Effects of Impaired Glucose Tolerance on Steady-State Expression Levels of Murine Cytohesin 2 and Cytohesin 3 mRNA in Liver

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List of Abbreviations

ACTH	adrenocorticotropic hormone
aPKC	atypical forms of protein kinase C
ARF	adenosine diphosphate ribosylation factor
AS160	Akt substrate of 160 kDa
AU	arbitrary units
BAD	bcl-2-associated death promoter
BMI	body mass index
bp	base pair
C	control (group)
CER	ceramide
CHF	Swiss francs
CT	cycle threshold
DAG	diacylglycerol
DIO	diet-induced obesity
EDTA	ethylenediamintetraacetic acid
ERK	extra cellular signal regulated kinase
FFA	free fatty acid
FOXO1	forkhead box protein O1
FOXO3	forkhead box protein O3
HF	high-fat diet (group)
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GLUT4	glucose transporter type 4
GRB2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
HDL	high density lipoprotein
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IL-1	interleukin 1
IL-6	interleukin 6
IR	insulin receptor
IRS	insulin receptor substrate
kDa	kilo Dalton
LDL	low density lipoprotein
MAPK	mitogen-activated protein kinase
m-pscd	murine pleckstrin homology, Sec7, and coiled-coil domains protein
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MUFA	monounsaturated fatty acid
NGDP	national gross domestic product
NP-40	nonyl phenoxypolyethoxylethanol
PDK1	3-phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKC	protein kinase C

PTEN	phosphatase and tensin homologue
PUFA	polyunsaturated fatty acid
qRT-PCR	quantitative real-time polymerase chain reaction
RIPA	radioimmunoprecipitation assay
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamid gel electrophoresis
SEM	standard error of the mean
SFA	saturated fatty acid
SH2	Src homology 2
SHC	Src homology 2 domain containing transforming protein
SHIP	SH2 domain-containing inositol 5'phosphatase
SOS	son of sevenless homolog
SREBP1c	sterol regulatory element-binding protein 1c
T2DM	type 2 diabetes mellitus
TFA	trans-fatty acid
TNFα	tumor necrosis factor alpha
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
TSC	tuberous sclerosis complex
VHF	very high-fat diet (group)

1. Summary

Impaired fasting glucose and impaired glucose tolerance constitute pre-diabetic states that can continuously proceed to insulin resistance, the pre-eminent condition in type 2 diabetes mellitus (T2DM). Further deterioration of glucose homeostasis can manifest itself as T2DM. The liver, the predominant source of glucose production, plays a central role in glucose homeostasis. Hepatic insulin resistance and steatosis result from elevated free fatty acid (FFA) blood levels and lead to increased hepatic glucose output. A simple way of increasing FFA blood levels is ensuring excessive caloric intake for the organism. Thus, diet-induced obesity is being used as a model for insulin resistance. Insulin regulates the carbohydrate and fat metabolism of the body. The complexity of the insulin signal transduction is reflected in the vast amount and diversity of involved signaling components. Mechanisms of insulin signaling are described including the beginning of insulin signaling, where the signaling molecules cytohesin 2 and cytohesin 3 come into play. Cytohesins are a subfamily of small guanine nucleotide exchange factors (GEF) that share a common domain organization. Inhibition of cytohesin 3, a regulator of hepatic insulin signaling, was shown to lead to hepatic insulin resistance.

This dissertation examines effects of high-fat diet feeding on cytohesin 2 and cytohesin 3 mRNA steady-state expression levels and cytohesin 3 protein expression levels in murine liver tissue using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. I analyzed livers of mice derived from a study in which effects of high-fat diet on the cardiovascular system was analyzed in a mouse model. Mice were fed control (C), high-fat (HF), and very high-fat (VHF) diets for 15 weeks, which resulted in overweight and impaired glucose tolerance in the groups with elevated fat intake. Relative expression levels for cytohesin 2, cytohesin 3 and β2 microglobulin (as house keeping gene) were determined by qRT-PCR. Correct primer

design for cytohesin 2 was controlled by sequencing of the qRT-PCR product. The primer product of cytohesin 3 had to be inserted into a plasmid before it could be sequenced successfully. Proteins were also extracted from the mouse liver samples, separated on SDS-PAGE gel and immunoblotted with anti-cytohesin 2 and cytohesin 3 antibodies and anti-p44/42 antibodies.

I found that there is approximately seven times more cytohesin 2 than cytohesin 3 mRNA expressed in murine livers. Only the VHF group exhibited significant but small increases in cytohesin 2 and cytohesin 3 mRNA expression levels compared with control diet fed mice (24 and 37 %, respectively). Cytohesin 3 protein expression levels were not affected by high-fat diet feeding.

In summary in light of current T2DM treatment recommendations and fading scientific interest in cytohesins with respect to insulin signaling, my data support the notion that expressional control of cytohesins is not essential for insulin signaling in the conditions analyzed. Literature data suggest that ectopic fat accumulation (especially in the liver) and chronic low-grade inflammation generated by adipose tissue macrophages are important factors that contribute to high-fat diet-induced impaired glucose tolerance and later stages of T2DM.

2. Introduction

2.0. Type 2 Diabetes Mellitus

All mammalian cells use glucose as a fuel for their basic functions [1]. In order to maintain stable glucose levels, an intricate system of neural, hormonal and direct nutrient responses are initiated after stress (exercise or hypoglycemia) or a meal [1]. Diabetes mellitus is a syndrome encompassing heterogeneous diseases that share a disorder of glucose metabolism with chronic hyperglycemia as consequence of absolute or relative, i.e. functional, insulin insufficiency [2]. Impaired fasting glucose and impaired glucose tolerance constitute pre-diabetic states that can proceed to insulin resistance, which means that target tissues do not respond effectively to normal insulin levels. Inhibition of the insulin signaling pathway leads to insulin resistance. Diabetes mellitus is mainly divided in type 1 and type 2 diabetes. Type 1 diabetes is characterized by primary destruction of pancreatic Langerhans β-cells, the production and secretory site of insulin [2]. Insulin resistance is the pre-eminent condition in type 2 diabetes mellitus (T2DM) and can lead to secondary failure of Langerhans β-cells. Diabetes is generally associated with insulin resistance in the main target tissues, including liver, muscle, and fat but the hypothalamus is involved, also [3]. The etiology of T2DM consists of multiple genetic and environmental factors (foremost overnutrition and lack of exercise) [2, 4].

2.0.1. Obesity, Liver and T2DM

Obesity is defined in the ICD-10-code by a body mass index (BMI) greater than 30 kg/m², whereas overweight starts at a BMI greater than 25 kg/m². The BMI is calculated by dividing body weight in *kg* by body height in *m* squared. The etiology or cause of obesity is an imbalance between the energy ingested and the energy expended [5]. The excess energy is stored in fat cells, adipocytes, that enlarge and/or increase in

number [5]. It is this hyperplasia and hypertrophy of fat cells that is the pathological lesion of obesity [5]. The weight or mass of extra fat, and the increased secretion of free fatty acids (FFA) and numerous peptides from enlarged adipocytes contribute to the clinical problems associated with obesity [5]. Among endocrine changes associated with obesity are decreased levels of growth hormone and adiponectin and increases in levels of insulin, leptin, thyroid stimulating hormone (in the upper normal range), androgens, progesterone, interleukin 6 (IL-6), ACTH/cortisol, and sympathetic nervous system activity [5]. IL-6, secreted in adipose tissue, is an important mediator of hepatic insulin resistance [6]. Obesity and increased fat mass are associated with adipose tissue inflammation [7]. Obese subjects have generally been found to be leptin resistant, thus the promoting of satiation in the hypothalamus is dysfunctional [8]. An intact functional leptin axis is necessary to prevent accumulation of lipids in non-adipose tissues that are not adapted for triglyceride storage [9]. Still, weight loss is associated with a reduction in adipocyte size, macrophage infiltration, and secretion of inflammatory factors, and an increase in adiponectin secretion [9]. Besides increased insulin secretion, insulin resistance results from obesity [5]. But increased insulin levels are found only before the endocrine production of insulin by the pancreas fails.

T2DM is strongly associated with overweight in both genders in all ethnic groups and typically is preceded by obesity [5, 10]. The risk of T2DM increases with the degree and duration of overweight [5]. Weight loss or moderating weight gain over years reduces the risk of developing diabetes [5]. There is evidence that obese individuals differ substantially during rest in brain networks related to reward and food regulation compared with lean subjects because obesity and insulin levels influence brain function [11]. Studies demonstrate that increasing lipid oxidation in the liver prevents hepatic steatosis and insulin resistance [12]. Important aspects in T2DM treatment are lifestyle changes: foremost weight reduction through calorie reduction in overweight

patients and increased, appropriately prescribed physical exercise which increases insulin sensitivity in skeletal muscles [13, 14].

The amount of fat in a meal required to result in significant elevations in plasma triglycerides, the hallmark of postprandial lipemia, is in the order of 30–50 g, well within the range of many usual meals [15]. It seems that the higher the BMI the greater the postprandial lipemic response [16]. Interestingly, fat quality appears to have a greater impact on postprandial lipemia than fat quantity, because ingested saturated fatty acids (SFA) have a greater impact on the postprandial triglyceride response than monounsaturated fatty acids (MUFA) and n-6 polyunsaturated fatty acids (PUFA), which contribute more to lipemia than n-3 PUFA [15]. But in the usual diet SFA and MUFA, not PUFA, are the most abundant fatty acids [15]. Reducing intake of TFA, as is recommended by the American Diabetes Association, lowers LDL cholesterol and increases HDL cholesterol [14]. Therefore, improving fat quality should be considered part of a dietary lifestyle strategy to prevent or manage T2DM [17].

The magnitude of the risk of diabetes contributed by obesity appears to be much greater than that imparted by physical inactivity [15]. A one-year long lifestyle (diet and exercise) intervention in T2DM patients lead to greatly improved levels of peripheral insulin sensitivity, fasting glucose, and FFA in parallel to significant weight and fat loss, improvement in adipose tissue distribution, and decrease in hepatic fat [18]. It appears that the degree of adipose tissue inflammation rather than the level of adiposity is more closely related to liver fat [9]. The changes in peripheral insulin sensitivity were best predicted by the overall change in weight and fat mass; interestingly, the only regional fat measure independently predicting metabolic improvements was the decrease in hepatic fat [18].

The liver is the predominant source of glucose production, with small contributions by the kidney and the gut while peripheral tissues (skeletal muscle and fat) and the central nervous system remove glucose from the plasma especially [1]. While an insulin sensitive

glucose transporter (GLUT4) is rate limiting in several peripheral tissues (muscle, fat), in the liver, however, glucose moves freely across the plasma membrane in either direction [1]. A positive correlation between anti-apoptotic mediators and hepatic mRNA levels of insulin receptor substrate 1 and 2 (IRS1 and IRS2) was noted, indicating that enhanced hepatocyte apoptosis in non-alcoholic steatohepatitis (NASH) and chronic hepatitis C virus (HCV) infection is closely associated with impairment of the hepatic insulin signaling pathway [19]. Moreover, there is a negative correlation between hepatic mRNA levels of IRS1/IRS2 and type I collagen, indicating that disruption of hepatic insulin signaling is associated with increased liver fibrogenesis in non-alcoholic fatty liver disease (NAFLD) and HCV infection [19]. Lemoine and Serfati consider insulin resistance being the cornerstone in the development of NAFLD, which includes simple steatosis and NASH [20]. But Valverde and Gonzalez-Rodriguez demonstrated that the hepatic insulin signaling cascade is normal in patients with simple steatosis; this could indicate that hepatic fat accumulation precedes disruption of hepatic insulin signaling at the molecular level [19]. The insulin-resistant state that is so common in human obesity probably reflects the effects of increased release of FFA from fat cells that are then stored in the liver or muscle [5]. Further was shown that fat accumulation in the liver is associated with features of the metabolic syndrome, regardless of intra-abdominal fat accumulation, fat accumulation in skeletal muscle, or BMI [21-23]. Hypertension, a widespread disease, is strongly associated with T2DM, impaired glucose tolerance, hypertriglyceridemia, and hypercholesterolemia [5].

The leading hypothesized mechanisms to explain insulin resistance and islet β-cell dysfunction in T2DM have been oxidative stress, endoplasmic reticulum stress, amyloid deposition in the pancreas, ectopic lipid deposition in the muscle, liver and pancreas, and lipotoxicity (toxic effects of elevated levels of FFA) and glucotoxicity (toxic effects of hyperglycemia) [24]. All of these stresses can be caused by overnutrition [24]. It is noteworthy, that each of these cellular stresses is also thought to either induce an

inflammatory response or to be exacerbated by or associated with inflammation [24]. Thus, both, obesity and T2DM, have an inflammatory component [24].

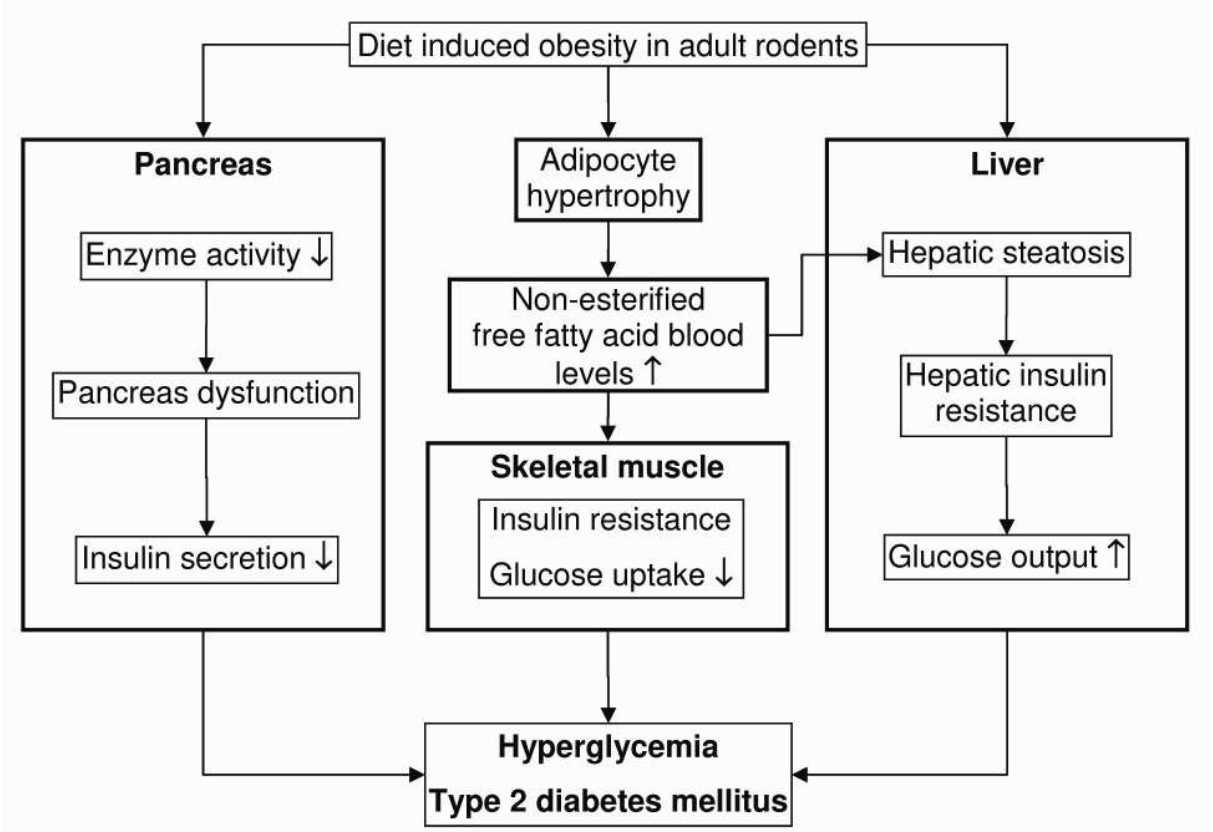

Figure 1. Effects of diet-induced obesity leading to T2DM in adult rodents. Adapted from [10].

The effects of diet-induced obesity (DIO) in rodents are summarized in figure 1: In the pancreas, DIO leads to a decreased pancreatic enzyme activity, followed by pancreatic dysfunction and decreased insulin secretion. Finally the progression to hyperglycemia and T2DM ensues.

Obesity leads to adipocyte hypertrophy and elevated blood concentrations of non-esterified FFA. These FFA are deposited in the liver and in skeletal muscle, leading to steatosis and insulin resistance of these tissues. The insulin resistance and the reduced glucose uptake in skeletal muscle are followed by hyperglycemia and T2DM.

DIO results in hepatic steatosis, also. This is succeeded by hepatic insulin resistance and an increased hepatic glucose output, which is followed by hyperglycemia and T2DM.

2.0.2. Metabolic Syndrome and T2DM

Diabetes mellitus is constituent of the metabolic syndrome. But internationally no agreement exists upon the criteria for metabolic syndrome [25]. Even the inclusion of diabetes mellitus in these criteria is being questioned [25]. Moreover, the concept of metabolic syndrome itself is subject to limitations, because the treatment of metabolic syndrome is no different than the treatment for each of its components, and the medical value of diagnosing it is unclear [25].

2.0.3. Epidemiology and Costs of T2DM

Type 1 diabetes affects 5% of the overall diabetic patients; the rest of diabetic syndromes, of which T2DM forms the major group, represent 95% of the entire diabetic population [26]. T2DM affects over 5% of the population in Western societies [26]. T2DM used to be referred to as adult-onset diabetes. Today this name has become obsolete, since the condition is being diagnosed in children more frequently in recent years, especially in obese children [27, 28].

Between 1992 and 2007 there was a considerable increase from 30.3% to 37.3% of the Swiss population that suffered from overweight [29]. Based on data from 2007 and 2008 the prevalence of adult overweight in the USA was 68.3% – almost doubling the prevalence in the Swiss population [30]. The most recent estimate for overweight prevalence in the U.S. amounted to 69.2% [31]. The total (obesity-linked) disease costs of overweight and obesity in Switzerland more than doubled from CHF 2.648 billions (cost basis 2001) to CHF 5.755 billions (cost basis 2006) [29]. For the cost estimate reported in 2004 T2DM contributed 54.5% of the total obesity-linked disease costs [29].The total attributable cost of T2DM amounted to CHF 2.070 billions in 2006 (36% of the total obesity-linked disease costs) [29]. From 26 reported obesity-associated diseases, T2DM is the leading comorbidity carrying the highest risk (relative risk ≥ 3.4) of developing in the course of body weight increase [29]. Clearly, the growing prevalence

of obesity is the major factor driving the increasing prevalence of T2DM [29]. Consequently, the number of adults with T2DM is predicted to almost double on a worldwide scale, from approximately 171 million in 2000 to 366 million by the year 2030 [29]. It can be expected that the prevalence of T2DM in Switzerland in the coming years will increase in parallel with the observed increase in prevalence of overweight and obesity in the period from 1992 to 2007 [29]. In Switzerland health care costs amounted to 10.8% of the national gross domestic product (NGDP) of CHF 486.2 billions in 2006 [29]. The total costs of T2DM added up to 4.3‰ of the NGDP or 2.3% of health care costs [29]. In comparison, in the USA prevalence of diabetes continues to grow, too, leading to a higher economic burden [32]. There, approximately 10% of health care costs were attributed to diabetes in 2007 [32]. This relatively high quota in health care costs can be explained, at least in part, with the high prevalence in overweight.

2.0.4. T2DM and Pregnancy

As obesity reaches pandemic proportions, of particular concern is the increase in obesity among women of reproductive age [33]. Because there is strong evidence from both human and animal studies that the early environment and in particular early nutrition play an important role [34]. So individuals exposed *in utero* to either maternal obesity or gestational diabetes mellitus have increased risk of developing T2DM, hypertension, obesity and other features of metabolic syndrome in later life [33]. But poor growth *in utero* is associated with increased risk of developing diseases such as T2DM in later life, also [34]. Additionally to T2DM, similar relationships have been observed linking birth weight to other conditions such as cardiovascular disease, insulin resistance, and other features of metabolic syndrome [34]. Interestingly, the detrimental effects of poor fetal growth on long-term metabolic health appear to be exaggerated if followed by accelerated postnatal growth and/or obesity [10, 34]. Concerning the risk of obesity, accelerated postnatal growth appears to be particularly detrimental even in individuals

who had a normal birth weight [34]. Human twin studies provide strong evidence for the importance of a nongenetic intrauterine factor in the development of T2DM in later life [34]. It has been shown that nutritional influences in early life can induce permanent alterations in epigenetic and histone modifications [34]. Studies revealed that individuals who were exposed to famine *in utero* during the Dutch Hunger Winter had altered methylation of the insulin-like growth factor 2 gene in white blood cells in adulthood – thus maternal diet can influence epigenetic marks in humans [34]. An alternative mechanism by which environmental factors at critical periods of development could have long-term phenotypic consequences is through permanent structural changes in key organs [34]. If a certain nutrient or hormone is essential at a critical period of development for growth and differentiation of a tissue, inappropriate levels of this factor will have permanent structural consequences [34]. Taken together, there is now little doubt that fetal and early postnatal life are important time periods for the determination of future risk of T2DM, obesity, and other features of the metabolic syndrome [34].

2.1. Insulin Signaling

2.1.1. Insulin

Insulin, an anabolic hormone, is produced and secreted into the bloodstream by the Langerhans β-cells in the endocrine pancreas islets. The most important stimulus for insulin synthesis and secretion is an increase in blood sugar level [35]. Its function constitutes the regulation of the carbohydrate and the fat metabolism of the body. It promotes processes that affect a decrease of blood sugar level, such as stimulating glucose uptake in skeletal muscle and the liver, supporting glycogenesis and inhibiting glycogenolysis, promoting lipogenesis and inhibiting lipolysis, and stimulating protein synthesis (figure 2). Insulin stimulates lipogenesis in fat tissue, as well as the liver, by activating acetyl-coA carboxylase, the rate-limiting enzyme in fatty acid synthesis [8]. Upon fasting, lipogenesis is inhibited and acetyl-coA carboxylase is inactivated as a result of low insulin levels, as well as the effects of catecholamines and glucagon [8].

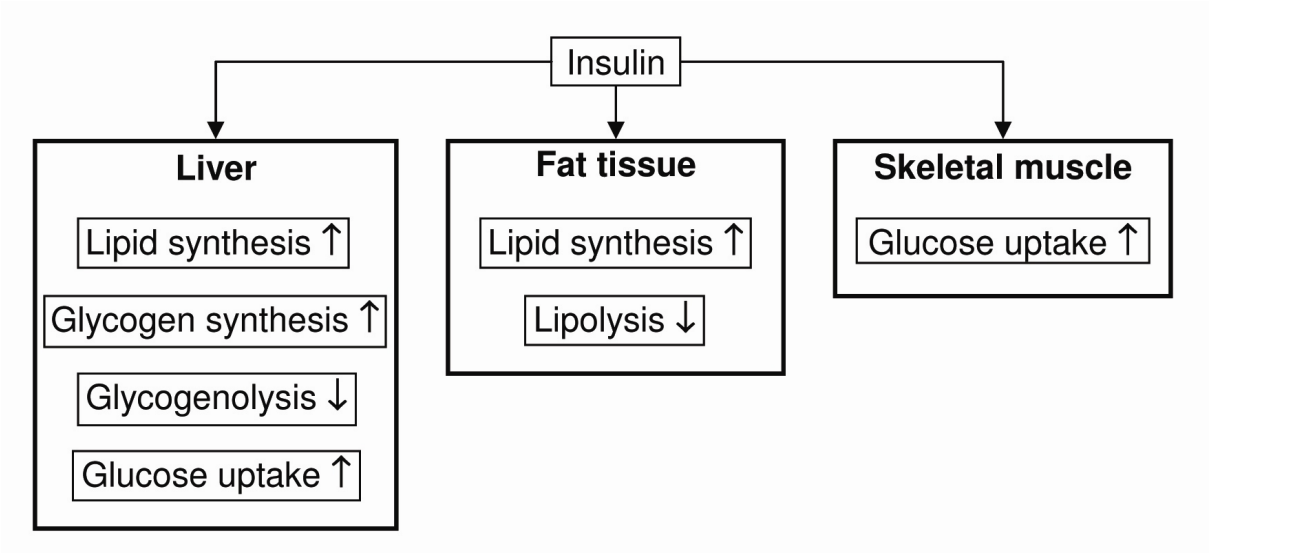

Figure 2. Insulin action in main target tissues. ↑up-regulation, ↓down-regulation.

Insulin has a higher potency in suppressing glycogenolysis versus gluconeogenesis, yet increased gluconeogenesis is the major contributor to fasting hyperglycemia in humans who have T2DM [36]. Insulin also regulates nonmetabolic processes such as cell growth and differentiation and is a growth factor during fetal development [3, 8]. Insulin signaling has

been found to regulate dopamine neurotransmission and to affect the ability of drugs that target the dopamine system to exert their neurochemical and behavioral effects [37]. Main target tissues of insulin are skeletal muscles, fat tissue and the liver, but other tissues, i.e. hypothalamus, are influenced by insulin, also [6, 35]. The effects of insulin on glycogenesis and glycogenolysis are antagonised by glucagon [35]. In contrast to other mammals (such as humans and guinea pigs), murine rodents express two insulin genes instead of one [38].

Oral glucose uptake in a healthy mammal is followed by typical changes in blood glucose concentration: A rise in blood glucose in the absorptive phase stimulates the release of insulin from pancreatic β-cells and hepatic glucose output is inhibited through the Akt/FOXO1 signaling pathway by suppressing transcriptional activation of genes encoding enzymes in the gluconeogenic pathway [1, 8, 19]. Half an hour to an hour after glucose ingestion the blood glucose zenith is reached [1]. Glucose removal processes operating at maximal rates combined with inhibited hepatic glucose output lead to a rapid fall of blood glucose, reaching levels below the original baseline and finally settling at baseline level [1]. This hypoglycemic phase is due to the inertia of the regulatory mechanisms because, in general, the higher the glycemia the greater the subsequent hypoglycemia [1].

2.1.2. Mechanisms of Insulin Signaling

The insulin signaling cascade is complexly build, which is illustrated by the fact that even in the first three signaling steps in the metabolic pathway of insulin action (from insulin receptor (IR) to insulin receptor substrate (IRS) to Akt), there are at least 288 combinations of signaling molecules [3]. Taking into account other pathways and those not yet discovered, the number of signaling combinations probably exceeds 1000 [3]. Thus, in theory, diminishing the activity of any single component of the insulin signaling cascade or any combination of components may produce a distinct type of insulin resistance with its own molecular signature [3]. Further, all signaling components can undergo posttranslational modifications that decrease or increase their activity [3]. On top of that the signaling pathways of insulin

and insulin-like growth factor 1 (IGF1) are very similar [8]. This is reflected in the existence of insulin/IGF hybrid receptors [39].

There are several known insulin signaling pathways. For all of them, the first step in insulin signaling is the docking of insulin to the IR [19]. This induces a conformational change, which activates the tyrosine kinase activity on the cytoplasmic part of the cell and results in autophosphorylation of the IR and phosphorylation of other protein molecules [8, 19]. The activation of the IR by insulin results in the tyrosine phosphorylation of the immediate substrates of the insulin receptor kinase, members of the IRS family, IRS1 and IRS2 (figure 3, in red) [6, 8, 19, 40]. This initiates two major signaling cascades: the mitogenic and the metabolic pathways (figure 3, green and blue pathways, respectively) [33, 41]. The IR and the IRS proteins share common mechanisms of regulation: they are activated by tyrosine phosphorylation, and they are negatively regulated by protein tyrosine phosphatases (PTP), serine phosphorylation and ligand-induced down-regulation [41].

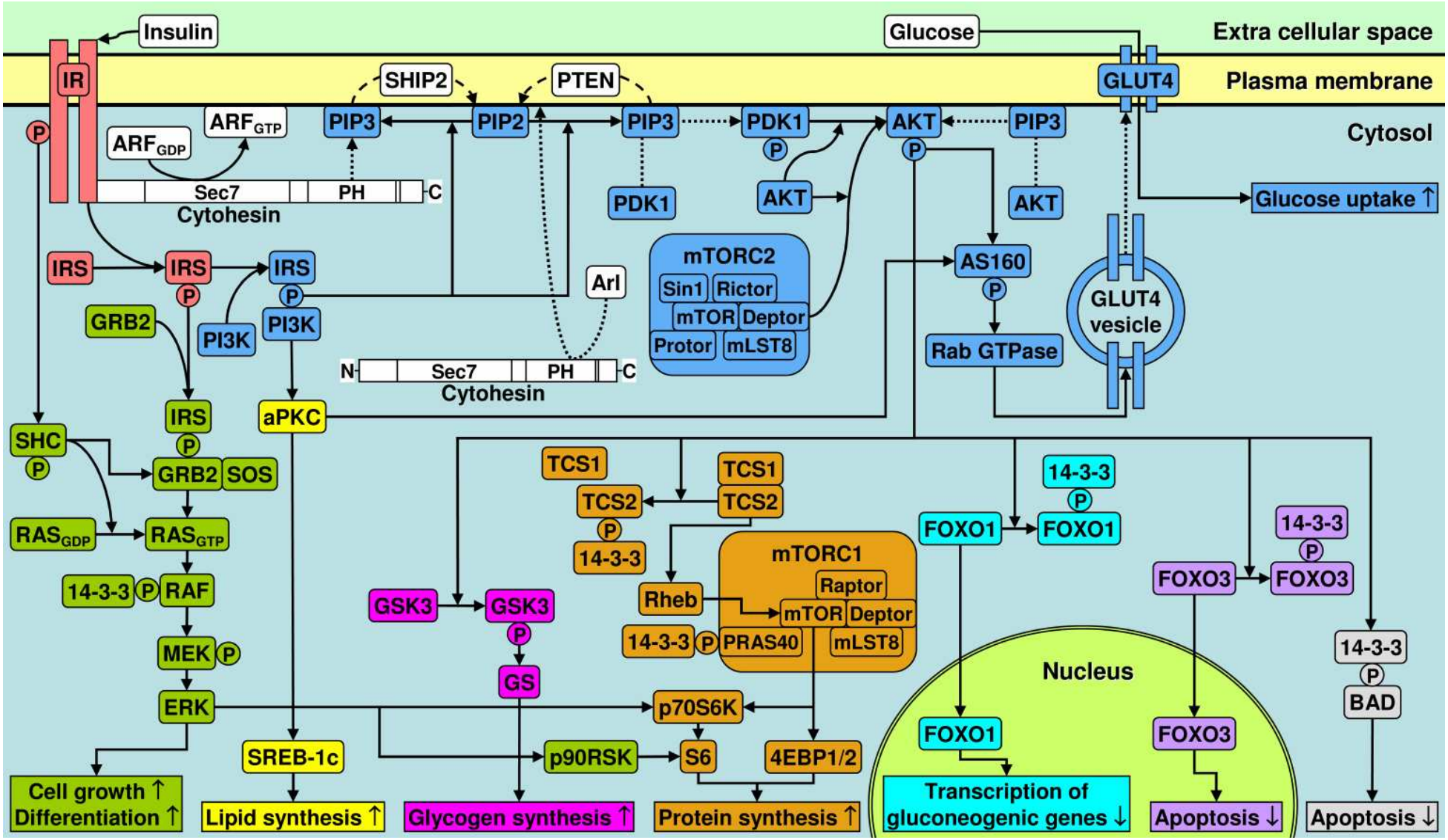

Figure 3. Signaling pathways for the main effects of insulin. Unbroken arrows stand for signal transduction. Dotted arrows imply translocation to the plasma membrane. Interrupted lines signify signaling inhibition. Cytohesin domains are explained in figure 4. ↑ up-regulation, ↓ down-regulation. Adapted from [3, 8, 39, 42-44]

2.1.2.1. Increasing Glucose Uptake

Insulin stimulates cellular glucose uptake through the following pathway: Phosphorylation of IRS proteins on tyrosine residues results in the recruitment of signaling molecules to these proteins [8]. This recruitment is typically mediated by phosphotyrosine binding motifs such as SH2 (Src homology 2) domains and allows the binding and activation of the phosphatidylinositol 3-kinase (PI3K) [3, 6, 8, 19]. Active PI3K exists as a heterodimer, consisting of a p110 catalytic subunit, of which there are three isoforms, and a regulatory subunit, of which there are eight known isoforms, p85α being the most abundant and ubiquitously expressed of them [3]. If the regulatory subunits of PI3K (p50α, p55α, p85α and β) are increased above physiological levels, they can act as inhibitors of the normal dimeric form of the enzyme [3]. p85α contains multiple domains that may mediate interactions with other proteins, including a src homology region 3 (SH3) domain, a bcr homology domain flanked by two proline-rich regions, and two SH2 domains that flank the p110 catalytic subunit-binding region [3]. In response to insulin, IRS1 and IRS2, as well as the p85 subunit of PI3K, migrate from the intracellular membranes to the cytosol [43]. Recruitment of PI3K to IRS1 results in the phosphorylation of membrane phosphoinositides, generating the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3) in the cell, which leads to membrane recruitment through its pleckstrin homology (PH) domain and phosphorylation and activation of a downstream signaling kinase, the serine/ threonine kinase Akt, via the 3-phosphoinositide-dependent protein kinase 1 (PDK1) [3, 6, 8, 26, 39-41]. In mammalian cells, PIP_3 is transiently accumulated in plasma membranes following activation of PI3K [45]. Negative regulation of the PI3K pathway is primarily accomplished through the action of the phosphatase and tensin homologue (PTEN) tumor suppressor proteins, but other phosphatases, such as SH2 domain-containing inositol 5'phosphatase (SHIP)-1 and SHIP-2, play a role, too [46]. PDK1 and PDK2 are recruited to the plasma membrane by PIP_3 and they are activated by

phosphatidylinositol 3-phosphates, particularly PIP3 [3, 26, 43]. Akt contains an amino-terminal PH domain that serves to target the protein to the membrane for activation, which depends on the phosphorylation of two of its residues [46]. Akt is not only phosphorylated and thusly activated by PDK1 but by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2), also, which used to be referred to as PDK2 [46]. Once activated, Akt leaves the cell membrane to phosphorylate intracellular substrates, which can be located in the nucleus [46]. One of these substrates is Akt substrate of 160 kDa (AS160), also known as TBC1D4 [3, 39, 47]. Phosphorylation by Akt inhibits the Rab-GTPase-activating protein AS160, resulting in activation of Rab small GTPases required for translocation of GLUT4, the predominant glucose transporter isoform expressed in muscle and fat tissues, from GLUT4 storage vesicles to the plasma membrane [40, 43]. Docking and fusion of GLUT4 storage vesicles with the plasma membrane is regulated by insulin [40]. An increase in the amount of GLUT4 at the plasma membrane results in increased glucose uptake (figure 3, blue pathway).

In adipocytes it is suggested that the protein pleckstrin homology-like domain family B member 1 (PHLDB1) is an insulin signaling component involved in the regulation of Akt activation and its downstream signaling (not shown in figure 3) [48]. The levels of PHLDB1 protein influence a major biological effect of insulin in adipocytes, GLUT4 translocation and glucose uptake [48]. These effects appear to be exerted at the level of Akt activation downstream of the IR/IRS1/PI3K pathway activated by insulin [48].

There is an alternative pathway resulting in enhanced glucose uptake: PI3K also activates the atypical forms of protein kinase C (aPKC), including PKCλ and PKCζ [3, 33]. Like Akt, the aPKC appear to be important in mediating insulin-stimulated glucose uptake, since their over-expression results in increased translocation of GLUT4 to the plasma membrane [3]. aPKC phosphorylate and thus inhibit AS160, which leads to enhanced GLUT4 incorporation into the plasma membrane as described above (figure 3) [39].

2.1.2.2. Increasing Lipid Synthesis

But aPKC also activate the transcription factor sterol regulatory element-binding protein (SREBP1c) (figure 3, yellow pathway) [3]. It is the dominant isoform in liver and adipose tissue of the SREBP family of three nuclear transcription factors [3]. Many of insulin's effects on lipogenesis appear to be mediated via SREBP1c [3]. SREBP1c can activate the entire program of MUFA synthesis (resulting in increased lipid synthesis), appears to be involved (to a lesser extent than FOXO1) in the regulation of gluconeogenic genes, and can suppress transcription of IRS2 [3]. Mixed insulin sensitivity and resistance in the liver – that is, decreasing activation of Akt and the ability of insulin to suppress glucose production, while increasing activation of SREBP1c and lipogenesis – may produce glucose intolerance and hepatic steatosis [3].

2.1.2.3. Increasing Glycogen Synthesis

Activated Akt controls a variety of downstream responses depending on cell type and substrate (figure 3) [39]. Glycogen synthase kinase (GSK) and mTOR, which lead to stimulation of glycogen and protein synthesis, respectively, are downstream targets of Akt [3, 6, 8]. Akt phosphorylates glycogen synthase kinase 3 (GSK3), deactivating it [3]. Deactivation of GSK3 leads to derepression, i.e. activation of glycogen synthase, which results in a stimulation of glycogen synthesis (figure 3, pink pathway) [3]. But it was found that allosteric regulation of glycogen synthase by glucose 6-phosphate, rather than covalent regulation by the Akt/GSK3 cascade, is the major mechanism by which insulin stimulates muscle glycogen synthesis *in vivo* [39].

2.1.2.4. Increasing Protein Synthesis

Tuberous sclerosis complex 2 (TSC2), when complexed with its binding partner TSC1, acts as a GTPase activating protein (GAP) for the RAS-related small G protein Rheb, which is a potent activator of mTOR Complex 1 (mTORC1) [49]. Akt mediated direct phosphorylation of TSC2 within an intracellular membrane compartment and subsequent translocation to the cytosol, where it is bound by 14-3-3, relieves its ability to

act as a GAP for Rheb within cells (through spatial segregation but without loss of its intrinsic GAP activity toward Rheb), allowing Rheb-GTP to accumulate and activate mTORC1 (figure 3, brown pathway) [46, 49, 50]. Thus, Akt-induced multi-site phosphorylation and destabilization (inhibition) of the Rheb GTPase activating TSC1/TSC2 complex, which inhibits mTOR, is necessary for mTORC1 activation [3, 6, 39, 49]. Akt activates mTORC1 by phosphorylating TSC2 and PRAS40 on sites that mediate 14-3-3 binding and that relieve the inhibitory effects of these proteins [44]. The TSC1/TSC2 complex dissociates after phosphorylation by Akt [50]. In mTORC1, mTOR associates with two highly conserved proteins Raptor (Regulatory associated protein of mTOR), a protein scaffold that recruits downstream substrates of mTOR, such as the S6 kinase and 4EBP1, and mLST8, a member of the Lethal-with-Sec-Thirteen gene family, plus PRAS40, a repressor of mTOR activity, and Deptor [44, 46, 51]. The mTOR pathway integrates insulin and amino acids/nutrient signaling pathways [6]. There is evidence that mTORC1 activation is both necessary and sufficient to stimulate expression of the adipogenic transcription factor PPARγ (peroxisome proliferator-activated receptor γ) and adipocyte differentiation [49]. While mTORC1 is clearly downstream of Akt, its activation can lead to feedback inhibition of insulin signaling to Akt through effects on the IRS proteins and the ensuing attenuation of PI3K activation (not shown in figure 3) [49]. mTORC1-mediated phosphorylation of S6K1/2 (ribosomal protein S6 kinase, also known as p70 S6 kinase or p70S6K) and 4EBP1/2 (eukaryotic translation initiation factor 4E-binding protein 1 and 2) stimulates mRNA translation and, ultimately, anabolic processes, such as cell growth and proliferation (figure 3, brown pathway) [51]. p70 S6 kinase catalyzes the phosphorylation of the S6 protein, a component of the 40S subunit of eukaryotic ribosomes [52].

A second mTOR-containing protein complex (mTORC2) consists of mTOR, rictor (rapamycin-insensitive companion of mTOR; also known as mAVO3), Sin1 (stress-activated-protein-kinase-interacting protein 1, also known as mSin1 or MIP1),

mLST8, Deptor, and protor (protein observed with rictor) [51, 53, 54]. mTORC2 lies upstream of Akt and contributes to Akt activation by insulin and growth factors by serine phosphorylation in its hydrophobic regulatory motif (figure 3, blue pathway), besides it is also involved in the phosphorylation of a similar motif of protein kinase C α (PKCα) [49, 51]. The phosphorylation of a second conserved motif on Akt and PKCα, referred to as the turn motif, is also dependent on mTORC2 [51]. Thus, mTOR lies both upstream (mTORC2) and downstream (mTORC1) of Akt [51]. Experimental data demonstrate that the TSC1-TSC2 complex promotes mTORC2 activity in a manner that is independent of Rheb, mTORC1, and mTORC1-mediated feedback effects on PI3K [51]. By contributing to mTORC2 activation, and through its inhibition of mTORC1-dependent feedback mechanisms, the TSC1-TSC2 complex acts to divert Akt signaling from mTORC1 activation into its other downstream branches [51].

2.1.2.5. Inhibition of Gluconeogenesis and of Apoptosis by FOXO

Activated Akt can translocate into the nucleus where it phosphorylates the forkhead-box transcription factors FOXO1 and FOXO3 in vertebrates, leading to their deactivation and nuclear exclusion and eventual ubiquitination-dependent proteasomal degradation [3, 8, 55, 56]. FOXO1 is the molecular link between Akt phosphorylation and gluconeogenic gene expression [19]. FOXO3, in its unphosphorylated state, is a nuclear transcriptional regulator of genes involved in apoptosis, proliferation and the control of oxidative phosphorylation [56]. Phosphorylation of FOXO1 leads to its exclusion from the nucleus, preventing it from activating transcription of various genes, such as phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, two important enzymes in gluconeogenesis [3, 8, 57]. Nuclear export and cytoplasmic retention of FOXO proteins are facilitated by the binding to 14-3-3 proteins, which promote cell proliferation and survival (figure 3, cyan and lilac pathways, respectively) [44, 58].

2.1.2.6. Inhibition of Apoptosis by BAD

Also, failure of activation of Akt leads to the insulin signal impairment on BAD, a pro-apoptotic BCL-2 family member [26, 59]. Akt generates a 14-3-3 docking site on BAD [44]. BAD activity is antagonized by 14-3-3 proteins by retaining BAD in the cytoplasm and preventing its translocation to its site of action, the mitochondria (figure 3, gray pathway) [44]. Glucose deprivation results in dephosphorylation of BAD and BAD-dependent cell death [59]. Moreover, the phosphorylation status of BAD helps regulate glucokinase activity [59].

2.1.2.7. Increasing Transcription of Mitogenic Genes

The mitogenic effects of insulin are modulated by the mitogen-activated protein kinase (MAPK) pathway, which controls cell growth, differentiation and survival [8, 19, 33, 41]. Activation of this pathway occurs due to the ability of insulin-stimulated IRS1 to bind the SH2 domain of the adaptor molecule growth factor receptor-bound protein 2 (GRB2) [3, 8, 39]. GRB2 is recruited to the receptor via its SH2 domain [60]. This, in turn, recruits and activates the guanine nucleotide exchange factor SOS (son of sevenless homolog) to the plasma membrane next to membrane-bound G-proteins RAS, which it activates [3, 8, 52, 60]. Also, the pathway is initiated by recruitment of the adaptor/guanine nucleotide exchange factor complex GRB2/SOS to phosphorylated Src homology 2 domain containing transforming protein (SHC) (figure 3, green pathway) [39]. It is unclear whether SHC-bound and IRS-bound GRB2/SOS complexes are equally effective activators of RAS, given the differences in their abundance, subcellular localization and potential co-recruitment of additional components [39]. Activated (GTP-bound) RAS in turn sequentially phosphorylates and activates the kinase RAF, a MAPK kinase kinase (MKKK), which it recruits to the membrane, the MAPK kinase 1 and 2 (MEK1/2), and the MAPK ERK1 and ERK2 (extra cellular signal regulated kinase 1 and 2) [3, 39, 52, 60]. Binding of 14-3-3 proteins to a site in the carboxy-terminal region of RAF proteins is needed for full catalytic activity, which is responsible for

serine/threonine phosphorylation of MEK1 [44, 46]. MAPK can phosphorylate substrates in the cytoplasm (for example, increasing protein synthesis via p90RSK (p90 ribosomal protein S6 kinase) and p70S6K, respectively) or translocate to the nucleus to phosphorylate transcription factors such as Elk-1 [3]. Scaffold proteins play a role in coordinating this cascade, and may influence cellular responses through effects on signal intensity and duration, localization of complexes and recruitment of modulatory proteins such as phosphatases and ubiquitin ligases [39].

2.1.3. Insulin Receptor

The IR, a receptor tyrosine kinase consisting of a tetramer of extra cellular α-subunits (capable of insulin binding) and intracellular β-subunits (with intrinsic tyrosine kinase activity), exists as two isoforms differing by the presence (IR-B) or absence (IR-A) of 12 amino acids at the carboxyl terminus of the α-subunit, as a result of alternative splicing of the sequence encoded by exon 11 [3, 39-41]. In the type 1 insulin-like growth factor receptor (IGFR), the corresponding sequence is always lacking [39]. Ligand binding of the α-subunits allows the β-subunits to transphosphorylate one another, further increasing their kinase activity [3]. Serine phosphorylation of the IR decreases its kinase activity [3]. IR-B is the more abundant isoform in muscle, liver and fat tissue [39]. Each isoform has a different affinity for insulin and IGF1 [41]. Insulin binds with similar affinity to both isoforms, but IGF, and particularly IGF2, have greater affinity for IR-A than IR-B, such that IR-A is a significant mediator of IGF2 action at physiological concentrations [39]. Additionally, both IR isoforms can form hybrids with IGFR as well as with each other [39]. Hetero-dimerisation occurs with similar efficiency to homo-dimerisation, so that if one receptor is expressed in excess, the less abundant is assembled largely into hybrids [39]. Hybrids bind IGF with similar affinity to IGFR, but bind insulin with substantially lower affinity than IR [39]. It is unclear whether hybrid receptors have a distinct physiological role [39]. Despite the dimeric receptor structure,

only a single molecule of ligand can make all the contacts required to bind with high affinity and binding [39].

Functionally, the IR behaves like a classical allosteric enzyme in which the α-subunit, when not bound to insulin, inhibits the tyrosine-kinase activity that is intrinsic to the β-subunit [41]. As well as initiating intracellular signaling, ligand-induced auto-phosphorylation triggers internalization of ligand/receptor complexes, leading to dissociation and degradation of ligand in the intracellular endosome/lysosome system and inactivation and recycling of receptors [8, 39]. During this trafficking through the cell in endosomes, the receptor remains active and so increased or more rapid trafficking could result in attenuated signaling [8]. Interfering with the down-regulation of the receptor (as occurs in cells with mutant forms of the receptor) or the ubiquitin protein ligase (thus preventing their interaction), results in increased levels of the receptor [8]. However, receptor internalization may also play an active role in signaling, particularly via the MAPK pathway [39]. Internalized receptors are inactivated by phosphotyrosine-specific phosphatases, particularly PTP1B, which is localized to the cytosolic face of the endoplasmic reticulum [39].

The IR plays a direct role in the β-cell growth (it is critical in triggering β-cell hyperplasia in response to hepatic insulin resistance), and it is part of the onset of the glucose sensing machinery involved in the insulin secretion [26]. Besides of the IRS family targets of the IR tyrosine kinase include SHC, CBL (Casitas B-lineage lymphoma protooncogene), p62dok (Docking protein 1), and GRB2-associated binder-1 (Gab-1) [3]. Once phosphorylated, these substrates act as docking molecules for proteins that contain SH2 domains, which in turn become activated or associate with other downstream signaling molecules [3].

The IR is a polymorphic gene in humans with several known mutations [26]. But mutations in the IR account for only 1% of the non-insulin-dependent diabetics [26]. Thus these pathologies that result in the lack of functioning IR, which among others

generates insulin resistance, do not appear to be essential in the pathogenesis of insulin resistance in the vast majority of diabetic patients [26].

2.1.4. Insulin Receptor Substrate Proteins

At least 11 intracellular substrates of the IR and IGFR kinases have been identified [41]. Six of these belong to the family of IRS proteins, and have been termed IRS1-6 [41]. IRS proteins are a family of adaptor molecules, with IRS1 and IRS2 acting as central integrators of insulin signaling to downstream effector molecules [56]. In rodents IRS1 and IRS2 are widely distributed, whereas IRS3 is largely limited to the adipocytes and brain (it is probably not expressed in humans), and IRS4 is expressed primarily in embryonic tissues or cell lines [41, 43, 57]. The role of IRS4 in the metabolic effects of insulin is probably limited because IRS4-null mice have nearly normal glucose homeostasis [43]. There are five IRS proteins expressed in humans: IRS1, IRS2 and IRS4-6 [56]. IRS5 and IRS6 seem to have limited tissue expression and function in signaling [41]. IRS family members vary in their tissue distribution and subcellular localization, and in their relative importance in different cells and tissues and therefore can mediate specific actions of insulin, despite their structural similarities [3, 8, 56].

IRS proteins are characterized by amino-terminal PH domains and phosphotyrosine-binding (PTB) domains that enable the binding of IRS1 to PI3K, and central and carboxy-terminal numerous tyrosine residues that can undergo phosphorylation by the insulin receptor tyrosine kinase, thereby enabling the binding to intracellular molecules that contain SH2 domains [3, 6, 40, 41]. The PH domain brings IRS proteins to the membrane in proximity to the IR [6]. The PTB domain of IRS binds to a phosphorylated tyrosine located in the juxtamembrane region of the IR [3, 6]. IRS proteins are also substrates for many serine/threonine kinases downstream of other signaling networks and become serine phosphorylated in response to various conditions such as inflammation, stress and over-nutrients [61]. Thus, IRS proteins coordinate signals from the insulin and IGFR tyrosine kinases with those generated by

proinflammatory cytokines and nutrients [62]. Tyrosine-phosphorylated IRS1 and IRS2 are upstream mediators of Akt activation whereas serine phosphorylation negatively modulates IRS1-mediated insulin signaling [19]. Degradation of IRS proteins can be a contributing factor in the development of insulin resistance [8].

Insulin induces the phosphorylation of a complex array of sites (under physiological conditions in time-controlled manner) that allows the fine-tuning of its signaling [6]. Positive regulatory sites are phosphorylated first by kinases in the insulin signaling pathway such as Akt and PKCζ [6]. This phosphorylation allows a correct tyrosine phosphorylation of IRS1 and the propagation of the insulin signal [6]. Inhibitory sites are phosphorylated later on by PKCζ and mTOR/S6K and decrease the tyrosine phosphorylation of IRS1 leading to the termination of the insulin signal [6]. Serine phosphorylation of the IRS proteins impairs their ability to be tyrosine phosphorylated, which increases their association with 14-3-3 proteins, removing them from the insulin signaling cascade [3]. The MAPK pathway also mediates down-regulation of IRS1 expression induced by inflammatory cytokines [6]. Taken together, IRS protein signaling is highly regulated by processes including tyrosine and serine/threonine phosphorylation/ dephosphorylation and ubiquitination-mediated proteasomal degradation, which might be an important mechanism of insulin resistance during acute injury and infection, or chronic stress associated with aging or obesity [5, 8, 56, 61, 62]. These processes control the magnitude and duration of the response to insulin at the level of the IRS protein [56]. Chronic glucose, insulin or IGF1 exposure of various cell lines leads to increased serine/ threonine phosphorylation mediated proteasomal degradation of IRS2 induced by a mTOR-dependent down-regulation of PI3K/Akt/mTOR activity [56].

2.1.4.1. Differential Roles of IRS Proteins

Decreased hepatic IRS1 correlates with the increased expression of genes that are involved in gluconeogenesis, whereas down-regulation of hepatic IRS2 results in

enhanced expression of genes that are involved in lipogenesis [41]. IRS1 has robust interaction with GRB2, leading to activation of the RAS-MAPK pathway, whereas IRS2 interacts only weakly with GRB2, suggesting that, in this context at least, IRS1 segregates best with mitogenic, and IRS2 with metabolic outcomes [43]. At least in muscle cells, IRS1 and IRS2 seem to participate differentially in the mitogenic and metabolic signals of events downstream of Akt, ERK and p38 MAPK [43]. IRS1 regulates actin remodeling, Akt1 and Akt2 phosphorylation, GLUT4 translocation and glucose uptake, whereas IRS2 contributes to Akt2 activation but makes a minimal contribution to GLUT4 or glucose uptake [43]. Conversely, ERK is primarily downstream of IRS2, whereas p38 MAPK is activated equally and complementarily via IRS1 and IRS2 [43]. IRS1 seems to be the main mediator of insulin signaling in skeletal muscle, adipose tissue, and pancreatic β-cells [57]. IRS1 emerged from studies using cells in culture, animal models and human studies as the most relevant isoform regulating metabolic outcomes in muscle and adipose tissues, whereas in liver, IRS1 and IRS2 have complementary roles in insulin signaling and metabolism [43]. IRS2 is important for β-cell proliferation besides liver metabolism [57]. The loss of IRS1 or IRS2 has a negative effect on glucose homeostasis in mice, whereas the loss of IRS3 has a neutral and the loss of IRS4 only a slightly negative effect [3].

2.1.5. Insulin Resistance

Insulin resistance is a pathological condition in which insulin becomes less effective at lowering blood glucose levels [24]. Essentially, inhibition of the insulin signaling pathway leads to insulin resistance [42]. The pancreatic islets respond to insulin resistance by enhancing their cell mass and insulin secretory activity which, over time, leads to the exhaustion of these compensatory reactions, and insulin deficiency and ultimately T2DM develop [24]. Insulin resistance is typically present throughout the progression from pre-diabetes to the later stages of T2DM [24, 42]. By contrast, the onset

of T2DM and its progression are largely determined by the progressive failure of β-cells to produce sufficient levels of insulin [24].

Insulin resistance is an important pathophysiological factor in the development of cardiovascular and liver diseases [6]. Concerning insulin resistance, it is clear that not all insulin-regulated processes and tissues become equally resistant to insulin [3]. Moreover, the hyperinsulinemia that evolves with insulin resistance may lead to increased insulin action in tissues or pathways that are not as resistant as those related to glucose metabolism [3]. Most cases of insulin resistance are due to a combination of factors acting to create partial resistance in some, but not necessarily all, pathways of insulin action [3]. The phenotype of insulin resistance depends on the exact components affected (the more proximal in the signaling pathway the more extensive the ramifications) and the exact tissues in which they are affected [3]. Candidates for insulin resistance genes are the IR, the IRS1 and IRS2, and the PI3K [26].

Insulin resistance results from a complex interplay between nutrient overload, systemic fatty acids excess (such as the sphingolipid ceramide (CER) and diacylglycerol (DAG)), inflammation of the adipose tissue, endoplasmic reticulum and oxidative stresses and hypoxia of the adipose tissue [6]. Partial loss of mitochondrial activity (responsible for fatty acid oxidation and respiration) goes hand in hand with the development of insulin resistance [4]. Accumulation of intracellular lipid metabolites, such as CER and DAG, was found to activate PKC and lead to dephosphorylation of Akt, thus interfering directly with the insulin signaling cascade, and so inducing hepatic insulin resistance, which is generally manifested by hyperglycemia [63]. Nitric oxide has been implicated in mediating insulin resistance [8]. A possible mechanism for this effects is demonstrated by the induction of IRS1 degradation by cytokine-inducible nitric oxide synthases and nitric oxide in cultured muscle cells [8]. The ubiquitin proteasome system has a role in down-regulating IRS proteins and thereby contributing to the two main defects in diabetes, insulin resistance and impaired insulin secretion [8].

Insulin resistance can also be produced by the interaction of inhibitory proteins with components of the insulin signaling cascade, further increases in the activity or amount of the enzymes that normally reverse insulin action, including the phosphotyrosine phosphatases that catalyze the dephosphorylation of tyrosine-phosphorylated proteins and are important negative regulators of tyrosine kinase receptor-mediated signaling, e.g., PTP1B or leukocyte antigen-related protein tyrosine phosphatase (LAR-PTP), and the PIP_3 phosphatases, e.g., PTEN and SHIP, can lead to insulin resistance (figure 3) [3, 19, 26]. PTP1B is a major negative regulator of insulin and leptin sensitivity; it interacts directly with the IR and dephosphorylates it and the leptin receptor-associated Janus kinase 2 (JAK2), thereby reducing their activity [19, 41]. High glucose enhances PTP1B transcription in human hepatocytes [19]. IR and IGFR are substrates for PTP1B [19]. In humans, PTP1B polymorphisms are associated with insulin resistance, obesity, and other characteristics of metabolic syndrome in some populations [19]. Serine phosphorylation of IRS1 in insulin resistant states is associated with decreased insulin-stimulated tyrosine phosphorylation of IRS1 resulting in the decreased ability of this protein to mediate signaling [8]. Indeed, reduced tyrosine phosphorylation of both IR and IRS proteins has been associated with the development of insulin resistance using *in vitro* and *in vivo* models [64].

Other proteins, such as suppressor of cytokine signalling-1 (SOCS1) and SOCS3, growth-factor-receptor bound protein 10 (Grb10) and plasma-cell-membrane glycoprotein-1 (PC1) down-regulate IR function by sterically blocking its interaction with the IRS proteins, or by modifying its kinase activity [41]. The SOCS proteins, induced by inflammatory cytokines, have been shown to be up-regulated in states of insulin resistance, such as obesity, and therefore might contribute to the pathophysiology of diabetes [8, 41]. Moreover, over-expression of these proteins stimulates degradation of IRS1 and IRS2 in cultured cells and in mouse liver [8]. The IR is also down-regulated at the protein level by ligand-stimulated internalization and degradation, which is a common

feature of most insulin-resistant, hyperinsulinemic states, including obesity and T2DM [41].

In obesity and diabetes, chronically activated mTORC1/S6K signaling promotes insulin resistance by a negative feedback mechanism involving serine phosphorylation and degradation of IRS1 as well as inhibition of IRS1 transcription [6]. Metabolic and inflammatory stresses derange the time controlled physiological feedback mechanism of IRS serine phosphorylation [6]. Several kinases including inhibitory-κ B kinase β (IKKβ), c-Jun amino-terminal kinases (JNK), ERK, mTOR, and p70S6 kinase are activated by these stresses that induce insulin resistance and phosphorylate IRS proteins on several serine residues in an uncontrolled manner [6]. The IKKβ is the master regulator of nuclear factor kappa B (NF-κB) activation in response to inflammatory stimuli [6]. IKKβ is activated by the inducers of insulin resistance such as the inflammatory cytokines and FFA, and IKKβ activity and/or expression are increased in obese patients [6]. In various insulin target cells, the inhibition of IKKβ activity prevents the deleterious effects of tumor necrosis factor alpha (TNFα) or fatty acids on insulin signaling [6]. The NF-κB and JNK pathways are activated in multiple tissues in obesity and T2DM, and have central roles in promoting tissue inflammation induced by overnutrition [6, 24]. Activation of IKKβ/NF-κB pathway in the liver and in myeloid cells, which can be inhibited by Aspirin® and salicylate, is a core mechanism that connects metabolic inflammation and insulin resistance, both in peripheral tissues and in the central nervous system [6].

2.1.5.1. Manifestation of Insulin Resistance in Different Tissues

Insulin resistance is associated with a spectrum of renal diseases (from nephrotic syndrome, mild renal dysfunction and focal segmental glomerulosclerosis in obesity to diabetic nephropathy) and also with significant changes in glucose and lipid homeostasis [64]. FFA are elevated in insulin-resistant states and are thought to play a critical role in the progression to T2DM [64]. Palmitate is the predominant circulating SFA, and elevated plasma FFA have been identified in both adult and childhood obesity

and are strongly implicated in the causation of insulin resistance [64]. Furthermore, high levels of palmitate correlate with increased CER production, and both endogenous and exogenous CER have been associated with the development of cellular insulin resistance, i.e. in podocytes and in skeletal muscles [64].

Studies in humans and in rodents have demonstrated that the mechanisms leading to an excess accumulation of hepatic triglycerides are mainly linked to increased delivery of FFA from peripheral adipose tissue to the liver and enhanced *de novo* lipid synthesis via the lipogenic pathway in the liver itself, while lipid disposal via β-oxidation and very low density lipoprotein (VLDL) export are only moderately affected [65]. Basal hyperinsulinemia and chronic high fat availability have also been shown to promote a condition in which incomplete fatty acid oxidation and accumulation of lipotoxic short chain fatty acids occur in peripheral tissues, ultimately leading to a complete loss of their insulin sensitivity [66]. An imbalance between fatty acid uptake and oxidation is believed to be responsible for accumulation of lipids (such as long chain acyl CoA, DAG, triacylglycerol, and CER), and is thought to be a major cause of insulin resistance in obesity and diabetes, due to lipid accumulation and inhibition of one or more steps in the insulin signaling cascade [67]. When fatty acid β-oxidation is increased in heart muscle, glucose oxidation is consequently decreased, a phenomenon that is termed the "Randle Cycle" and is proposed to contribute to insulin resistance in oxidative muscles (type 1 skeletal muscle and heart muscle) in general [67]. It is suggested that, besides increased fatty acid uptake, increased fatty acid β-oxidation also contributes to insulin resistance in the heart and skeletal muscle [67].

Muscle insulin resistance associated with obesity and T2DM in humans is associated with a dramatic decrease in signaling through IRS1 and PI3K, while signaling through MAPK is maintained [3]. Adipocytes from obese individuals show a decrease in IR tyrosine kinase activity and IRS1-associated PI3K activity; however, IRS2-associated PI3K activity is maintained at normal levels [3]. In liver, on the other hand, there is essentially no

change in the number of IR per hepatocyte, IR kinase activity, and the ability of insulin to inhibit glycogenolysis [3].

In C57BL/6J mice weaned on to a lard-based high-fat diet (45% energy from fat), whole-body insulin resistance was detected after 3 and 8 weeks on the high-fat diet and was entirely due to an inability of insulin to suppress endogenous glucose production from the liver, while muscle and adipose tissue and all other peripheral tissues retained full insulin sensitivity [68]. Further, hepatic insulin resistance was associated with accumulation of short- to medium-chain and depletion of long-chain fatty acylcarnitines [68]. More recent studies demonstrate that increasing lipid oxidation in the liver prevents hepatic steatosis and insulin resistance [12]. In a study, two weeks of high-fat feeding in male, 12–16 weeks old, C57Bl/6 mice resulted in hepatic insulin resistance in the presence of hepatic steatosis; the former reflected by decreased inhibition of hepatic glucose production without affecting muscle specific insulin sensitivity [69].

Hepatic insulin resistance can be defined as the failure of insulin to adequately suppress hepatic glucose production, further it leads to impaired glycogen synthesis and synthesis of inflammatory proteins; but the lipogenic actions of insulin are not compromised in insulin-resistant states [23, 33]. Hepatic insulin resistance is critical in triggering the progression to uncontrolled T2DM [26]. The failure of insulin to suppress hepatic glucose production is a key feature of insulin resistance and is a major factor contributing to hyperglycemia in diabetic states [8, 33]. Insulin exerts this effect by suppressing transcriptional activation of genes encoding enzymes in the gluconeogenic pathway [8]. Increased release of cortisol by visceral adipose tissue could play a role in hepatic insulin resistance by maintaining the expression of key enzymes of gluconeogenesis and, thus, hepatic glucose production, at a high level [65]. Chronic hyperglycemia also activates transcription of the insulin gene, resulting in *de novo* synthesis of insulin [8]. IRS2 and its downstream targets PI3K, Akt and FOXO1 are implicated as critical nodes of insulin

action in liver which suggests that impaired IRS2-mediated signaling may represent a major component of hepatic insulin resistance [19]. Besides insulin resistance can be promoted by excessive hepatic accumulation of fat [23]. The reduced adiponectin production by adipocytes is another potential factor in the development of insulin resistance [5].

2.2. Cytohesins

Cytohesins are a subfamily of small guanine nucleotide exchange factors (GEF) (in contrast to the large ARF-GEF of about 200 kDa, which also contain a Sec7 domain), that catalyze the activation of adenosine diphosphate ribosylation factors (ARF) through replacement of GDP with GTP; they are referred to as the Sec7 family, based on homology of their catalytic domains to the yeast ARF GEF, sec7p [70-74]. Cytohesins are resistant to the inhibitor brefeldin A, a fungal macrocyclic lactone, and have a molecular weight around 47 kDa [55, 71-73, 75-78]. All vertebrates express four cytohesin isoforms, cytohesin 1, cytohesin 2, cytohesin 3, and cytohesin 4, while only one isoform (named steppke) is present in Drosophila *melanogaster* (table 1) [71].

Name	Alternative titles	Accession No.
Cytohesin 1	Pleckstrin homology, Sec7, and coiled-coil domains protein 1 (PSCD1); SEC7; D17S811E	NM_011180
Cytohesin 2	Pleckstrin homology, Sec7, and coiled-coil domains protein 2 (PSCD2); ARF nucleotide-binding site opener (ARNO)	NM_011181
Cytohesin 3	Pleckstrin homology, Sec7, and coiled-coil domains protein 3 (PSCD3); ARF nucleotide-binding site opener 3 (ARNO3); General receptor of phosphoinositides 1 (GRP1)	NM_011182
Cytohesin 4	Pleckstrin homology, Sec7, and coiled-coil domains protein 4 (PSCD4); CYT4; DJ63G5.1	NM_028195

Table 1. Names of the cytohesin family members [79, 80].

While both cytohesin 2 and cytohesin 3 appear to be ubiquitously expressed, cytohesin 1 and cytohesin 4 are primarily found in leukocytes [71]. Human cytohesins exhibit at least 80% similarity in a pair wise comparison of their amino acid sequences and share a common domain organization [81]. Inactive cytohesins are located in the cytosol [76]. In cells, the cytohesins are distributed primarily to the cell periphery, and can be acutely recruited to the plasma membrane in response to PI3K signaling and phosphoinositides (not limited to products of PI3K) stimulate cytohesin GEF activity [71, 78]. Cytohesins participate in the regulation of diverse processes such as cytoskeletal organization, integrin activation or insulin signaling [55, 75, 82, 83].

Cytohesin 1 and 2 also interact with the cytoplasmic sequence of the LFA-1 β2 chain, CD18, which reflects a role of these proteins in signal complex assembly with the cytoplasmic tail of β2 integrins [82]. This interaction results in an increased avidity of integrin substrate binding, and thereby cytohesins mediate β2 integrin-dependent adhesion of T-cells [84]. The GEF activity of cytohesin 1 appears to play a minor role, if any, in this activity, and T-cell adhesion is only weakly inhibited by SecinH3 [84]. In a study, cytohesins were identified as ErbB receptor activators that enhance receptor activation by direct interaction with the cytoplasmic domain of the receptor [85]. In this, cytohesins do neither influence receptor dimerisation nor require receptor autophosphorylation for binding, but function as conformational activators of receptor dimers [85]. It is also indicated that the cytohesin-mediated activation of ErbB receptors does not involve the ARF proteins, nor does it require the GEF function of the Sec7 domain [85].

Beside the conserved, central catalytic, Sec7 domain, responsible for GEF activity, cytohesins contain a carboxy-terminal PH domain, mediating recruitment to the plasma membrane by binding to phosphatidylinositides, an amino-terminal coiled-coil (CC) region (containing heptad repeats) for protein-protein interactions and homo-dimerisation, a polybasic (PB) motif, a Sec7 and PH-linker (SP) domain, and PH and PB-linker domain (figure 4) [55, 70, 76, 78, 81, 86].

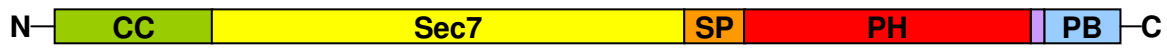

Figure 4. Schematic depiction of cytohesin domains. i) CC domain in green; ii) Sec7 domain in yellow; iii) Sec7/PH-linker (SP) in orange; iv) PH domain in red; v) PH/PB-linker in lilac; vi) PB-domain in blue. N amino terminus, C carboxy terminus. Adapted from [71, 81, 86].

The Sec7 domain consists of an elongated cylinder comprised of 10 transverse α-helices, separated into two sub domains by a deep, solvent-exposed hydrophobic groove [71]. A key feature of the catalytic mechanism is the presence of an invariant glutamate residue at the tip of a hydrophilic loop between helix 6 and 7, referred to as

'glutamic finger' [71]. In crystal structures of the Sec7 domain/ARF complex, this glutamate residue is inserted into the nucleotide-binding fold where it competes electrostatically with the β-phosphate of the bound nucleotide [71]. The Sec7 domain is proposed to act as a dual catalyst, facilitating both nucleotide release and conformational switching on ARF proteins [87]. Cytohesin 1 to 3 are strongly auto-inhibited through an intramolecular mechanism that requires the PB motif [86]. Further, a high-resolution crystal structure of cytohesin 3 (lacking the CC domain) revealed the regulatory importance of a structure connecting the Sec7 and PH-domains which, together with the PB domain, is involved in cytohesin auto-inhibition [81].

Several proteins have been identified that interact with the amino-terminal CC domain of cytohesins, including cytohesin interacting protein (Cytip, also known as CASP and Cybr), the related protein tamalin (also known as Grp1-associated scaffold protein, GRASP), and interacting protein for cytohesin exchange factors (IPCEF) [71]. These proteins function as adaptors, linking the cytohesins to other proteins and may provide a phosphoinositide independent mechanism for coupling receptor signaling to ARF activation [71]. For instance, tamalin has been shown to bind to metabotropic glutamate receptors and the neurotrophin receptor TrkC (tropomyosin-receptor-kinase C) via an amino-terminal PDZ (**P**SD-95, **D**iscs-large, **Z**O-I) domain, and associates with multiple cytohesins via its carboxyl terminus [71].

2.2.1.1. Cytohesin Isoforms

The cytohesin 1 gene appears to have an extra exon of only 3 base pairs (GAG) that results in the insertion of a single glycine in the variable loop between the first and second β-sheet of the PH domain [78]. This seems to be the case for the whole family since the existence of two splice variants for each cytohesin has been shown [76]. In human brain, the mRNA for cytohesin 1 and 2 were 80–90% GAG-positive, whereas only 20–30% of cytohesin 3 mRNA contained the GAG exon [78]. There were no major differences between fetal and adult human brains in percentages of the two isoforms

of cytohesin 2 or 3 [78]. Cytohesins, regardless of their splice isoform, can be activated and recruited to the plasma membrane independently of PIP_3 by ARF-like GTPase 4 (Arl4) and phosphatidylinositol-4,5-bisphosphate (PIP_2) or in a PIP_3-dependent manner (figure 3), depending on the two splice variants [71, 76]. Both variants bind PIP_2, but only the diglycine form binds PIP_3 with high affinity [76]. However, the triglycine form has only micro molar affinity for both PIP_3 and PIP_2, affinities that are insufficient to confer membrane recruitment, raising the question of how the triglycine forms of cytohesins are regulated [76]. Although PIP_3 is only generated in response to external signals, PIP_2 is constitutively present at the plasma membrane, and thus this dual interaction may ensure that the recruitment of cytohesin 2 by the Arl4 family is tightly restricted to this location [76]. The PH domain of cytohesins is necessary and sufficient for relocation to the plasma membrane in response to Arl4a-d, indicating that the Arl4 family can control the localization of all four cytohesins [76]. The recruitment of diglycine cytohesin 2 to the plasma membrane by PIP_3 has been shown to cause the accumulation of ARF6 on the same membrane [76]. It was found that ARF6 could also be recruited to the plasma membrane when cytohesin 2 was co-expressed with Arl4c, confirming that the cytohesin 2 recruited by an Arl4 and PIP_2 was active rather than inhibited by the interaction with Arl4 [76]. This indicates that cytohesin 2 recruited to the plasma membrane by Arl4 is able to relocate ARF6 via GTP exchange [76].

2.2.1.2. ARF

ARF are a family of small, approximately 20 kDa heavy, ubiquitously expressed RAS-like GTPases that are central to many vesicular transport processes in eukaryotic cells [73, 77, 81, 88]. These proteins function as molecular switches and regulate a variety of cell biological events within both endocytotic and exocytotic trafficking pathways [81]. The ARF-family consists of six members that are categorized into three classes: class I (ARF_1, ARF_2, ARF_3), class II (ARF_4, ARF_5) and class III, which is represented by ARF6 [77, 81, 88]. Insulinotropic concentration of glucose stimulates ARF6

activation [89]. The activation of ARF ("on"-state in the GTP-bound form) is driven by GEF, while their deactivation ("off"-state in the GDP-bound form) is catalyzed by GTPase-activating proteins (GAP) [81, 88]. Since GDP/GTP-exchange is coupled to high-affinity membrane binding of ARF, ARF-GEF (i.e. cytohesins) are also responsible for recruiting ARF to specific membrane domains (figure 3) and are, thus, capable of regulating ARF activity in a spatial manner [81]. The cytohesin family of ARF-GEF, like ARF themselves, cycle between inactive and active conformations, resulting in even more tight regulation of ARF activation [81].

2.2.2. Cytohesin 1

Cytohesin 1 was initially characterized as an activator of β2-integrin-mediated adhesion (hence the name cytohesin) [71]. Localized at the plasma membrane, its substrates are ARF1 and ARF domain protein 1 (ARD1) while interaction protein for cytohesin exchange factors-1 (IPCEF1), β2 integrin, adenosine diphosphate ribosylation factor-related protein (ARP), Arl4, and CASP constitute binding partners [71, 88]. Cytohesin 1 is predominantly expressed in hematopoietic cells, where it binds specifically to the cytoplasmic tail of the integrin β2 integrin subunit CD18 and regulates adhesion (i.e. regulating LFA-1 activation leading to adhesion of T cells to ICAM-1 (intercellular adhesion molecule 1) which is presented on endothelial cell surfaces) and migration of leukocytes, through mechanisms that require both catalytic and non-catalytic functions of the protein [71, 72, 82, 90-93]. The common denominator of both functions is the positioning of the protein upstream of the GTPase RhoA activation [92]. The exchange activity of cytohesin 1 is not required for integrin activation, but it is required for spreading, suggesting that cytohesin 1 plays different roles in inside-out versus outside-in T cell integrin signaling [90, 91]. A study suggested involvement of cytohesin 1 in the regulation of the functional responses of human polymorphonuclear neutrophils (PMN) and linked these events, in part at least, to the activation of ARF6 [94]. Cytohesin 1 is a

key regulatory molecule in the mediation of inflammatory processes and plays a role in synaptic transmission [88, 95].

2.2.3. Cytohesin 2

Cytohesin 2 is implicated in the remodeling of the actin cytoskeleton, G-protein receptor desensitization, and activation of calcium-regulated exocytosis [71, 88]. Also, it has been shown to promote the migration of epithelial cells and the outgrowth and branching of neurites through a mechanism that requires its catalytic activity [71, 88]. In both contexts, activation of ARF6 by cytohesin 2 leads to the downstream activation of the Rho family GTPase Rac1, which is necessary for both cell migration and neurite outgrowth [71]. It has been reported that cytohesin 2 is part of a GTPase signaling cascade in which integrin-mediated activation of R-RAS leads to recruitment of cytohesin 2 through an interaction with the R-RAS effector protein RLIP76/RalBP1 [71]. The resulting activation of ARF6 again leads to downstream activation of Rac1 and the resulting extension of lamellipodia [71]. Pharmacological or molecular biological inhibition of cytohesin 2/Arf6 inhibits glucose-induced activation of Cdc42 and Rac1 [89]. Glucose promotes association between cytohesin 2 and Arf6 and induces Arf6 activation which is inhibited by SecinH3 or siRNA-cytohesin 2, suggesting a critical involvement of cytohesin 2/Arf6 in insulin secretion in the pancreas [89]. Involvement in vascular endothelial growth factor signaling in endothelial cells was recently demonstrated for cytohesin 2 [96].

Cytohesin 2 has also been implicated in several distinct aspects of vesicular transport including the docking and fusion of secretory granules in adrenal chromaffin and neuroendocrine cells, the endocytosis/desensitization of a subset of G-protein-coupled receptors through an interaction with β-arrestins, and the regulation of post-endocytic trafficking via an interaction with the vacuolar adenosine triphosphatase (V-ATPase) [71]. It has been shown that the V-ATPase functions as an endosomal pH-sensor that recruits ARF6 and cytohesin 2 to the endosomal membrane and provides

a regulatory link between intra-endosomal acidification and protein trafficking in the endosomal respectively lysosomal protein degradative pathways [71, 81]. Further was discovered that cytohesin 2 binds directly to all a-subunit isoforms of the V-ATPase, while ARF6 interacts with the c-subunit isoform, and that both interactions require an acidic pH in the endosomal lumen [71, 81, 97]. Its other binding partners are GRASP, IPCEF1, A2A adenosine receptor, Arl4, ARF6, and CASP [71, 88]. ARF1-3 and ARF6 function as substrates for cytohesin 2 [71, 88]. It is localized at the plasma membrane and the endosomal membrane [71, 88]. The ARF-GEF activity of cytohesin 2 is regulated by serine-phosphorylation of its PB domain by protein kinase C (PKC) [81].

2.2.4. Cytohesin 3

Cytohesin 3 plays a role in growth-factor signaling (i.e. insulin, epidermal growth factor, nerve growth factor) [88]. It is an essential component of the PI3K pathway in insulin signal transduction of Drosophila, mouse, and human cells and is localized at the plasma membrane [55, 71, 72, 75]. While ARF1 acts as its substrate, ARF6 is its binding partner as well [71, 88]. Other binding partners are IPCEF1, GRASP, GRP1 signaling partner-1 (GRSP1), thyroid hormone receptor, Arl4, and CASP [71, 88].

The PH domain of cytohesin 3 exhibits selectivity for PIP_3 over phosphatidylinositol-3,4-bisphosphate and PIP2 but it has significant affinity for phosphatidylinositol-3,4-bisphosphate-containing vesicles [98, 99]. The PH domain of cytohesin 3 binds to PIP_3 and, to a much lesser extent, to phosphatidylinositol-3,4-bisphosphate [99]. In contrast to the PH domain of cytohesin 3, the PH domain of cytohesin 2, which is known to specifically bind PIP_3, showed two-fold higher affinity for phosphatidylinositol-3,4-bisphosphate vesicles than for PIP_3 vesicles [98]. The PH domain of the two glycine isoform of cytohesin 2 has modest selectivity for phosphatidylinositol-3,4-bisphosphate over PIP_3 and a lower affinity to PIP_3-containing vesicles than the PH domain of cytohesin 3 [98]. Measurements showed that the PH domain of Akt to have a 1.7-fold preference for phosphatidylinositol-3,4-bisphosphate over PIP_3 [98]. The PH domain of

cytohesin 3 translocates faster to the plasma membrane than the PH domain of cytohesin 2 and both show similar monolayer penetration behaviors but the PH domain of cytohesin 2 dissociates from model and cellular membranes more slowly than the PH domain of cytohesin 3 [98].

2.2.5. Cytohesin 4

The amino acid sequence of the Sec7 domain of cytohesin 4 is 77% identical to those of cytohesins 1, 2, and 3, which are more than 86% identical to each other [78]. Cytohesin 4 mRNA is present predominantly in peripheral blood leukocytes and not in bone marrow cells [78]. Cytohesin 4 mRNA is abundant in both CD31/CD41 (helper) and CD31/CD81 (cytotoxic) T-cells [78]. Most CD33+ cells (largely monocytes), 80% of CD2+ cells (NK, helper, and cytotoxic T-cells), and 10–20% of CD19+ cells (B-cells) also contain cytohesin 4 mRNA, whereas CD15+ cells (mostly granulocytes) do not [78]. It appears that cytohesin 4 is expressed in the more mature cells of T-cell and myelomonocytic lineages (and some B-cells) [78]. Cytohesin-4 exhibits GEF activity *in vitro* with both ARF1 and ARF5 but is inactive with ARF6 [78]. Known binding partners of cytohesin 4 are IPCEF1 and CASP [88].

2.3. Validity of the Diet-Induced Obesity Model in Animals

Data from a study in 1988 showed that exposure to a high-fat, high-simple-carbohydrate, low-fiber diet produced obesity in mice, and suggested that the C57BL/6J mouse carries a genetic predisposition to develop non-insulin-dependent (type 2) diabetes [100]. A deletion in the genetic locus of nicotinamide nucleotide transhydrogenase only observed in C57BL/6J mice was shown to account for their glucose insensitivity and reduced insulin secretion [101, 102]. Male C57BL/6J mice developed fasting hyperglycemia and hyperinsulinemia after 6 weeks on the Surwit Diet (which was used in my setup) [100, 103]. High-fat feeding will readily induce obesity and diabetes in C57bl/6 mice, which are frequently used as animal models of overfeeding [8, 104]. Post weaning consumption of diets rich in SFA results in the progressive development of insulin resistance, leptin resistance, and obesity in C57BL/6J mice [66]. Independent of the precise nature of the diet, DIO in rodents results in many of the same changes as seen in human obesity, including the development of central and peripheral leptin and insulin resistance, and in the altered expression of adipokines (adiponectin and resistin) which are known to contribute to the regulation of peripheral insulin sensitivity [10]. DIO was shown to be associated with a reduced pancreatic abundance of enzymes involved in clearance of reactive oxygen species, providing a potential mechanism for the deterioration of pancreatic function seen in the later stages of T2DM, with adipocyte hypertrophy and the induction of hepatic steatosis and hepatic insulin resistance, which again resembles the human obese state (figure 1) [10].

Oxidative stress has emerged as an important factor in the pathogenesis of obesity and T2DM [33]. High-fat feeding in rats has been shown to increase hepatic oxidative stress, and reactive oxygen species synthesis has been proposed as an initial event that precedes the onset of insulin resistance in mice fed a high-fat diet [33].

DIO can not only be observed in rodents but also in primates: In more than 50% of nonhuman primates obesity develops over years as they age [5]. Nearly half of these obese animals subsequently develop diabetes, as a greater BMI correlates with greater insulin secretion [5]. Once the animals gain weight, impaired glucose removal from the blood stream and increased insulin resistance are detectable [5]. The hyperinsulinemia, in turn, leads to an increase of hepatic VLDL triglyceride synthesis and secretion, increased plasminogen activator inhibitor-1 synthesis, increased sympathetic nervous system activity, and increased renal sodium reabsorption [5].

2.4. Aim of the Dissertation

Cytohesins are important regulators of hepatic insulin signaling *in vivo* at the level between IR and IRS [71]. Specific inhibition of cytohesin 3, a protein involved in the intracellular part of the insulin signaling pathway, by SecinH3, a small organic inhibitor (a 1-, 2-, 4-substituted triazole), leads to hepatic insulin resistance [75, 84]. SecinH3 is known to inhibit nucleotide exchange, insulin signaling, and to a part cell adhesion [84]. This dissertation examines effects of high-fat diet feeding (which lead to impaired glucose tolerance) on cytohesin 2 and cytohesin 3 mRNA steady-state expression levels and cytohesin 3 protein expression levels in murine liver using quantitative real-time PCR (qRT-PCR) and Western blot analysis.

3. Methods

3.0. Animals and Dietary Treatments

In this dissertation I analyzed livers of mice derived from a study in which effects of high-fat diet on the cardiovascular system was analyzed in a mouse model. Parts of the results were published in the manuscript with the title "Fat intake modifies vascular responsiveness and receptor expression of vasoconstrictors: Implications for diet-induced obesity" by Mundy et alii [105]. These data indicated that a higher fat quota in chow leads to impaired glucose tolerance [105].

Diet	C	HF	VHF
Protein	22.4	17	16.4
Carbohydrate	65.4	43	25.5
Fat	12.3	41	58

Table 2. Major macronutrient constituents of diets given in percent of kcal. Taken from [105].

For that study healthy male mice (C57BL/6, Charles River, Sulzfeld Germany) were housed in the institutional animal facilities on a 12:12-hours light-dark cycle, and animals had free access to food and water [105]. Housing facilities and experimental protocols were approved by the local authorities for animal research (Kommission für Tierversuche des Kantons Zürich, Switzerland) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) [105]. Mice were randomly assigned to one of the following diets (n = 10–12 mice/group, but only for 8 mice/group cytohesin levels were determined): control (C, 12.3% of total kcal from fat, KLIBA NAFAG 3430, Kaiseraugst, Switzerland), high fat (HF, 41% of total kcal from fat, Research Diets D12079B, New Brunswick, NJ), and a diet containing very high amounts of fat (VHF, 58% of total kcal from fat, Research Diets D12331) for 15 weeks [105]. The macronutrient compositions of the three diets are reported in table 2. At the end of the treatment, mice were anesthetized (xylazine: 100 mg/kg body weight; ketamine: 23 mg/kg body weight; and

acepromazine: 3.0 mg/kg body weight, intraperitoneal), and exsanguinated via cardiac puncture [105].

3.1. Quantification of mRNA and Protein Expression

3.1.1. RNA Isolation and qRT-PCR

Murine liver tissue was snap-frozen in liquid nitrogen immediately after dissection and stored at –80 °C. Tissue was disrupted in Buffer RLT® (Qiagen AG, Hombrechtikon, Switzerland) and homogenized using a Qiagen TissueLyser (Retsch, Haan, Germany). Total RNA was extracted using the RNeasy® Mini Kit (Qiagen AG, Hombrechtikon, Switzerland). 1 µg of RNA was reverse transcribed using the Quantitect® Reverse Transcription Kit (Qiagen AG, Hombrechtikon, Switzerland), which includes a genomic DNA elimination reaction. Relative expression levels of murine genes encoding for cytohesin 2 and cytohesin 3 were determined by qRT-PCR using murine β2 microglobulin as a housekeeping gene. After analyzing preliminary results, β2 microglobulin was chosen over β actin as housekeeping gene, because it yielded less variation of the mean cycle threshold (CT) values (table 4). qRT-PCR experiments were run on a iCycler™ Thermal Cycler (Bio-Rad Laboratories AG, Reinach, Switzerland) with iCycler iQ™ Multi Color Real time PCR Detection System (Bio-Rad Laboratories AG, Reinach, Switzerland) using specific cDNA primers (Microsynth AG, Balgach, Switzerland). The same apparatus was used for all reactions. The reaction for the qRT-PCR consisted of cDNA template, forward and reverse primers (400nM final concentration, primer sequences are shown in Table 3), and iQ SYBR Green Supermix (Bio-Rad Laboratories AG, Reinach, Switzerland) in a total volume of 25 µl. qRT-PCR was analyzed using Bio-Rad iCycler iQ™ Optical System Software (version 3.1.7050), following the manual provided by the supplier, and Microsoft® Excel.

In all reactions containing β2 microglobulin the CT value was set at 1900 arbitrary units (AU) with base line range of cycle 2 to 11. Above the threshold value the increase in

fluorescence, and therefore cDNA, is exponential. For cytohesin 2 reactions a threshold value of 1949.7 AU was used with a base line range of cycle 2 to 17. In the case of cytohesin 3 reactions a threshold value of 2034.9 AU was used with a base line range of cycle 2 to 22. All reactions were performed in duplicate; specific reactions with differences in cycle threshold values over 0.5 cycles were repeated in order to acquire consistent data. Samples from all three groups were used for each 96-well PCR plate setup. Every group consisted of eight samples. The validity of the obtained PCR cDNA products was controlled by melting curve analysis and sequencing.

Primer	Primer sequences (5'–3')	Accession No.
m-pscd2	For: ACCTCTACGACAGCATCC (position 898, 18bp)	NM_011181
	Rev: CCGCTTCCAAGTCTTCAC (position 1037, 18bp)	
m-pscd3	For: GGTCTTCCAGTCCACAGATAC (position 739, 21bp)	NM_011182
	Rev: CTCGTTGATGCCTCGGTTC (position 874, 19bp)	
β_2 micro-globulin	For: GGTCGCTTCAGTCGTCAG (position 33, 18bp)	NM_009735
	Rev: TGTTCGGCTTCCCATTCTC (position 75, 19bp)	

Table 3. Primers used for amplification of specific cDNA fragments encoding for cytohesin 2, cytohesin 3 and β_2 microglobulin [80].

3.1.2. Sequencing

Correct primer design for m-pscd2 was controlled by sequencing of the qRT-PCR product by Microsynth AG. The m-pscd3 primer product could not be sequenced by Microsynth AG. Because Microsynth AG stated that PCR products <100 bases were often problematic for sequencing; incorporating these PCR products into a plasmid vector instead of sequencing them directly was recommended. The cytohesin primers used were chosen to yield approximately the same product size. Since the shorter product could not be sequenced, I decided to prolong it. In order to increase the length of the PCR product to be sequenced, the m-pscd3 primer product was inserted into a plasmid using the TOPO® TA Cloning® Kit for Sequencing (with pCR™4-TOPO® vector) with One Shot® TOP10 Chemically Competent E. coli and PureLink™ Quick Plasmid Miniprep Kit (Invitrogen AG, Basel, Switzerland). In a first step I produced the m-pscd3 primer PCR product following the TOPO® TA Cloning® Kit manual. In order to incorporate the PCR product

into plasmids it had to be mixed with the supplied TOPO® vector, amongst others. One Shot® TOP10 Chemically Competent E. coli were transformed using the TOPO® Cloning reaction. A few colonies were cultured overnight. Using the PureLink™ Quick Plasmid Miniprep Kit cells were lysed, the plasmid DNA was isolated and sent to be sequenced by Microsynth AG (table 5).

3.1.3. Western Blot Analysis

From the above mentioned, at –80 °C stored, mouse liver samples about 30 mg of liver tissue were taken without thawing of the samples. The tissue samples were disrupted in RIPA lysis buffer and homogenized using a Qiagen TissueLyser (Retsch, Haan, Germany). Supernatant was separated from tissue detritus by centrifugation. Protein concentration of each sample was determined by Bradfort Protein Assay. Equal amounts of protein lysates were separated on an 8–16% SDS-PAGE gel and immunoblotted with anti-cytohesin 2 antibodies (cytohesin-2 (N-20): sc-9727 1:500, Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-cytohesin 3 antibodies (GRP1 (N-17): sc-9730 1:500, Santa Cruz Biotechnology Inc., Heidelberg, Germany) and anti-p44/42 antibodies (rabbit anti pospho-Erk1/2 (9102) 1:1000, and rabbit anti total-Erk1/2 (9102) 1:1000, Cell Signaling, Danvers, MA, USA). Densitometric analysis of Western blots was performed using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA).

3.2. Calculations and Statistical Analysis

For statistical calculations and graphs, the statistic program StatView® 5.0.1 (SAS Institute Inc., North Carolina, USA) was used. For comparison between two groups, the unpaired Student's t-test was performed. All data are expressed as mean ± standard error of the mean (SEM). *P* values <0.05 were considered statistically significant.

4. Results

4.0. Presentation of Mice Used, House Keeper Testing and Primer Sequences

As indicated in the method section, a study using the mice of the present study has already been published. The authors showed that the mice of the HF and VHF groups gained significantly more weight compared to group C (17±1 g respectively 21±1 g vs. 11±1 g) [105]. In addition, glucose clearance was significantly lowered in the HF and the VHF groups indicating impaired glucose tolerance [105].

4.0.1. House Keeper Testing

To begin with, two house keeping primers sets, amplifying about 140 bp stretches of β actin and β2 microglobulin sequences respectively, were tested for stability. β2 microglobulin was chosen over β actin because of 45% less variation in CT values (table 4).

Gene	mean CT value
β actin	19.63±0.236 (18.30; 21.10)
β_2 microglobulin	17.90±0.132 (17.25; 18.65)

Table 4. House keeper comparison. Mean CT values ± SEM; lowest, respectively highest, mean CT values in brackets. n = 13 per gene.

4.0.2. Cytohesin 2 and Cytohesin 3 Amplicon Sequencing in Murine Liver

Verified primer product sequences for cytohesin 2 and cytohesin 3 are shown in table 5.

Gene	Product sequence (5'–3')	Size
m-pscd2 (cytohesin 2) NM_011181	ACCTCTACGACAGCATCCGGAACGAGCCCTTCAAAATCCCA GAGGATGATGGGAATGATCTCACCCACACCTTCTTCAACCC AGACCGAGAGGGCTGGCTCCTAAAGCTGGGAGGGGGCCG GGTGAAGACTTGGAAGCGG	140 bp
m-pscd3 (cytohesin 3) NM_011182	GGTCTTCCAGTCCACAGATACCTGCTACGTGTTGTCCTTTG CCGTGATCATGCTGAACACCAGCCTTCACAATCCCAATTCA GGGACAAGCCGGGCCTGGAGCGCTTTGTGGCCATGAACC GAGGCATCAACGAG	136 bp

Table 5. Verified primer product sequences.

4.1. Effects of High-Fat Diet on mRNA Steady-State Expression Levels of Cytohesin 2 and Cytohesin 3 in Murine Liver

In order to determine effects of high-fat diet feeding on the expression of cytohesins, I investigated mRNA expression levels of cytohesin 2 and cytohesin 3 in murine livers by qRT-PCR. In the control samples cytohesin 2 had a seven times higher mRNA expression level than cytohesin 3 (table 6). This indicates that there is seven times more cytohesin 2 than cytohesin 3 mRNA expressed in murine liver.

Gene	C
Cytohesin 2	532±41
Cytohesin 3	75.4±9

Table 6. Cytohesin 2 and cytohesin 3 mRNA steady-state expression levels. mRNA expression levels were measured with qRT-PCR with β_2 microglobulin as a house keeper using the ΔCT-method. n = 8 per group. Values stated in arbitrary units. Data represent means ± SEM.

4.1.1. Effects of High-Fat Diet on mRNA Expression Levels of Cytohesin 2

In mice fed a VHF diet cytohesin 2 mRNA steady-state expression levels in liver tissue were significantly increased compared to control diet fed mice (24%, $P<0.05$ VHF vs. C, figure 5). However, mice fed a HF diet showed no effect in gene expressions of cytohesin 2 compared to mice of group C. A significant difference in gene expression was shown for cytohesin 2 in mice fed a VHF diet compared to mice fed a HF diet (27%, $P<0.05$ VHF vs. HF).

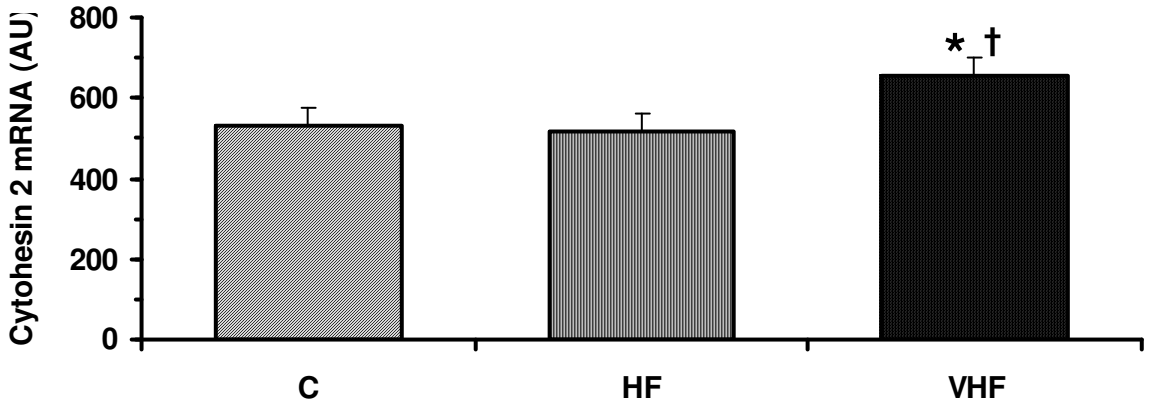

*Figure 5. Effects of dietary fat content on relative mRNA steady-state expression levels of cytohesin 2 in mouse liver. mRNA expression levels were measured with qRT-PCR with β_2 microglobulin as a house keeper using the ΔCT-method. n = 8 per group. AU = arbitrary units. Data represent means ± SEM. * P<0.05 vs. C; †P<0.05 vs. HF.*

4.1.2. Effects of High-Fat Diet on mRNA Expression Levels of Cytohesin 3

In livers of mice fed a VHF diet cytohesin 3 gene expression levels were significantly increased compared to control diet fed mice (37%, *P*<0.05 VHF vs. C, figure 6). However, mice fed a HF diet showed no effect in gene expressions of cytohesin 3 compared to the control group. A significant difference in gene expression was shown for cytohesin 3 in the VHF group compared to mice fed a HF diet (85% (*P*<0.001 VHF vs. HF).

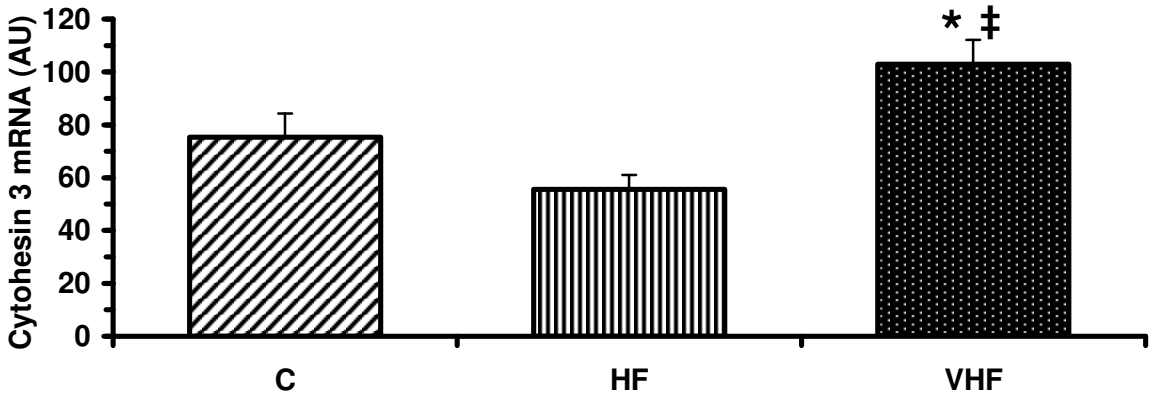

*Figure 6. Effects of dietary fat content on relative mRNA steady-state expression levels of cytohesin 3 in mouse liver. mRNA expression levels were measured with qRT-PCR with β_2 microglobulin as a house keeper using the ΔCT-method. n = 8 per group. AU = arbitrary units. Data represent means ± SEM. *P<0.05 vs. C; ‡P<0.001 vs. HF.*

4.2. Effects of High-Fat Diet on Protein Steady-State Expression Levels of Cytohesin 3 in Murine Liver

In order to determine effects of high-fat diet feeding on the expression of cytohesin 3 in liver tissue, I investigated protein level of cytohesin 3 in murine livers by Western blotting and subsequent analysis of the integrated density of the blots (figure 7).

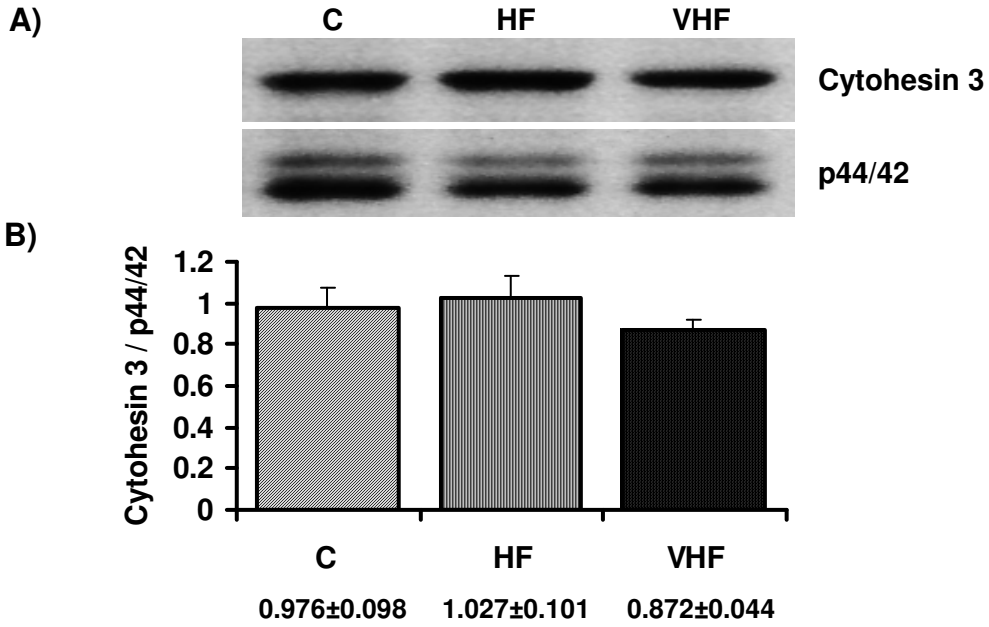

Figure 7. Steady-state protein expression analysis of p44/42 and cytohesin 3 in mouse livers. A) Effects of dietary fat content on protein levels of p44/42 and cytohesin 3. Samples of blots used for densiometric analysis. B) Cytohesin 3 to p44/42 integrated density ratios. n = 8 per group. Data represent means ± SEM.

Compared to the C group, the reference protein p44/42 yielded a non-significant decrease in protein expression levels of less than 2% in the HF group and less than 4% in the VHF group (data not shown). Cytohesin 3 protein expression levels showed a non-significant increase of 5% in the HF group and a decrease of 11% in the VHF, when compared to the C group. Thus, I detected that elevated fat intake had no significant effect on protein expression levels of p44/42 and cytohesin 3.

5. Discussion

This dissertation was prompted by the publication of an article about cytohesin inhibition in Nature by Hafner et alii in 2006. At the time cytohesins were being associated with the insulin signaling cascade.

5.0. Cytohesins and Insulin Signaling

I determined that cytohesin 2 mRNA expression levels were seven times higher than cytohesin 3 mRNA levels in male C57BL/6 mice on a normal diet. Also, I found that impaired glucose tolerance induced by high-fat feeding in mice was not accompanied by significant changes in cytohesin 2 and cytohesin 3 mRNA steady-state expression levels and by changes in cytohesin 3 protein expression levels in murine liver tissue. Only the VHF group exhibited significant increases in cytohesin 2 and cytohesin 3 mRNA expression levels compared with control diet fed mice. But cytohesin 3 mRNA and protein expression levels seemed having a different translational control, because increased mRNA expression levels were not accompanied by increased protein expression levels. A possible explanation for this could be found in the coarse sensitivity of the Western blot analysis in comparison to the relatively high sensitivity of qRT-PCR. Inhibition of cytohesin 3 by SecinH3 leads to hepatic insulin resistance [75]. Knockdown of either cytohesin 2 or cytohesin 3 (but not cytohesin 1), or of ARF6 (but not ARF1) also inhibited insulin signaling in HepG2 cells [71]. Despite that, it appears that high-fat diet-induced impaired glucose tolerance was not caused by cytohesin 3 expression inhibition in the mouse liver, at least not in a (to our setup) relevant extent. Although autophosphorylation of the IR itself is not affected by cytohesin inhibition, subsequent tyrosine phosphorylation of IRS1, the next step in the pathway, was shown to be significantly impaired [71]. Cytohesins appear to act at a point in the insulin signaling pathway that precedes the recruitment of PI3K, suggesting that their association with

the IR is not dependent upon local phosphoinositide synthesis [71]. Experiments have shown that in cells the IR is physically associated with cytohesin 2 and cytohesin 3 [42]. That suggests that, at least these two, cytohesins are involved at the very beginning of cellular insulin signaling. If cytohesins precede PI3K in the insulin pathway the very beginning of the cellular insulin signaling remains only partially illuminated; nevertheless they appear to be part of the metabolic insulin pathway.

There are several possible mechanisms of how insulin signal transduction could be inhibited at the level of cytohesins: i) Cytohesin gene expression is suppressed, which would lead to decreased cytohesin mRNA expression levels and accordingly to low cytohesin protein levels. ii) Gene expression of cytohesin encodes for dysfunctional cytohesin mRNA and protein, respectively. iii) Given normal cytohesin mRNA expression levels, post-transcriptional regulation could lead to decreased cytohesin protein levels. iv) Cytohesin proteins are inhibited in their functions through an antagonist and/or have to compete for substrate. v) Cytohesin proteins are spatially inhibited through retention in the cytosol.

Impaired glucose tolerance is a pre-diabetic condition which can continuously proceed to insulin resistance. Possibly, cytohesins contribute to hepatic insulin resistance in later stages or their effects in earlier stages could not be detected by our experimental setup. An explanation for our findings could be sought in the complexity of insulin signaling cascade. Insulin signaling is very complex when looking at all the involved components of which many come in several subtypes with different effects. Different defects or combination of defects may have the same phenotypic consequences. This appears to be the case in T2DM. Because cytohesins come in to play at or near the onset of cellular insulin signaling, the effects of their inhibition are phenotypically evident. One might argue that would be the case for any inhibition of components at the beginning of signal transduction. But the interesting point is not that cytohesins can cause insulin resistance but if this actually plays a role in the genesis of insulin resistance *in vivo*. In

light of our experiments, it appears that cytohesin inhibition is not relevantly involved in the pathology of impaired glucose tolerance in mice in the setup used for the present study.

5.1. Mechanisms of High-Fat Diet-Induced Impaired Glucose Tolerance

If involvement of cytohesin 3 in the pathogenesis of high-fat diet-induced impaired glucose tolerance seems irrelevant – what is relevant?

While long chain fatty acids suppress hepatic glucose output via the hypothalamus, they increase hepatic glucose production through a direct effect on the liver [66]. There is indication that FFA, triglycerides and lipid molecules act locally in the brain to influence leptin transport, food intake and peripheral glucose metabolism, and as such provide additional mechanisms through which the chronic consumption of a high-fat diet might be compromising body weight and glucose homeostasis [66]. But hypothalamic involvement in hepatic insulin resistance is proposed to be marginal, as a study investigated the effects of a two-week high-fat diet in 12–16 weeks old, C57Bl/6 mice on mRNA expression levels in hypothalamic neuropeptides involved in regulation of food intake and fuel homeostasis [69]. It suggests that the hypothalamus does not play a major role in the primacy of hepatic insulin resistance during a high-fat diet in mice [69]. Further was proposed that hepatic steatosis could be either a consequence of metabolic alterations elsewhere in the body and/or an active player in the pathophysiology of the metabolic syndrome [69]. Generally accumulation of lipid in tissues not designed for storage is a key initial step in the progression towards insulin resistance [66, 106, 107]. Chronic consumption of HF diets results in an oversupply of lipids to metabolic organs that is temporally related to periods of rapid weight gain, and associated with decreased insulin sensitivity and impaired glucose homeostasis [66, 106].

Findings from a high-fat diet mouse study indicate that hepatic steatosis is susceptible to oxidative stress induced by transient hypoxia for six hours and obstructive sleep apnea is potentially a trigger of development of NASH [108].

Hypothalamic control of liver metabolism can also participate in generating a proinflammatory milieu, at least in the liver [36]. TNFα is able to inhibit insulin- and leptin-induced PI3K signaling in the hypothalamus [36]. Of note, weight loss, the single most important lifestyle modification to prevent T2DM in overweight individuals, is strongly and linearly associated with reduction in C-reactive protein levels, which are indicative of inflammation [15]. In rodents the hypothalamus is able to sense and respond to hormones, cytokines, and nutrients and modulate hepatic glucose production accordingly [36]. Scientific evidence indicates that hepatic insulin resistance is caused, in part, by a failure of hypothalamic insulin, leptin, and nutrient sensing [36].

Macrophage accumulation in adipose tissue (recruitment, proliferation and/or retention of resident macrophages) as well as macrophage differentiation are the results of distinct mechanisms involving adipocyte-derived products, adipokines, chemokines and FFA, as well as local adipose tissue hypoxia and nutrient-induced metabolic endotoxemia [109]. Accumulation of adipose tissue macrophages, in turn, locally affects adipose tissue plasticity and also exerts systemic effects by favoring the release of FFA into the circulation and ectopic lipid storage in non-adipose tissue [109]. It seems evident that chronic low-grade inflammation is an important contributor to the etiology of insulin resistance associated with obesity, and that adipose tissue macrophages may play a key role in orchestrating the tissue inflammatory response [109].

Aside from fat intake, hepatic sugar excess inflicts a metabolic burden on the hepatocyte that selectively increases phosphorylation of MKK7 (MAPK kinase), activation of JNK (MAPK) and association of JNK with IRS proteins and the scaffold protein JIP1 [110]. Association of JNK with IRS1 reduces tyrosine phosphorylation of IRS1

and, thus, insulin signaling [110]. Fructose delivery also reduces tyrosine phosphorylation of IRS2 [110].

Differences observed in metabolic outcomes in C57Bl/6 mice on high-fat diets are, in part, generated by genetic differences among C57Bl/6 substrains which were generally not indicated in past studies [111]. One example shall demonstrate what is meant: Schreyer et aliae used C57Bl/6 mice, among others, and indicated that they replicated the induction of diabetes and obesity showed by Surwit et alii in such mice [104]. But Surwit et alii reported using C57Bl/6J and other substrains and not C57Bl/6 [100, 103, 112]. Additionally was shown that genetic predisposition can be more important in determining the degree of obesity in response to a high-fat diet than caloric intake [112].

Mice and rats on very high-fat (55–60 kcal%) diets produce a rapid deterioration (3 weeks) in whole body insulin sensitivity that coincides with a simultaneous reduction in insulin-dependent glucose uptake in peripheral tissues coupled with a failure of insulin to suppress hepatic glucose production [66]. In contrast, consuming high-fat diets with 45% of fat for one to five weeks resulted in loss of suppression of hepatic glucose production by insulin without any change in insulin-sensitive glucose uptake in peripheral tissue [66]. Taken together, this suggests that loss of insulin sensitivity in liver is responsible for the initial deterioration in whole body insulin sensitivity [66]. Differences in metabolic outcomes among studies seem likely to be due to amount, source, and saturation of dietary fat, length of exposure and age of animals upon introduction of test diets [66]. But ambient temperature and rearing conditions may also affect the progression of metabolic outcomes, as was shown for the progression of diet-induced leptin resistance using a 45% high-fat diet in C57BL/6 mice [66].

Further, a study shows that short-term high-fat treatment of mice seems to worsen their spatial learning tasks performance through impairment of hippocampus dependent cognition, and provides evidence that, independently of insulin, dietary intake could

impair learning processes by itself [113]. This would mean that (at least some) nutrients are capable of influencing behavior to a degree. Moreover, it was demonstrated that cognitive deficits arise prior to the development of hyperglycemia or hyperinsulinemia [113]. Possibly, dietary impairment of learning processes could lead to T2DM promoting behavior.

Taken together, several factors seem relevant and contribute to high-fat diet-induced impaired glucose tolerance or later stages of T2DM: Ectopic fat accumulation (especially in the liver) and chronic low-grade inflammation generated by adipose tissues macrophages appear to be central, though partially, the brain appears to be involved in several ways, also.

5.2. Protein Expression Levels of Cytohesin 2

I attempted to demonstrate the effects of high-fat diet on protein steady-state expression levels of cytohesin 2 in murine liver but encountered problems technical in nature: Western blot staining with the cytohesin 2 antibodies yielded repeatedly diffuse blots that did not allow a secure identification of the correct bands (data not shown).

5.3. Fat Composition of the Diets Used

Fat content of the fodder used for group C accounted for 85% vegetable oil (soybean oil) and 15% animal fat (milk fat) [114, 115]. Chow for HF diet, which is described in the product sheet as RD Western Diet, contained predominantly animal fat: 1.9% of total kcal was corn oil versus 38% milk fat [116]. Whereas VHF diet, also named after its designer Surwit Diet, had only vegetable oil (54% of total kcal hydrogenated coconut oil and 4% soybean oil) incorporated [117].

Taking into account the different origins of fat used, the question might arise if these are relevant to the comparison of weight gain and glucose tolerance. But despite this variation in the amount and source of dietary fat, in mice all high-fat diets are

associated with the emergence of hyperphagia, rapid weight gain and excess body fat accumulation [10]. Thus, fat types used to feed the mice whose livers were used in this dissertation do not seem to play a significant role with respect to weight gain.
When considering that both high-fat diets (with 17% difference in fat content) elicited approximately the same degree of glucose tolerance impairment, quantitative differential effects of animal fat and vegetable oil on glucose tolerance seem probable.

A study using healthy Swedish adults compared rapeseed (or canola) oil diet with dairy fat diet and found no differences between the diets with regard to insulin sensitivity or fasting glucose concentrations even though clinically relevant beneficial effects on the serum lipid profile were observed in subjects that replaced SFA with unsaturated fats of rapeseed oil for 3 weeks [118]. Observational studies and dietary trials suggest that increased consumption of trans-fatty acids (TFA, products of partial hydrogenation of vegetable oils, also occurring naturally in meat and dairy products from ruminants) is associated with increased triglycerides, LDL cholesterol, total to HDL cholesterol ratio, apolipoprotein B to apolipoprotein A ratio, and cardiovascular mortality and morbidity [119, 120]. Further, high intake of TFA has been associated with an increased risk of coronary heart disease and prospective cohort studies have shown that dietary TFA promote abdominal obesity and weight gain [120]. There is less clear or even conflicting evidence from observational studies regarding the effect of TFA on systemic inflammation, endothelial function, insulin resistance, and T2DM [119]. In addition, the consumption of TFA appears to be associated with the development of insulin resistance and T2DM [120].
It seems that not only the quantity and source but also the processing of fat can have an impact on health. Partially hydrogenated sunflower oil contains 20% TFA, whereas unprocessed sunflower oil contains only traces of TFA [121]. Not hydrogenated coconut oil contains 91% SFA; hydrogenation increases TFA and SFA contents slightly [121]. Thus, the VHF group had a higher intake of SFA than the HF group and a comparable intake

of TFA. Generally, elevated SFA intake is associated with a higher risk of insulin resistance, but lauric acid, a medium chain SFA, may improve total to HDL cholesterol ratio [122]. Lauric acid constitutes approximately 49% of total fatty acid content of coconuts (depending on the cultivar) and may protect against diabetes-induced dyslipidemia [123, 124]. That could explain the comparable degree of glucose tolerance impairment in both high-fat diets even though the VHF diet, despite containing no animal fat, appeared to contain more nocuous fatty acids than the HF diet.

5.4. T2DM Treatment

At first T2DM treatment should involve lifestyle interventions and administration of antidiabetic agents [14]. Aim is to achieve HbA1c plasma concentration below 6.5% [14]. In case of insufficient target glycemic control through non-insulin monotherapy a second oral agent or insulin is added [14]. Mechanisms of action of major antidiabetic agents are described shortly:

Metformin, a biguanide, leads to a reduction in hepatic glucose production mainly through transient inhibition of respiratory chain complex I [125]. Further, it activates AMP-activated protein kinase [14, 125]. Meglitinides and sulfonylureas increase insulin secretion through closing of K_{ATP} channels on β-cell plasma membranes [14]. Glitazones activate nuclear transcription factor PPAR-γ (peroxisome proliferators-activated receptor) which leads to increased glucose uptake through increased insulin sensitivity [14, 126]. α-Glucosidase inhibitors slow intestinal carbohydrate digestion [14]. Activation of the GLP-1 receptor by incretins results in increased cAMP production leading to increased synthesis and secretion of insulin and decreased glucagon secretion [14, 126]. That can also be achieved by inhibition of dipeptidyl-peptidase-4, an enzyme responsible for degradation of endogenous incretins [14, 126].

No information on the involvement of cytohesins in the mechanisms of action of antidiabetic agents was found at the time of writing. A hypothetical antidiabetic

substance targeting cytohesins would have to specifically activate/increase or mimic the action of cytohesins. But even if the action of cytohesins in the insulin signaling pathway could be enhanced, the practical value might be limited. Because of the complexity of the insulin signaling cascade, cytohesin's involvement at the intra cellular beginning of this pathway, and the high probability of interference with insulin signaling at later stages of the cascade, an antidiabetic agent centering on the action of cytohesins does not seem likely to be developed. So far cytohesin research has yielded no clinical implications for treatment of T2DM.

5.4.1. Diet Recommendations

Excess body weight is the most important modifiable risk factor for the development of diabetes [15]. There is also an indication that lean, physically active subjects can adjust to the postprandial glucose challenge following a high-glycemic-index meal by increasing insulin sensitivity, while obese, inactive subjects must increase their insulin secretion in order to re-establish glucose homeostasis [15].

Unfavorable effects of a high animal fat and protein diet, low on carbohydrates, were demonstrated in a cohort of male health professionals, where this diet was positively associated with the risk of T2DM [127]. This association was mainly due to intake of red and processed meat [127]. Conversely, a score representing a diet low in carbohydrate and high in vegetable protein and fat was not significantly associated with T2DM [127]. Interestingly, in a cohort of American adult men studied longitudinally for 7 years, it was observed that intakes of total fat and saturated fat were higher, with higher intake of animal protein and lower at higher levels of vegetable protein intake [128]. Further, animal protein intake was positively related to and vegetable protein intake was inversely associated with body mass and overweight/obesity, independent of other dietary macronutrients [128]. Thus animal and vegetable protein play different roles in obesity development, suggesting that replacement of animal protein with vegetable

protein in the diet may offer promise in future interventions aimed at prevention/control of overweight and obesity [128].

Fat quality and quantity are not the only factors influencing weight gain: Long-term supplementation (45 weeks) of a high-fat Western-style diet with soluble highly fermentable guar fiber resulted in an obese phenotype in C57BL/6J mice [129]. In contrast, supplementing the same diet with moderately fermentable insoluble cereal fiber prevented a high-fat diet-induced obese phenotype in these animals [129]. Although dietary energy intake was comparable, weight gain and estimates of insulin resistance were significantly different between groups [129].

After four weeks of diet intervention in male C57BL/6J mice, weight loss with normal fat diet (12.7% of kcal from fat), but not with high-fat diet (42.0% of kcal from fat) restriction, markedly alleviated hepatic steatosis and inflammation as assessed by hepatic triglyceride and chemokine and CD11c+ expression [7]. Normal fat diet-induced weight loss normalized adiponectin expression in adipose tissue and tended to attenuate adipose tissue expression of chemokines and CD11c+, whereas high-fat diet restriction-induced weight loss significantly attenuated adipose tissue expression of chemokines and CD11c+ and tended to increase adipose tissue adiponectin [7]. Improvements in glucose tolerance and insulin resistance were not significantly different between the diets in the short observation period, but the effects of a long-term weight loss on normal fat diet are expected to be more beneficial considering its ability to alleviate hepatic inflammation and steatosis [7].

Taken together, foods rich in SFA and simple sugars (as is the case for both, HF and VHF, diets) are clearly deleterious for obesity and diabetes, and the best replacements are MUFA and fiber-rich carbohydrate foods [15]. Or differently phrased, replacing fats from red meats and butter with non-hydrogenated vegetable oils, margarines, nuts and seeds rich in MUFA and/or PUFA should be encouraged to improve insulin sensitivity, reduce diabetes risk, and lower cardiovascular risk by reducing the serum LDL/HDL ratio and

triacylglycerols [17]. This is reflected in the macronutrient recommendations for diabetes management where SFA intake should be below 7% of total calories [14].

5.5. Conclusion

Today, cytohesin research is concerned mainly with cancer and immunology research [130]. A search for cytohesin on PubMed.gov yielded 187 search results compared to 18 hits for cytohesin and insulin [130, 131]. Only about a third of the latter addressed insulin signaling in depth and most of these were published in 2006 and 2007, when this dissertation started. Thus today, it appears, there is little scientific attention directed at the details of cytohesins participation in insulin signaling.

This study could not demonstrate any impact of diet-induced obesity (leading to impaired glucose tolerance) on steady-state expression levels of cytohesin 2 and cytohesin 3 mRNA in murine liver. If cytohesins are involved in the pathogenesis of impaired glucose tolerance and/or T2DM *in vivo*, they seem to play a subordinate role.

6. References

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8. Appendix

Preparation of RIPA Buffer: The following amounts of the constituents listed below were mixed in a beaker and filled up to a volume of 100 ml with distilled water.

10 ml NP-40 [10%], 0.1 g SDS, 3 ml NaCl [5M], 5 ml Tris-HCl [1M], 200 µl EDTA [1mM], 10 ml Glycerol.

9. Curriculum Vitae

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