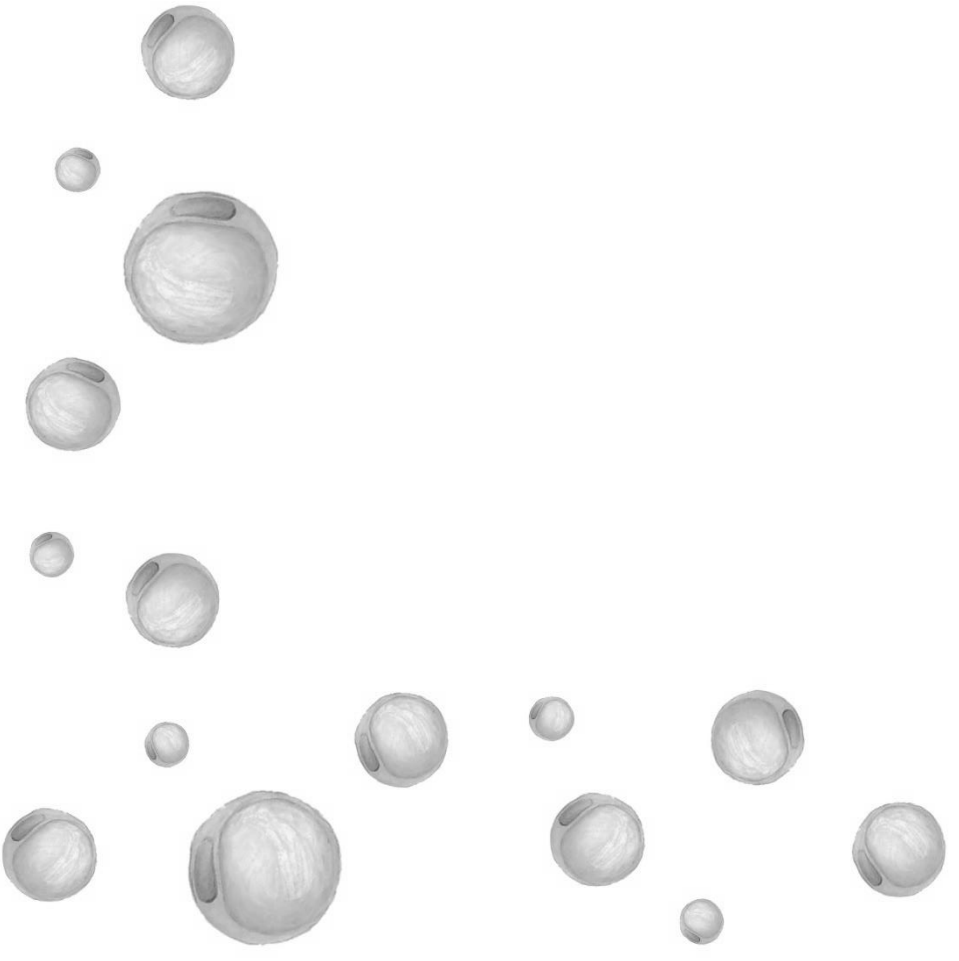
To confirm the relevance of the *in vitro* and *ex vivo* data on the regulation of circulating adiponectin levels *in vivo*, mice were daily injected with CL (1 mg/kg) or vehicle (saline) for 2 days. The total adiponectin levels were not affected by the daily injections with either CL or saline. Interestingly, the circulating HMW adiponectin levels were elevated by almost 40% in the CL-treated group compare to control *(figure 7B and 7C*

in paper III). Moreover, there was a sign of improved insulin sensitivity in CL-injected mice, showing a tendency of reduced serum insulin (P=0.06), despite the slightly higher blood glucose levels in both groups.

Next, we investigated if daily injection with CL over a longer time can restore circulating HMW adiponectin levels in obese/T2D mice. Mice that have been fed with HFD for 8 weeks, were injected daily with CL or saline for two additional weeks, while kept on the same diet. Daily CL-injection within two weeks did not affect serum total adiponectin levels in either chow or HFD groups. As expected, after 8 weeks on HFD, mice displayed reduced serum HMW adiponectin compared to chow-fed mice. However, the two additional weeks on CL-injection significantly improved serum HMW adiponectin in chow-fed mice compared to animals that received saline. HMW adiponectin continued to decline in saline-treated group of HFD-fed mice after further two weeks on the same diet. Interestingly, HMW adiponectin levels were improved in HFD-fed animals injected with CL for two weeks (*figure 8B in paper III*). Our data propose that circulating HMW adiponectin levels are regulated via β_3AR signaling pathway. Results also suggest that β_3AR agonists can improve serum HMW adiponectin over longer time-periods of days or weeks. Furthermore, treatment with the agonist can inhibit a further reduction of levels of circulating HMW adiponectin in HFD-fed mice.

To summaries, in paper III we investigated the role of insulin and catecholamine in the regulation of adiponectin release, specifically HMW adiponectin in healthy as well as obese state. Insulin induces the release of smaller forms of adiponectin only, involving PI3K- and PDE3B-dependent mechanisms, while HMW adiponectin is specifically released via an adrenergically stimulated mechanism involving β_3AR/Epac1-dependent signaling. Interestingly, the adiponectin release is blunted in obese/T2D condition in both mice and humans, and this was associated with reduced circulating HMW adiponectin. Daily injection with CL increase circulating HMW adiponectin during shorter time treatment as well as longer time-periods in chow-fed mice. However, the outcome of reduced HMW adiponectin in HFD-fed mice were inhibited in HFD-fed animals treated with CL.





WHERE ARE WE GOING?

In light of the global epidemic of obesity, the need to develop new anti-obesity drugs are necessary. Obesity can result in disturbed WAT function resulting in low plasma levels of adiponectin. Particular HMW adiponectin is associated to obesity-associated metabolic disease in both humans and rodents, confirming that there are a positive connection between metabolic health and maintained levels of HMW adiponectin (Blüher, 2014; Iwabu et al., 2019; Smith et al., 2019; Wang and Scherer, 2016). Even though the pathophysiological importance of adiponectin is well studied, the knowledge of the underlying mechanisms behind the adiponectin release is still unclear and need to be investigated.

The impact of sympathetic innervation on adiponectin release

The fact that the WAT is richly innervated with postganglionic neurons from the SNS co-releasing NA and ATP (Youngstrom and Bartness, 1995) suggest that sympathetic innervation might be a reasonable source as a physiological regulator of adiponectin release. Our findings in paper I show that NA stimulates adiponectin release via β_3AR/cAMP/Epac1 signaling pathway, and ATP induces adiponectin exocytosis via P2Y2R/PLC/IP3 signaling pathway elevating $[Ca^{2+}]_i$. In the view of this, we show that NA and ATP jointly control the release of white adipocyte adiponectin.

High concentrations of NA is more likely to trigger adiponectin exocytosis due to the close location of SNS to the WAT, compared to ADR originated from the adrenal medulla, located far from the WAT. Moreover, NA has higher affinity for this receptor compared to ADR (Tate et al., 1991). This is supported by the 80-fold higher concentrations of NA than ADR found in the quantification of catecholamine content in rat IWAT (Vargovic et al., 2011). Furthermore, catecholamines have the ability to also elevate $[Ca^{2+}]_i$ in the white adipocyte by acting on α_1ARs (Seydoux et al., 1996). Our group has shown that the modest elevation of $[Ca^{2+}]_i$ triggered by either ADR (Komai&Musovic. et al., 2016) or NA (*in paper I*) is without effect on adiponectin release. Therefore, the underlying signaling pathway behind the elevation of $[Ca^{2+}]_i$ in the release of adiponectin must involve other triggering molecules.

We have shown that the co-released ATP acts on P2Y2Rs, elevating $[Ca^{2+}]_i$, which induces further release of white adipocyte adiponectin. As demonstrated in paper I, adiponectin release was stimulated by extracellularly applied ATP in both early (30 min) as well as in later (60 min) incubations. Although, the release of adiponectin appears to be notable smaller in 30 min incubation with ATP. Interestingly, even though extracellularly applied ATP appear to augment catecholamine-induced adiponectin release, ATP alone have shown to stimulate the release of adiponectin in the absence of concomitant cAMP elevation via Ca^{2+}-independent effects.

The molecular characterization of the ATP signaling pathway has been a bit puzzling. It is notable to mention that the intracellular milieu of cells used for electrophysiological studies are controlled by the added solution in the pipette, while the primary white adipocytes used for adiponectin secretion measurements are metabolically intact and has the ability to produce endogenous cAMP as seen in paper I. It has been proposed that extracellularly applied ATP alone can also prompt the cAMP production in brain endothelial cells in Ca^{2+}-independent pathway (Anwar et al., 1999). Additionally, the ATP-induced Ca^{2+} increase may activate adenylyl cyclase (AC) leading to increased cAMP production (Halls and Cooper, 2011; Willoughby and Cooper, 2006).

We confirm previous results from our group that catecholamine-stimulated adiponectin release is blunted in obese and diabetic state, due to the reduced abundance of β_3ARs and Epac1 (Komai&Musovic. et al., 2016). In addition, we show that the purinergic signaling pathway is also disrupted in obese and diabetic mice, due to reduced abundance of P2Y2Rs and that this is associated with blunted ATP-triggered release of adiponectin, reduceing Ca^{2+} potentiation of adiponectin release. The obesity-associated changes of SNS activity is not completely understood. It has been discussed that SNS might contribute in the development of obesity and obesity-assiociated changes by either reducing or increasing the SNS sensitivity (Burnstock and Gentile, 2018). However, we propose that activation of both β_3ARs and P2Y2Rs are important for the pathophysiological control of adiponectin release, specifically serum HMW adiponectin. The involvement of different signaling pathways in adiponectin release

need further investigations in order to better understand the mechanism behind white adipocyte adiponectin release.

The role of β3AR in regulation of adiponectin release

Moving forward to paper II of this thesis, we show the central role of β_3AR in the regulation of white adipocyte adiponectin release. Furthermore, we show how β_3AR affect levels of circulating adiponectin and the metabolic health using mice that do not express β_3ARs in the whole body. This paper is a build-up study from previous study (Komai&Musovic. et al., 2016), showing that catecholamine-induced white adipocyte HMW adiponectin release depend on the activation of β_3AR, and that this release is abrogated in white adipocytes isolated from obese/T2D mice, in a state we refer to as *catecholamine resistance* (Komai&Musovic. et al., 2016).

Adiponectin secretion measurements, propose that the defect of adiponectin secretion, underlie the reduced serum adiponectin concentrations found in β_3AR-KO mice. We evaluated whether reduced adiponectin content in IWAT adipocyte could explain the blunted adiponectin release in KO mice, although adiponectin content in GWAT adipocytes were unaltered. Gene expression measurements of genes involved in β_3AR signaling pathway confirm the existence of an adiponectin secretory defect. Gene expression data of Adipoq (adiponectin) showed that both IWAT and GWAT adipocytes, displayed reduced mRNA levels of Adipoq and we confirmed the lack of expression of β_3AR in adipocytes isolated from KO mice. It has earlier been proposed that obese mice and humans are associated with reduced adiponectin gene and protein expressions (Hu et al., 1996). Moreover, this results are in agreement with previous findings in obese/T2D mice, where there were a ~30% reduced abundance of β_3ARs associated with blunted stimulation of adiponectin release, reduced circulating HMW adiponectin and unaltered adiponectin content in IWAT adipocyte isolated from obese and diabetic mice (Komai&Musovic. et al., 2016).

Some other possible explanations for the reduced adiponectin secretion could be a dysfunction of the exocytosis machinery within the adipocyte. A dysfunction in the synthesis of adiponectin and/or post-translational modification could lead to an

inhibition or degradation of the hormone within ER itself, which can result as blunted release (Hampe et al., 2015b; Liu et al., 2015; Mondal et al., 2012; Phillips et al., 2009; Wang et al., 2007; Zhou et al., 2010). However, gene expression data of the main chaperones (ERp44, Ero1-Lα and DsbA-L) involved in ER-located posttranslational modifications of adiponectin, were unaltered between the genotypes. We suggests that the production of adiponectin or trafficking in ER is unlikely to cause the secretion defect found in KO mice. Therefore, we find it possible the adiponectin may be trapped within the cell, affecting the adiponectin release via β_3AR-mediated signaling pathway. Moreover, the basal (unstimulated conditions) release of adiponectin is increased in the GWAT, but not IWAT primary adipocyte isolated from β_3AR null mice. This can probably be explained by the increased levels of cAMP in GWAT, but not IWAT β_3AR-KO mice. This results are similar with the findings in previous study, displaying an increase of basal adiponectin secretion in obese and diabetic mice (Komai&Musovic. et al., 2016).

The fact that ADR stimulates some of the adiponectin release in adipocyte isolated from KO mice are a bit surprising, since our previous research suggests that β_3AR is the key receptor among other βARs regulating adiponectin exocytosis. Genetic compensation of gene function during knockdown or gene mutations have been shown (El-Brolosy and Stainier, 2017). However, the ADR-stimulated adiponectin release does not seem to be related to a compensatory upregulation of gene expression of β_1- or β_2ARs. Thus, it is likely that in the absence of the β_3AR, other signaling mechanisms, either as adrenergic or non-adrenergic, may compensate in this mouse model (de Jong et al., 2017). Further, it is possible that the spatial location of β_1 and β_2 (together with their associated proteins) has been rearranged in the adipocytes lacking β_3ARs, leading to a participating of those receptors in the regulation of adiponectin release.

The current study suggest that lean KO mice are metabolically healthy and in fact more insulin sensitive than WT littermates, despite lower circulating levels of total and HMW adiponectin. This is a bit puzzling, since it is well known that in particular high levels of HMW adiponectin is associated with improved metabolic health, whereas

obesity is associated with lower levels of this molecular form (Iwabu et al., 2019; Tsao et al., 2003). Our data suggest that the reduced levels of adiponectin is not associated with reduced metabolic health in the lean state, and rather indicates improved metabolic health under those conditions. However, when challenged with HFD, KO mice become as insulin resistant as WT littermate on the same diet. It is worthy of note that the HMW/total adiponectin ratio remains largely unaltered in the lean KO mice. Studies suggest that the ratio between HMW and total adiponectin is more important for metabolic health than absolute concentrations of adiponectin molecular forms (Aso et al., 2006). This possibly explain the metabolic phenotype of lean β_3AR-KO mice.

The metabolic phenotype of KO mice may also be due to the observed "browning" of IWAT. The development of browning adipocytes in WAT has been mentioned to reduce adverse effects of AT and to improve metabolic health (Bartelt and Heeren, 2014; Kaisanlahti and Glumoff, 2019). Recent study has reported the requirement of β_3AR in cold-induced "browning/beigeing" of WAT (Barbatelli et al., 2010). However, studies using the same mouse model, suggested that β_3AR is nonessential for the browning process in WAT (de Jong et al., 2017; Mattsson et al., 2011; Susulic et al., 1995). The increase of IWAT adipocyte number, propose that KO mice have increased adipogenesis and angiogenesis, which was indeed supported by mRNA data. It has been reported that when adipocyte increase in size (hypertrophy) via adipogenesis and concomitant angiogenesis, it counterbalance the negative metabolic effects of obesity (Ghaben and Scherer, 2019).

Adrenergic signaling stimulates release of HMW adiponectin, whereas insulin trigger the release of smaller molecular forms of adiponectin

In the final work of this thesis (paper III), we show that β_3-adrenergic signaling pathway specifically triggers secretion of HMW, while insulin induces only the release of the smaller forms of adiponectin in primary mouse and human adipocytes. Our findings show that the release of different molecular forms of adiponectin is stimulated by two opposing signaling pathways. This may appear to be a bit puzzling from a physiological point of view. Insulin (decreases cAMP) is an anabolic hormone, increases during time of food intake, responsible for glucose and FFA uptake in

peripherial tissues for later energy consumptions (Chang et al., 2004; Rutkowski et al., 2015). In contrast, catecholamines (elevates cAMP) are included among the catabolic hormones, and are the main physiological activators of lipolysis during time of fasting/energy deficiency (Duncan et al., 2007; Hellström et al., 1997). We suggest that there may be two different explanations for how the release of different molecular forms of adiponectin can occur.

Firstly, the different molecular forms of adiponectin might be released from various pool of vesicles, which can be regulated by either catecholamine or insulin. Second option suggest that all forms of adiponectin may be contained within the same vesicle, and that the release of adiponectin-contained vesicles are controlled at the level of fusion of the adiponectin-containing vesicle membrane with the plasma membrane of the white adipocyte, allowing or limiting the release of the largest form of adiponectin (Hastoy et al., 2017; Rothman, 2014). We believe that the latter explanation is more likely to occur due to the fact that upon adrenergic-stimulation (in the absence of insulin), HMW and smaller forms of adiponectin are released together. Moreover, observation with TIRF microscope shows that insulin-induced adiponectin release appears to have a slower time-kinetics compared to ADR-induced adiponectin release, which showed a rapid exocytotic event in the TIRF zone. This may be due to the limited level of vesicle fusion with the plasma membrane, allowing only the smaller forms of adiponectin to be released upon insulin stimulation. Furthermore, electrophysiological and biological secretion measurements confirmed our TIRF data, showing that insulin-induced adiponectin release had slower time-kinetics compared to ADR-triggered rapid adiponectin release. Additionally, ADR could trigger adiponectin exocytosis in adipocytes that have already been pre-exposed to insulin.

In paper III, we observed that catecholamine-triggered and insulin-induced adiponectin release was disturbed in obesity and diabetes as a consequence of catecholamine resistance coupled with insulin resistance. Disruption in the secretory/release process, caused by a defect in the synthesis and/or oligo- and multimerisation of adiponectin in metabolically unhealthy state (Liu et al., 2008; Waki

et al., 2003; Wang et al., 2006), may also affect adiponectin secretion. ER stress, in which proteins are not fully folded or misfolded, can cause a reduced production of adiponectin and/or decreased assembly of adiponectin oligomers (Zhou et al., 2010). However, the unaltered gene expression of different ER chaperone protein in obese and diabetic mice, indicates that there is no problem with the synthesis of the hormone.

Interestingly, we show that HMW adiponectin release is stimulated by β_3AR agonist also in primary human adipocytes. This is interesting due to the fact that the physiological role of β_3AR in human white adipocytes have been debated, and β_3ARs abundance has been observed to be low (Berkowitz et al., 1995; Krief et al., 1993; Revelli et al., 1993). Moreover, the abundance of β_3ARs are reduced, associated with blunted adrenergic-triggered total and HMW adiponectin release in human primary white adipocytes isolated from individuals with obesity/T2D. Furthermore, daily injection with CL increases circulating HMW adiponectin in obese and diabetic mice. These findings jointly suggest that β_3AR is the main signaling pathway for the release of the higher order form of adiponectin, and may therefore be a promising therapeutic target to combat obesity-associated metabolic disease.

The current understanding of how white adipocyte adiponectin of different molecular forms, particularly HMW adiponectin release is yet incomplete. β_3AR is the one of the least studied receptor among the beta adrenergic receptors. Additionally, the regulation of adiponectin release have only been investigated in few studies and the molecular regulation of adiponectin release has gained little attention. However, the discovery of adiponectin as the most abundant peptide hormone secreted by white adipocytes have opened up new doors due to its important and protective roles in metabolic health. Therefore, adiponectin replacement therapy has been suggested as a promising pharmacological treatment of obesity and obesity-related diseases. However, the development of recombinant adiponectin or synthetic analogs have some complications.

Adiponectin is a quite complex polypeptide molecule and can therefore not be administrated orally. It is difficult to obtain a stable multimeric adiponectin form and

intravenous administration is challenging to maintain since adiponectin has a short biological half-life of ~75 – 150 min (Halberg et al., 2009; Hoffstedt et al., 2004; Pajvani et al., 2003). The probability to instead increase the endogenous adiponectin is thereby desired. The pharmacological elevation of circulating adiponectin have shown to improve metabolic status and propose to be a promising therapeutic strategy for treatment of metabolic disease (Pajvani et al., 2004; Yamauchi and Kadowaki, 2008). However, it is essential to understand the cellular and molecular regulation of endogenous adiponectin release in order to define suitable targets. All three papers included in this thesis, jointly suggests that there is a secretory defect behind the reduced serum HMW adiponectin observed in obesity and obesity-related diseases. Our studies clarify mechanisms regulating adiponectin release under normal physiological conditions and in disturbed metabolic conditions, opening up the possibilities for future treatments.

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REFERENCES

Anwar, Z., Albert, J.L., Gubby, S.E., Boyle, J.P., Roberts, J.A., Webb, T.E., and Boarder, M.R. (1999). Regulation of cyclic AMP by extracellular ATP in cultured brain capillary endothelial cells. British Journal of Pharmacology *128*, 465-471.

Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., et al. (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun *257*, 79-83.

Aso, Y., Yamamoto, R., Wakabayashi, S., Uchida, T., Takayanagi, K., Takebayashi, K., Okuno, T., Inoue, T., Node, K., Tobe, T., et al. (2006). Comparison of serum high-molecular weight (HMW) adiponectin with total adiponectin concentrations in type 2 diabetic patients with coronary artery disease using a novel enzyme-linked immunosorbent assay to detect HMW adiponectin. Diabetes *55*, 1954-1960.

Barbatelli, G., Murano, I., Madsen, L., Hao, Q., Jimenez, M., Kristiansen, K., Giacobino, J.P., De Matteis, R., and Cinti, S. (2010). The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. American journal of physiology. Endocrinology and metabolism *298*, E1244-1253.

Barg, S., Ma, X., Eliasson, L., Galvanovskis, J., Göpel, S.O., Obermüller, S., Platzer, J., Renström, E., Trus, M., Atlas, D., et al. (2001). Fast exocytosis with few Ca(2+) channels in insulin-secreting mouse pancreatic B cells. Biophysical journal *81*, 3308-3323.

Bartelt, A., and Heeren, J. (2014). Adipose tissue browning and metabolic health. Nature reviews. Endocrinology *10*, 24-36.

Bartness, T.J., Liu, Y., Shrestha, Y.B., and Ryu, V. (2014). Neural innervation of white adipose tissue and the control of lipolysis. Frontiers in Neuroendocrinology *35*, 473-493.

Bartness, T.J., Shrestha, Y.B., Vaughan, C.H., Schwartz, G.J., and Song, C.K. (2010). Sensory and sympathetic nervous system control of white adipose tissue lipolysis. Molecular and cellular endocrinology *318*, 34-43.

Becherer, U., Moser, T., Stühmer, W., and Oheim, M. (2003). Calcium regulates exocytosis at the level of single vesicles. Nature Neuroscience *6*, 846-853.

Berg, A.H., Combs, T.P., Du, X., Brownlee, M., and Scherer, P.E. (2001). The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nature medicine *7*, 947-953.

Berkowitz, D.E., Nardone, N.A., Smiley, R.M., Price, D.T., Kreutter, D.K., Fremeau, R.T., and Schwinn, D.A. (1995). Distribution of beta 3-adrenoceptor mRNA in human tissues. European journal of pharmacology *289*, 223-228.

Blüher, M. (2014). Are metabolically healthy obese individuals really healthy? European journal of endocrinology *171*, R209-219.

Blümer, R.M., van Roomen, C.P., Meijer, A.J., Houben-Weerts, J.H., Sauerwein, H.P., and Dubbelhuis, P.F. (2008). Regulation of adiponectin secretion by insulin and amino acids in 3T3-L1 adipocytes. Metabolism *57*, 1655-1662.

Bogan, J.S., and Lodish, H.F. (1999). Two compartments for insulin-stimulated exocytosis in 3T3-L1 adipocytes defined by endogenous ACRP30 and GLUT4. The Journal of cell biology *146*, 609-620.

Burgoyne, R.D., and Morgan, A. (2003). Secretory granule exocytosis. Physiol Rev *83*, 581-632.

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev *87*, 659-797.

Burnstock, G. (2014). Purinergic signalling in endocrine organs. Purinergic Signalling *10*, 189-231.

Burnstock, G., and Gentile, D. (2018). The involvement of purinergic signalling in obesity. Purinergic Signal *14*, 97-108.

CANNON, B., and NEDERGAARD, J. (2004). Brown Adipose Tissue: Function and Physiological Significance. Physiological Reviews *84*, 277-359.

Chang, L., Chiang, S.-H., and Saltiel, A.R. (2004). Insulin Signaling and the Regulation of Glucose Transport. Molecular Medicine *10*, 65-71.

Cheng, X., Ji, Z., Tsalkova, T., and Mei, F. (2008). Epac and PKA: a tale of two intracellular cAMP receptors. Acta biochimica et biophysica Sinica *40*, 651-662.

Chusyd, D.E., Wang, D., Huffman, D.M., and Nagy, T.R. (2016). Relationships between Rodent White Adipose Fat Pads and Human White Adipose Fat Depots. Frontiers in nutrition *3*, 10.

Cinti, S. (2012). The adipose organ at a glance. Dis Model Mech *5*, 588-594.

Cnop, M., Havel, P.J., Utzschneider, K.M., Carr, D.B., Sinha, M.K., Boyko, E.J., Retzlaff, B.M., Knopp, R.H., Brunzell, J.D., and Kahn, S.E. (2003). Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia *46*, 459-469.

Collins, S. (2012). β-Adrenoceptor Signaling Networks in Adipocytes for Recruiting Stored Fat and Energy Expenditure. Frontiers in endocrinology *2*, 102-102.

Combs, T.P., Berg, A.H., Obici, S., Scherer, P.E., and Rossetti, L. (2001). Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. The Journal of clinical investigation *108*, 1875-1881.

Cong, L., Chen, K., Li, J., Gao, P., Li, Q., Mi, S., Wu, X., and Zhao, A.Z. (2007). Regulation of adiponectin and leptin secretion and expression by insulin through a PI3K-PDE3B dependent mechanism in rat primary adipocytes. Biochem J *403*, 519-525.

de Jong, J.M.A., Wouters, R.T.F., Boulet, N., Cannon, B., Nedergaard, J., and Petrovic, N. (2017). The beta3-adrenergic receptor is dispensable for browning of adipose tissues. American journal of physiology. Endocrinology and metabolism *312*, E508-e518.

de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A., and Bos, J.L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature *396*, 474-477.

Degerman, E., Ahmad, F., Chung, Y.W., Guirguis, E., Omar, B., Stenson, L., and Manganiello, V. (2011). From PDE3B to the regulation of energy homeostasis. Current Opinion in Pharmacology *11*, 676-682.

Dehvari, N., da Silva Junior, E.D., Bengtsson, T., and Hutchinson, D.S. (2018). Mirabegron: potential off target effects and uses beyond the bladder. Br J Pharmacol *175*, 4072-4082.

Denker, A., and Rizzoli, S.O. (2010). Synaptic vesicle pools: an update. Front Synaptic Neurosci *2*, 135-135.

Ding, G., Qin, Q., He, N., Francis-David, S.C., Hou, J., Liu, J., Ricks, E., and Yang, Q. (2007). Adiponectin and its receptors are expressed in adult ventricular cardiomyocytes and upregulated by activation of peroxisome proliferator-activated receptor gamma. J Mol Cell Cardiol *43*, 73-84.

Duncan, R.E., Ahmadian, M., Jaworski, K., Sarkadi-Nagy, E., and Sul, H.S. (2007). Regulation of Lipolysis in Adipocytes. Annual Review of Nutrition *27*, 79-101.

El-Brolosy, M.A., and Stainier, D.Y.R. (2017). Genetic compensation: A phenomenon in search of mechanisms. PLoS genetics *13*, e1006780.

El Hachmane, M.F., Ermund, A., Brannmark, C., and Olofsson, C.S. (2018). Extracellular ATP activates store-operated Ca(2+) entry in white adipocytes: functional evidence for STIM1 and ORAI1. Biochem J *475*, 691-704.

Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M., and Paschke, R. (2002). Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. Biochem Biophys Res Commun *290*, 1084-1089.

Fruebis, J., Tsao, T.S., Javorschi, S., Ebbets-Reed, D., Erickson, M.R., Yen, F.T., Bihain, B.E., and Lodish, H.F. (2001). Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proceedings of the National Academy of Sciences of the United States of America *98*, 2005-2010.

Gauthier, C., Tavernier, G., Charpentier, F., Langin, D., and Le Marec, H. (1996). Functional beta3-adrenoceptor in the human heart. The Journal of clinical investigation *98*, 556-562.

Ghaben, A.L., and Scherer, P.E. (2019). Adipogenesis and metabolic health. Nature reviews. Molecular cell biology *20*, 242-258.

Grujic, D., Susulic, V.S., Harper, M.E., Himms-Hagen, J., Cunningham, B.A., Corkey, B.E., and Lowell, B.B. (1997). Beta3-adrenergic receptors on white and brown adipocytes mediate beta3-selective agonist-induced effects on energy expenditure, insulin secretion, and food intake. A study using transgenic and gene knockout mice. The Journal of biological chemistry *272*, 17686-17693.

Halberg, N., Schraw, T.D., Wang, Z.V., Kim, J.Y., Yi, J., Hamilton, M.P., Luby-Phelps, K., and Scherer, P.E. (2009). Systemic fate of the adipocyte-derived factor adiponectin. Diabetes *58*, 1961-1970.

Halls, M.L., and Cooper, D.M. (2011). Regulation by Ca2+-signaling pathways of adenylyl cyclases. Cold Spring Harbor perspectives in biology *3*, a004143.

Hampe, L., Radjainia, M., Xu, C., Harris, P.W.R., Bashiri, G., Goldstone, D.C., Brimble, M.A., Wang, Y., and Mitra, A.K. (2015a). Regulation and Quality Control of Adiponectin Assembly by Endoplasmic Reticulum Chaperone ERp44. The Journal of biological chemistry *290*, 18111-18123.

Hampe, L., Radjainia, M., Xu, C., Harris, P.W.R., Bashiri, G., Goldstone, D.C., Brimble, M.A., Wang, Y., and Mitra, A.K. (2015b). Regulation and Quality Control of Adiponectin Assembly by Endoplasmic Reticulum Chaperone ERp44 *. J. Biol. Chem. *290*, 18111-18123.

Hara, K., Horikoshi, M., Yamauchi, T., Yago, H., Miyazaki, O., Ebinuma, H., Imai, Y., Nagai, R., and Kadowaki, T. (2006). Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. Diabetes care *29*, 1357-1362.

Hastoy, B., Scotti, P.A., Milochau, A., Fezoua-Boubegtiten, Z., Rodas, J., Megret, R., Desbat, B., Laguerre, M., Castano, S., Perrais, D., et al. (2017). A Central Small Amino Acid in the VAMP2 Transmembrane Domain Regulates the Fusion Pore in Exocytosis. Scientific reports *7*, 2835.

Hellström, L., Rössner, S., Hagström-Toft, E., and Reynisdottir, S. (1997). Lipolytic catecholamine resistance linked to alpha 2-adrenoceptor sensitivity--a metabolic predictor of

weight loss in obese subjects. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity *21*, 314-320.

Hoffstedt, J., Arvidsson, E., Sjölin, E., Wåhlén, K., and Arner, P. (2004). Adipose tissue adiponectin production and adiponectin serum concentration in human obesity and insulin resistance. The Journal of clinical endocrinology and metabolism *89*, 1391-1396.

Holz, G.G., Kang, G., Harbeck, M., Roe, M.W., and Chepurny, O.G. (2006). Cell physiology of cAMP sensor Epac. J Physiol *577*, 5-15.

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., et al. (2000). Plasma Concentrations of a Novel, Adipose-Specific Protein, Adiponectin, in Type 2 Diabetic Patients. Arteriosclerosis, Thrombosis, and Vascular Biology *20*, 1595-1599.

Hu, E., Liang, P., and Spiegelman, B.M. (1996). AdipoQ is a novel adipose-specific gene dysregulated in obesity. The Journal of biological chemistry *271*, 10697-10703.

Hug, C., Wang, J., Ahmad, N.S., Bogan, J.S., Tsao, T.-S., and Lodish, H.F. (2004). T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. Proceedings of the National Academy of Sciences *101*, 10308-10313.

Ibrahim, M.M. (2010). Subcutaneous and visceral adipose tissue: structural and functional differences. Obesity reviews : an official journal of the International Association for the Study of Obesity *11*, 11-18.

Iwabu, M., Okada-Iwabu, M., Yamauchi, T., and Kadowaki, T. (2019). Adiponectin/AdipoR Research and Its Implications for Lifestyle-Related Diseases. Frontiers in cardiovascular medicine *6*, 116.

Jo, J., Gavrilova, O., Pack, S., Jou, W., Mullen, S., Sumner, A.E., Cushman, S.W., and Periwal, V. (2009). Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. PLoS computational biology *5*, e1000324.

Kaisanlahti, A., and Glumoff, T. (2019). Browning of white fat: agents and implications for beige adipose tissue to type 2 diabetes. Journal of physiology and biochemistry *75*, 1-10.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E., and Graybiel, A.M. (1998). A family of cAMP-binding proteins that directly activate Rap1. Science (New York, N.Y.) *282*, 2275-2279.

Kelly, K.L., Deeney, J.T., and Corkey, B.E. (1989). Cytosolic Free Calcium in Adipocytes: Distinct mechanisms of regulation and effects on insulin action. J. Biol. Chem. *264*, 12754-12757.

Kershaw, E.E., and Flier, J.S. (2004). Adipose tissue as an endocrine organ. The Journal of clinical endocrinology and metabolism *89*, 2548-2556.

Kharroubi, I., Rasschaert, J., Eizirik, D.L., and Cnop, M. (2003). Expression of adiponectin receptors in pancreatic β cells. Biochemical and Biophysical Research Communications *312*, 1118-1122.

Kits, K.S., and Mansvelder, H.D. (2000). Regulation of exocytosis in neuroendocrine cells: spatial organization of channels and vesicles, stimulus-secretion coupling, calcium buffers and modulation. Brain Research Reviews *33*, 78-94.

Komai, A.M., Brännmark, C., Musovic, S., and Olofsson, C.S. (2014). PKA-independent cAMP stimulation of white adipocyte exocytosis and adipokine secretion: modulations by Ca2+ and ATP. The Journal of Physiology *592*, 5169-5186.

Komai, A.M., Musovic, S., Peris, E., Alrifaiy, A., El Hachmane, M.F., Johansson, M., Wernstedt Asterholm, I., and Olofsson, C.S. (2016). White Adipocyte Adiponectin Exocytosis Is Stimulated via beta3-Adrenergic Signaling and Activation of Epac1: Catecholamine Resistance in Obesity and Type 2 Diabetes. Diabetes *65*, 3301-3313.

Komai&Musovic., Komai, A.M., Musovic, S., Peris, E., Alrifaiy, A., El Hachmane, M.F., Johansson, M., Wernstedt Asterholm, I., and Olofsson, C.S. (2016). White Adipocyte Adiponectin Exocytosis Is Stimulated via beta3-Adrenergic Signaling and Activation of Epac1: Catecholamine Resistance in Obesity and Type 2 Diabetes. Diabetes *65*, 3301-3313.

Krief, S., Lönnqvist, F., Raimbault, S., Baude, B., Van Spronsen, A., Arner, P., Strosberg, A.D., Ricquier, D., and Emorine, L.J. (1993). Tissue distribution of beta 3-adrenergic receptor mRNA in man. The Journal of clinical investigation *91*, 344-349.

Laplante, M.-A., Monassier, L., Freund, M., Bousquet, P., and Gachet, C. (2010). The Purinergic P2Y1 Receptor Supports Leptin Secretion in Adipose Tissue. Endocrinology *151*, 2060-2070.

Lavie, C.J., Milani, R.V., and Ventura, H.O. (2009). Obesity and cardiovascular disease: risk factor, paradox, and impact of weight loss. Journal of the American College of Cardiology *53*, 1925-1932.

Lee, H., Jun, D.-J., Suh, B.-C., Choi, B.-H., Lee, J.-H., Do, M.-S., Suh, B.-S., Ha, H., and Kim, K.-T. (2005). Dual Roles of P2 Purinergic Receptors in Insulin-stimulated Leptin Production and Lipolysis in Differentiated Rat White Adipocytes. J. Biol. Chem. *280*, 28556-28563.

Lee, M.-J., Wu, Y., and Fried, S.K. (2013). Adipose tissue heterogeneity: Implication of depot differences in adipose tissue for obesity complications. Molecular Aspects of Medicine *34*, 1-11.

Lim, C.-Y., Hong, W., and Han, W. (2015). Adiponectin is released via a unique regulated exocytosis pathway from a pre-formed vesicle pool on insulin stimulation. Biochemical Journal *471*, 381-389.

Liu, M., Chen, H., Wei, L., Hu, D., Dong, K., Jia, W., Dong, L.Q., and Liu, F. (2015). Endoplasmic reticulum (ER) localization is critical for DsbA-L protein to suppress ER stress and adiponectin down-regulation in adipocytes. The Journal of biological chemistry *290*, 10143-10148.

Liu, M., Zhou, L., Xu, A., Lam, K.S.L., Wetzel, M.D., Xiang, R., Zhang, J., Xin, X., Dong, L.Q., and Liu, F. (2008). A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. Proceedings of the National Academy of Sciences *105*, 18302-18307.

Lohse, M.J., Engelhardt, S., and Eschenhagen, T. (2003). What Is the Role of β-Adrenergic Signaling in Heart Failure? Circulation research *93*, 896-906.

Mattsson, C.L., Csikasz, R.I., Chernogubova, E., Yamamoto, D.L., Hogberg, H.T., Amri, E.Z., Hutchinson, D.S., and Bengtsson, T. (2011). β_1-Adrenergic receptors increase UCP1 in human MADS brown adipocytes and rescue cold-acclimated β_3-adrenergic receptor-knockout mice via nonshivering thermogenesis. American journal of physiology. Endocrinology and metabolism *301*, E1108-1118.

Meyer, L.K., Ciaraldi, T.P., Henry, R.R., Wittgrove, A.C., and Phillips, S.A. (2013). Adipose tissue depot and cell size dependency of adiponectin synthesis and secretion in human obesity. Adipocyte *2*, 217-226.

Mondal, A.K., Das, S.K., Varma, V., Nolen, G.T., McGehee, R.E., Elbein, S.C., Wei, J.Y., and Ranganathan, G. (2012). Effect of endoplasmic reticulum stress on inflammation and

adiponectin regulation in human adipocytes. Metabolic syndrome and related disorders *10*, 297-306.

Musovic, S., and Olofsson, C.S. (2019). Adrenergic stimulation of adiponectin secretion in visceral mouse adipocytes is blunted in high-fat diet induced obesity. Scientific reports *9*, 10680.

Ouchi, N., Kihara, S., Funahashi, T., Nakamura, T., Nishida, M., Kumada, M., Okamoto, Y., Ohashi, K., Nagaretani, H., Kishida, K., et al. (2003). Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. Circulation *107*, 671-674.

Pajvani, U.B., Du, X., Combs, T.P., Berg, A.H., Rajala, M.W., Schulthess, T., Engel, J., Brownlee, M., and Scherer, P.E. (2003). Structure-Function Studies of the Adipocyte-secreted Hormone Acrp30/Adiponectin: IMPLICATIONS FOR METABOLIC REGULATION AND BIOACTIVITY *. J. Biol. Chem. *278*, 9073-9085.

Pajvani, U.B., Hawkins, M., Combs, T.P., Rajala, M.W., Doebber, T., Berger, J.P., Wagner, J.A., Wu, M., Knopps, A., Xiang, A.H., et al. (2004). Complex Distribution, Not Absolute Amount of Adiponectin, Correlates with Thiazolidinedione-mediated Improvement in Insulin Sensitivity *. J. Biol. Chem. *279*, 12152-12162.

Parsons, T.D., Coorssen, J.R., Horstmann, H., and Almers, W. (1995). Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. Neuron *15*, 1085-1096.

Phillips, S.A., Kung, J., Ciaraldi, T.P., Choe, C., Christiansen, L., Mudaliar, S., and Henry, R.R. (2009). Selective regulation of cellular and secreted multimeric adiponectin by antidiabetic therapies in humans. American Journal of Physiology-Endocrinology and Metabolism *297*, E767-E773.

Qiang, L., Wang, H., and Farmer, S.R. (2007). Adiponectin secretion is regulated by SIRT1 and the endoplasmic reticulum oxidoreductase Ero1-L alpha. Molecular and cellular biology *27*, 4698-4707.

Rasmussen, M.S., Lihn, A.S., Pedersen, S.B., Bruun, J.M., Rasmussen, M., and Richelsen, B. (2006). Adiponectin receptors in human adipose tissue: effects of obesity, weight loss, and fat depots. Obesity *14*, 28-35.

Revelli, J.P., Muzzin, P., Paoloni, A., Moinat, M., and Giacobino, J.P. (1993). Expression of the beta 3-adrenergic receptor in human white adipose tissue. Journal of molecular endocrinology *10*, 193-197.

Reynisdottir, S., Wahrenberg, H., Carlström, K., Rössner, S., and Arner, P. (1994). Catecholamine resistance in fat cells of women with upper-body obesity due to decreased expression of beta 2-adrenoceptors. Diabetologia *37*, 428-435.

Rothman, J.E. (2014). The Principle of Membrane Fusion in the Cell (Nobel Lecture). Angewandte Chemie International Edition *53*, 12676-12694.

Rutkowski, J.M., Stern, J.H., and Scherer, P.E. (2015). The cell biology of fat expansion. The Journal of cell biology *208*, 501-512.

Scherer, P.E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H.F. (1995). A Novel Serum Protein Similar to C1q, Produced Exclusively in Adipocytes (*). J. Biol. Chem. *270*, 26746-26749.

Schraw, T., Wang, Z.V., Halberg, N., Hawkins, M., and Scherer, P.E. (2008). Plasma Adiponectin Complexes Have Distinct Biochemical Characteristics. Endocrinology *149*, 2270-2282.

Seino, S., and Shibasaki, T. (2005). PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev *85*, 1303-1342.

Seydoux, J., Muzzin, P., Moinat, M., Pralong, W., Girardier, L., and Giacobino, J.P. (1996). Adrenoceptor heterogeneity in human white adipocytes differentiated in culture as assessed by cytosolic free calcium measurements. Cellular signalling *8*, 117-122.

Shrestha, M.M., Barg, S., and Olofsson, C.S. (2022). Real-time TIRF imaging of exocytosis in 3T3-L1 adipocytes stably expressing mCherry fused to human adiponectin. bioRxiv, 2022.2009.2013.507762.

Smith, G.I., Mittendorfer, B., and Klein, S. (2019). Metabolically healthy obesity: facts and fantasies. The Journal of clinical investigation *129*, 3978-3989.

Spranger, J., Kroke, A., Möhlig, M., Bergmann, M.M., Ristow, M., Boeing, H., and Pfeiffer, A.F. (2003). Adiponectin and protection against type 2 diabetes mellitus. Lancet *361*, 226-228.

Stahl, A., Evans, J.G., Pattel, S., Hirsch, D., and Lodish, H.F. (2002). Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. Developmental cell *2*, 477-488.

Stenkula, K.G., and Erlanson-Albertsson, C. (2018). Adipose cell size: importance in health and disease. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology *315*, R284-R295.

Susulic, V.S., Frederich, R.C., Lawitts, J., Tozzo, E., Kahn, B.B., Harper, M.E., Himms-Hagen, J., Flier, J.S., and Lowell, B.B. (1995). Targeted disruption of the beta 3-adrenergic receptor gene. The Journal of biological chemistry *270*, 29483-29492.

Szkudelski, T., Nogowski, L., and Szkudelska, K. (2011). Short-term regulation of adiponectin secretion in rat adipocytes. Physiological research *60*, 521-530.

Tate, K.M., Briend-Sutren, M.M., Emorine, L.J., Delavier-Klutchko, C., Marullo, S., and Strosberg, A.D. (1991). Expression of three human beta-adrenergic-receptor subtypes in transfected Chinese hamster ovary cells. Eur J Biochem *196*, 357-361.

Taylor, C.W., and Tovey, S.C. (2010). IP(3) receptors: toward understanding their activation. Cold Spring Harbor perspectives in biology *2*, a004010.

Tozzi, M., Hansen, J.B., and Novak, I. (2020). Pannexin-1 mediated ATP release in adipocytes is sensitive to glucose and insulin and modulates lipolysis and macrophage migration. Acta physiologica (Oxford, England) *228*, e13360.

Tozzi, M., and Novak, I. (2017). Purinergic Receptors in Adipose Tissue As Potential Targets in Metabolic Disorders. Frontiers in Pharmacology *8*.

Trujillo, M.E., and Scherer, P.E. (2006). Adipose tissue-derived factors: impact on health and disease. Endocrine reviews *27*, 762-778.

Tsao, T.S., Tomas, E., Murrey, H.E., Hug, C., Lee, D.H., Ruderman, N.B., Heuser, J.E., and Lodish, H.F. (2003). Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. The Journal of biological chemistry *278*, 50810-50817.

Vargovic, P., Ukropec, J., Laukova, M., Cleary, S., Manz, B., Pacak, K., and Kvetnansky, R. (2011). Adipocytes as a new source of catecholamine production. FEBS Letters *585*, 2279-2284.

Waki, H., Yamauchi, T., Kamon, J., Ito, Y., Uchida, S., Kita, S., Hara, K., Hada, Y., Vasseur, F., Froguel, P., et al. (2003). Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. The Journal of biological chemistry *278*, 40352-40363.

Wang, Y., Lam, K.S., Chan, L., Chan, K.W., Lam, J.B., Lam, M.C., Hoo, R.C., Mak, W.W., Cooper, G.J., and Xu, A. (2006). Post-translational modifications of the four conserved lysine

residues within the collagenous domain of adiponectin are required for the formation of its high molecular weight oligomeric complex. The Journal of biological chemistry *281*, 16391-16400.

Wang, Y., Xu, A., Knight, C., Xu, L.Y., and Cooper, G.J. (2002). Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. The Journal of biological chemistry *277*, 19521-19529.

Wang, Z.V., and Scherer, P.E. (2016). Adiponectin, the past two decades. J Mol Cell Biol *8*, 93-100.

Wang, Z.V., Schraw, T.D., Kim, J.Y., Khan, T., Rajala, M.W., Follenzi, A., and Scherer, P.E. (2007). Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. Molecular and cellular biology *27*, 3716-3731.

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R.E., and Tataranni, P.A. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. The Journal of clinical endocrinology and metabolism *86*, 1930-1935.

Willoughby, D., and Cooper, D.M. (2006). Ca2+ stimulation of adenylyl cyclase generates dynamic oscillations in cyclic AMP. J Cell Sci *119*, 828-836.

Xie, L., O'Reilly, C.P., Chapes, S.K., and Mora, S. (2008). Adiponectin and leptin are secreted through distinct trafficking pathways in adipocytes. Biochimica et biophysica acta *1782*, 99-108.

Yamauchi, T., and Kadowaki, T. (2008). Physiological and pathophysiological roles of adiponectin and adiponectin receptors in the integrated regulation of metabolic and cardiovascular diseases. Int J Obes (Lond) *32 Suppl 7*, S13-18.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., et al. (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature *423*, 762-769.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., et al. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nature medicine *8*, 1288-1295.

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., et al. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nature medicine *7*, 941-946.

Youngstrom, T.G., and Bartness, T.J. (1995). Catecholaminergic innervation of white adipose tissue in Siberian hamsters. The American journal of physiology *268*, R744-751.

Zhou, L., Liu, M., Zhang, J., Chen, H., Dong, L.Q., and Liu, F. (2010). DsbA-L Alleviates Endoplasmic Reticulum Stress–Induced Adiponectin Downregulation. Diabetes *59*, 2809-2816.