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Omics Techniques Applied to Diabetes Research - Focus on HSL-Null Mice and Clonal β -Cells

Fernandez, Celine

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PO Box 117
221 00 Lund
+46 46-222 00 00

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Abstract

Diabetes mellitus is a disease characterized by increased blood glucose levels. For overt type 2 diabetes to develop, both insulin action and insulin secretion must be perturbed. In the work presented in this thesis, two models were analyzed: hormone-sensitive lipase (HSL) null mice and a pancreatic clonal β -cell line to study the mechanisms underlying insulin resistance and insulin secretion perturbations, respectively. To achieve this, techniques allowing a global analysis of the transcriptome, proteome and metabolome were used.

HSL is best known as the enzyme hydrolyzing acylglycerides stored in adipose tissue. But HSL has broad substrate specificity and is widely expressed elsewhere than in adipose tissue. Its role in non-adipose tissues is not completely understood, but the phenotype revealed by the characterization of several independently generated HSL-null mouse lines during the recent years suggests that HSL has several functions in addition to its role in adipocyte lipolysis. The role of HSL in the liver was studied in this thesis. More specifically, the liver phenotype of HSL-null mice was investigated at the transcriptome (Paper I and IV) and proteome (Paper IV) levels. The obtained results didn't allow the obvious identification of possible mechanisms behind the hepatic insulin resistance observed in our HSL-null mouse strain. However, our results show that HSL plays an important role as a cholesteryl ester hydrolase in the liver and that HSL influences overall cholesterol homeostasis by indirectly controlling hepatic HDL-cholesterol clearance. We also demonstrated the importance of a cross-talk between white adipose tissue and liver that regulates cholesterol homeostasis via the type of non-esterified fatty acids (NEFA) released. Moreover, changes in expression of proteins involved in polyamine metabolism were observed in the liver of HSL-null mice, which could be responsible for the increased liver weight characterizing HSL-null mice. The physiological response of HSL-null mice to aerobic treadmill exercise was also investigated (Paper II). HSL was shown to play an important role during aerobic exercise in controlling the mobilization of lipid stores from white adipose tissue, a function which cannot be fully compensated by any other acylglyceride lipases.

Proteome and metabolome analyses were performed in Paper III to study glucose-stimulated insulin secretion in a β -cell line cultured in presence of normal or toxic glucose concentrations. A metabolite fingerprint which is characteristic for β -cells cultured at high glucose concentrations was obtained.

List of Papers

This thesis is based upon the following papers, which are referred to in the text by their Roman numerals (I-IV). The papers can be found as appendices at the end of the book.

- I Fernandez, C., Lindholm, M., Krogh, M., Lucas, S., Larsson, S., Osmark, P., Borén, J. Fielding, B., Frayn, K. and Holm, C. Upregulation of hepatic ABCA1 and disturbances in cholesterol homeostasis in hormone-sensitive lipase null mice (Submitted to Am. J. Physiol. - Endocrinol. Metab.)
- II Fernandez, C.*, Hansson, O.*, Nevsten, P., Holm, C. and Klint, C. Hormone-sensitive lipase is necessary for normal mobilization of lipids during sub-maximal exercise (Submitted to Am. J. Physiol. - Endocrinol. Metab.)
- III Fernandez C, Fransson U, Hallgard E, Spégel P, Holm C, Krogh M, Wårell K, James P and Mulder H. Metabolomic and proteomic analysis of a clonal insulin-producing β -cell line (INS-1 832/13). J Proteome Res. 2008 Jan;7(1):400-11. Epub 2007 Dec 7.
- IV Fernandez C, Krogh M, Wårell K, James P and Holm, C. Transcriptome and proteome analysis of liver of hormone-sensitive lipase null mice (Manuscript).

* shared first authorship

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Abbreviations

2-D	Two-Dimensional
ADA	ArylAcetamide DeAcetylase
ABCA1	ATP-Binding Cassette A1
ACAT	Acyl-CoA:Cholesterol AcylTransferase
ADRP	Adipose Differentiation-Related Protein
Apo	Apolipoprotein
ATGL	Adipose Triglyceride Lipase
CE	Cholesteryl Ester
CEH	Cholesteryl Ester Hydrolase
CETP	Cholesteryl Ester Transfer Protein
DGAT	Diacylglycerol AcylTransferase
EDL	Extensor Digitorum Longus
FC Free	Cholesterol
GC	Gas Chromatography
GLUT	Glucose Transporter
HDL	High Density Lipoprotein
HFD	High Fat Diet
HMG-CoA	HydroxyMethylGlutaryl- CoA
HSL	Hormone-Sensitive Lipase
IDL	Intermediate Density Lipoprotein
IMTG	IntraMyocellular TriGlyceride
LCAT	Lecithin-Cholesterol AcylTransferase
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
LRP	LDL Receptor-related Protein
LXR	Liver X Receptor
MALDI	Matrix Assisted LASER Desorption Ionisation
MS	Mass Spectrometry
MTP	Microsomal triacylglycerol Transfer Protein
ND	Normal Diet
NEFA	Non-Esterified Fatty Acid
PMF	Peptide Mass Fingerprint
PKA	Protein Kinase A
PL	PhosphoLipids
Q	Quadrupole
RXR	Retinoid X Receptor
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SR-BI	Scavenger ReceptorClass B type I
SREBP	Sterol Regulatory Element Binding Protein
TCA	TriCarboxylic Acid
TG	TriGlycerides
TGH	TriGlycerides Hydrolase

Abbreviations

TIP47	Tail-Interacting Protein of 47 kDa
TNF α	Tumor Necrosis Factor α
TOF	Time Of Flight
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue

Introduction

Energy Homeostasis

Energy homeostasis in mammals is about maintaining an equilibrium between the energy input and output in order to keep stable blood glucose level (from 3 to 9 mM in humans) (117) and body weight. Energy input originates from the diet whereas energy output is the sum of energy consumed by the basal metabolism, during physical activity and by adaptive thermogenesis in response to diet or cold (65). In order to maintain this balance, several organs are involved in a coordinated manner through the action of hormones.

Key Players

Liver

Liver Metabolism

The liver is the first organ to sense when actions need to be taken to maintain energy homeostasis because of its vascularization by the portal vein which carries the water soluble nutrients absorbed from the diet and the pancreatic hormones insulin and glucagon to the liver before their entry into the general circulation. Right after a meal (postprandial state), when the insulin levels are high, the role of the liver is to store energy. Blood glucose will be taken up by the glucose transporter 2 (GLUT2), which is not responsive to insulin, and stored in the liver as glycogen (glycogenesis) and/or converted to fatty acids that are then esterified to triglycerides (lipogenesis). Plasma non-esterified fatty acids (NEFA) and lipids associated to chylomicron remnants will be taken up by the liver and stored as triglycerides (TG). When all nutrients from the latest meal have been absorbed (postabsorptive state), the task of the liver is to provide energy and more specifically glucose to the organs that are strictly relying on glucose to function i.e. the brain, the renal medulla, and red blood cells. To achieve this glycogen will be broken down (glycogenolysis) and *de novo* synthesis of glucose (gluconeogenesis) will occur from non-carbohydrate substrates (glycerol, glucogenic amino acids and lactate) at the expense of beta-oxidation of fatty acids (20).

Introduction

Lipoproteins are particles enabling the transport of non-polar lipids in the blood. They typically consist of a highly hydrophobic core made of TG and cholesteryl esters surrounded by an amphipathic monolayer of phospholipids, free cholesterol and apolipoproteins. Lipoproteins are classified into chylomicrons, very low density lipoproteins (VLDL), low-density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicrons and VLDL are relatively enriched in TG; LDL and HDL are relatively enriched in cholesterol. In the fed state, chylomicrons are synthesized in the small intestine in several steps. First, dietary TG are emulsified by bile salts and hydrolyzed by pancreatic lipase within the intestinal lumen. The resulting monoglycerides and fatty acids are transported into the enterocytes where their re-esterification into TG occurs. The TG are packed with phospholipids, cholesteryl esters, free cholesterol and proteins into nascent chylomicrons, which are secreted via the lymphatic system into the circulation. In the circulation, chylomicron-TG are hydrolyzed to NEFA and glycerol by the endothelial-bound lipoprotein lipase (LPL) as they pass through capillaries of white adipose tissue and skeletal muscle. The obtained shrunken particles, known as chylomicron remnants, are rapidly cleared by the liver. In addition, the liver secretes very low density lipoprotein (VLDL) particles, predominantly in the fasted state. NEFA released by adipose tissue are the major substrates for hepatic VLDL production. Since VLDL-TG are also substrates for LPL, NEFA will be returned back to white adipose tissue thus forming an inter-tissue cycle. The resulting relatively cholesteryl ester-enriched particles can be taken up by low-density lipoprotein (LDL) receptors in the liver and other tissues, or they can remain in the circulation and undergo further LPL-mediated lipolysis with formation of intermediate density lipoproteins (IDL) and finally LDL particles, which will then be taken up via the LDL receptor in the liver and in extrahepatic tissues (20).

Lipoproteins

Apolipoproteins

The apolipoprotein (apo) composition, which is specific for the different lipoproteins, is an important characteristic since in addition to the solubilization and stabilization effects of apolipoproteins many of them function as ligands for cell surface receptors. ApoAI activates lecithin-cholesterol acyltransferase (LCAT), the enzyme that esterifies free cholesterol in HDL particles. ApoB is found in chylomicrons, VLDL and LDL particles and is present as a single

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molecule per particle which will stay with the particle throughout its lifetime. There are two isoforms of apoB called apoB100 and apoB48. ApoB48 is the major protein component of chylomicrons. In humans, apoB100 is the principal apolipoprotein for VLDL particles, but in the mouse VLDL particles contain both apoB100 and apoB48. ApoCII, which is necessary for the activation of LPL, is present in chylomicrons, VLDL and HDL particles. Finally apoE, which functions as a ligand for the LDL receptor and the LDL receptor-related protein (LRP), is present in chylomicrons, VLDL and HDL particles (20). The LDL receptor is also capable of binding apoB100 containing lipoproteins (76).

VLDL Synthesis

The working model of VLDL assembly is a two-step process taking place in the endoplasmic reticulum of the hepatocytes. In the first stage, a small quantity of TG becomes associated to apoB and results in the formation of an apoB-containing VLDL precursor. The second stage, or maturation phase, involves the fusion of the apoB-containing precursor with a larger droplet of TG. Both steps are dependent upon a lipid transfer protein called microsomal triacylglycerol transfer protein (MTP) (26). TG are not incorporated directly into VLDL particles from their storage pool. Rather, TG lipolysis followed by reesterification at the endoplasmic reticulum represents crucial steps in the VLDL assembly process. There are three potential sources of fatty acids that enter the TG storage pool in liver: de novo synthesized fatty acids, plasma NEFA (originating mainly from adipose tissue) and remnant lipoproteins (VLDL and chylomicron remnants) (25). Of these, plasma NEFA represent the main contributor to VLDL-TG both in the fed and fasted state (17). Thus, factors affecting plasma NEFA concentration will also influence VLDL generation.

Plasma NEFA enter the hepatocyte, are esterified into TG by diacylglycerol acyltransferase-1 (DGAT-1) and stored in the cytosol. TG from the cytosolic pool are not directly incorporated into VLDL. Rather they are hydrolyzed by arylacetamide deacetylase (AADA) and triglyceride hydrolase (TGH) (25). AADA is the only currently described mammalian protein that shows strong active site homology with hormone-sensitive lipase (HSL), but is associated with the endoplasmic reticulum (80). TGH is a neutral lipase that has been purified from hepatic endoplasmic reticulum (62). The lipolytic products of AADA and TGH are reesterified by DGAT-2 before channeling into a TG-rich VLDL precursor or returning back to

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the TG cytosolic pool in a futile cycle. Insulin, which increases the flux of newly reesterified TG back to the cytosolic pool, is among the factors that determine this partitioning (26). For TG mobilization and VLDL assembly see figure 1.

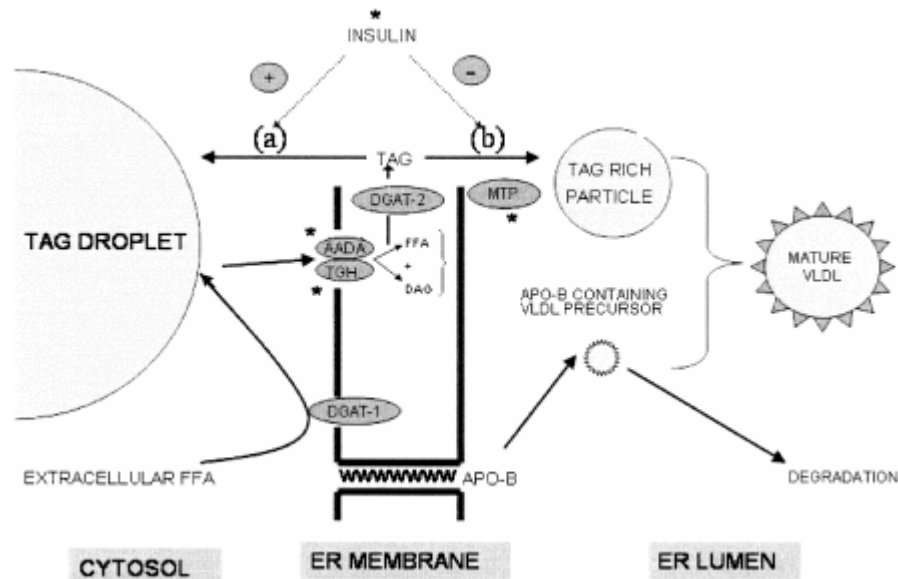


Figure 1. Schematic representation of VLDL assembly in hepatocytes. VLDL lipoproteins are assembled in two steps. The first step consists in the formation of a partially lipidated apoB particle. The second step involves the fusion of the apoB-containing VLDL precursor with a TG rich particle. TG required for VLDL synthesis are recruited from a cytoplasmic TG store that is hydrolyzed by the action of AADA and TGH. The products are then reesterified by DGAT2. The resulting TG are channeled into the TG rich VLDL precursor particle (pathway b) or recycled back to the cytosolic pool (pathway a). The latest is favored by insulin. (Modified from Gibbons et al. 2004).

HDL Synthesis

HDL are synthesized through a complex pathway. It begins with the interactions between apoA1, which is secreted predominantly by liver and intestine, and hepatic ATP-binding cassette transporter A1 (ABCA1), which transfers cellular phospholipids and cholesterol to lipid-free apoA1. Some of the excess surface lipid (free cholesterol and phospholipids) and apolipoproteins

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released by LPL action on TG-rich lipoproteins are also added, resulting in the formation of nascent pre- β HDL particles. Additional cholesterol and phospholipids are acquired from cells in extrahepatic tissues via ABCA1-mediated efflux, progressively generating particles that are more cholesterol enriched. The enzyme lecithin-cholesterol acyl transferase (LCAT), which is activated by apoA1, esterifies the free cholesterol molecules. Hydrophobic cholesteryl esters are retained in the HDL core forming larger mature α -migrating HDL particles, which can be sub-fractionated by ultracentrifugation into HDL₂ (larger particles) and HDL₃ (smaller particles) (20, 63). HDL particles undergo intravascular remodeling by the combined action of lipid transfer factors (cholesteryl ester transfer protein and phospholipid transfer protein) and of several lipolytic enzymes (LCAT, LPL, hepatic lipase) (Figure 2).

ABCA1 is a ubiquitous protein expressed abundantly in the liver, macrophages, brain and various other tissues (53, 60). Tangier disease patients, who are homozygous for functional mutations of ABCA1, and ABCA1 knockout mice fail to form discoidal or spherical HDL, have very low total plasma cholesterol and exhibit increased tissue and macrophage cholesterol accumulation (115). Targeted inactivation of hepatic ABCA1 in mice leads to a dramatic reduction of plasma HDL particles, indicating that hepatic ABCA1 is responsible for the formation of most of the plasma HDL particles (98). In support, overexpression by adenovirus delivery of ABCA1 in the liver of mice results in increased plasma HDL particles (4, 110). However, it was later demonstrated that hepatic ABCA1 plays an important role in the lipidation of lipid-free apoA1 while extrahepatic ABCA1 is critical to the further lipidation and maturation processes of the nascent HDL particles into mature HDL particles (93). The transcription of ABCA1 has been shown to be regulated by the liver X receptor (LXR)/retinoid X receptor (RXR) heterodimer with cholesterol-derived oxysterols (produced by sterol hydroxylases from cholesterol) as the physiological ligand (12, 91). In addition, unsaturated fatty acids have been reported to promote ABCA1 turnover as discussed further below.

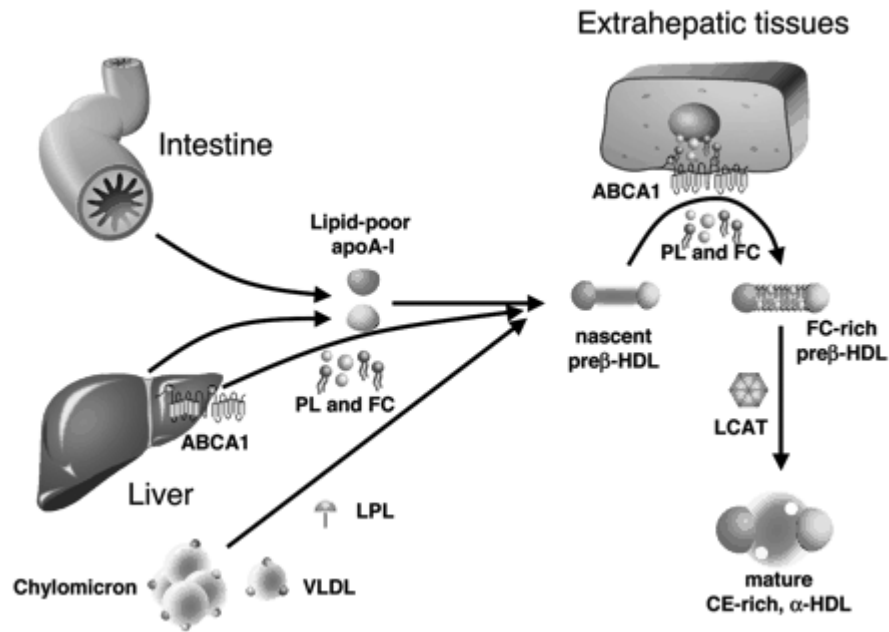


Figure 2. Schematic representation of HDL synthesis. Secreted lipid-poor apoA1 are lipidated via ABCA1-mediated efflux from the liver and by acquiring PL and FC shed from TG-rich lipoproteins during LPL-mediated lipolysis. The obtained nascent pre- β HDL particles receive further PL and FC from extrahepatic tissues, generating cholesterol enriched particles. Free cholesterol molecules in the particles are esterified by LCAT and migrate to the core of the particles forming mature α -migrating HDL particles.

ABCA1, ATP-binding cassette transporter A1; CE, cholesterol ester; FC, free cholesterol; LCAT, lecithin-cholesterol acyl transferase; LPL, lipoprotein lipase; PL, phospholipids. In Lewis GF et al. 2005.

Lipoprotein Clearance

The liver plays a central role in lipoprotein clearance. The catabolism of chylomicron remnants from the blood involves multiple components on the surface of hepatocytes, including hepatic lipase, and the recognition of apoE on chylomicron remnants by LDL receptor and LRP (37). LDL particle and VLDL remnant removal occur through interaction with the LDL receptor that mediates internalization by endocytosis and degradation of the particles. LDL receptors in the liver are responsible for clearing approximately 80% of the LDL cholesterol from the plasma while the one in the extrahepatic tissues clear the rest (Table 1, column D) (16). At the

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hepatocyte, mature HDL particle clearance takes place through the action of scavenger receptor class B type I (SR-BI). SR-BI is mainly expressed in the liver and steroidogenic glands (e.g. adrenal gland and gonads) and mediates the selective uptake of cholesteryl esters from HDL. The resulting cholesterol-depleted particles leave the receptor and are remodeled in the circulatory system to form HDL₂ particles. Moreover, SR-BI can off-load cholesteryl esters from LDL and mediates bidirectional unesterified cholesterol movement between HDL or LDL and cells (96, 97, 115).

Other factors involved in hepatic lipoprotein catabolism are LRP and hepatic lipase. LRP is involved in the uptake of cholesterol-enriched apoB-lipoproteins. Hepatic lipase is an extracellular lipolytic enzyme synthesized by hepatocytes. It hydrolyzes the phospholipids and less efficiently the triglycerides of apoB-containing lipoprotein remnants and large HDL particles (115). It converts larger HDL particles to smaller HDL remnants, pre- β HDL and lipid-poor or -free apoA1 (63).

Hepatic Cholesterol Metabolism

Cholesterol *de novo* Synthesis

Body cholesterol can originate from the diet or from *de novo* synthesis. On average, humans take in approximately 3 to 5 mg cholesterol /day / kg of body weight, which represents approximately 50% of *de novo* cholesterol synthesis (Table 1, column A and B) (56). On such a diet, most of the net input of cholesterol to the liver comes from *de novo* synthesis in the extrahepatic tissues rather than from the diet (Table 1, column C) (92).

Cholesterol synthesis from acetyl-CoA involves many enzymatic steps. The rate limiting enzyme of the pathway is hydroxymethylglutaryl CoA reductase (HMG-CoA reductase). Cholesterol is an end-product suppressor of the transcription of the genes involved in its own synthesis and of the gene encoding the LDL receptor. This regulation involves sterol regulatory element binding proteins (SREBPs), a family of membrane bound transcription factors. In the liver, SREBP-1c and SREBP2 predominate. The former preferentially enhances transcription of genes involved in fatty acid synthesis, while the latter regulates genes involved in cholesterol synthesis, such as HMG-CoA reductase and farnesyl diphosphate synthase (48). To act as a transcription factor, newly synthesized SREBP must be released from the membrane of the endoplasmic reticulum where it is inserted. At the endoplasmic reticulum SREBP is associated

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with a cholesterol sensor, the SREBP cleavage-activating protein (SCAP). When the cellular cholesterol content is low, proteolytic activation of SREBP takes place leading to the release of the NH₂-terminal domain of SREBP (nSREBP). nSREBP translocates to the nucleus where it binds to sterol response elements in the promoter regions of many genes (48).

Cholesterol Storage

Cellular cholesterol undergoes a continuous cycle of esterification and ester hydrolysis. The enzyme responsible for cholesterol esterification is acyl-CoA:cholesterol acyltransferase (ACAT). There are two isoforms of the enzyme, ACAT-1 and ACAT-2. In the liver, ACAT-2 is dominantly expressed and serves to esterify cholesterol to be included in VLDL particles (50). ACAT-2 does not appear to be transcriptionally regulated. ACAT-2 activity is instead determined by the hepatic fatty acids composition. Long chain unsaturated fatty acids are the preferred substrate of ACAT-2 and will affect hepatic intracellular cholesterol distribution favoring the esterified pool at the expense of the unesterified one thus restoring the transcription of the LDL receptors (113).

Several hepatic enzymes may be responsible for the cytosolic hydrolysis of cholesteryl esters. Carboxyl ester lipase (6), also known as bile salt-stimulated lipase, has been shown to be expressed at low levels in rat and human liver. However, it is located in endosomal compartments of hepatocytes or is directly secreted by hepatocytes thus ruling out its role as a cytosolic cholesteryl hydrolase (49). Hepatic neutral cytosolic cholesteryl ester hydrolase (CEH) is expressed in human liver and transient transfection of primary human hepatocytes with human liver CEH leads to an increase in bile acid synthesis (116). Another candidate is HSL since it has the capacity to hydrolyze cholesteryl esters and it has been shown that hepatic cholesteryl ester hydrolyze activity is decreased by 4.5 fold in the liver of HSL-null mice (72).

Reverse Cholesterol Transport

Cholesterol is an essential component of mammalian cell membranes and a substrate for steroid hormone synthesis. Most cholesterol is synthesized in the extrahepatic tissues (Table 1, column C) (16), but cells, with the exception of the liver and steroidogenic tissue, cannot metabolize cholesterol. Instead, excess cholesterol from peripheral organs is brought to the liver in the form of HDL particles for elimination from the body, a process called reverse cholesterol transport.

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However, reverse cholesterol transport is more than an unidirectional flux of cholesterol from extrahepatic tissues to the liver since a substantial flux of cholesterol from the liver occurs during the synthesis of nascent HDL particles, as previously discussed (63).

In addition to the transport of cholesterol to the liver by HDL humans, primates and a few other species possess another component of reverse cholesterol transport. In such species, a transfer of lipids between lipoprotein particles occurs via the action of cholesteryl ester transfer protein (CETP). Cholesteryl esters are transferred by CETP from HDL and LDL to chylomicrons and VLDL, which will then deliver their cholesterol loads through interaction with LDL receptors in the liver (37). In CETP possessing species LDL particles carry the majority of the plasma cholesterol (Table 1, column E) (67). Mice lack CETP activity, use HDL particles as their main plasma cholesterol transporters and have another class of HDL particles enriched in apoE, referred to as HDL₁, which can thus be cleared by the LDL receptors (67).

Excess cholesterol from peripheral cells that is transported to the liver is converted to bile acids and then excreted in the faeces or directly secreted into the bile and then disposed via the faeces. In mice and humans, those two pathways contribute almost equally to faeces cholesterol excretion (15). Cholesterol can be converted into bile salts via two pathways: the classic or neutral pathway and the alternative or acidic pathway. The key regulatory enzyme in the classic pathway is cholesterol 7 α -hydroxylase (CYP7A1) and in the alternative pathway sterol 27-hydroxylase (CYP27A) (50).

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Table 1. Cholesterol turnover in mice and humans. These values were obtained from mice and humans fed a diet where the cholesterol content ingested each day was less than 50% of the rate of whole body cholesterol de novo synthesis (16).

	A. Dietary cholesterol intake	B. Whole animal cholesterol synthesis rate	C. Liver contribution to whole animal synthesis	D. Liver contribution to LDL- cholesterol clearance	E. Steady- state LDL- cholesterol concentration
	mg/day/kg	mg/day/kg	% total	% total	mg/dl
Mouse (0.025 kg)	30	160	40	80	7
Human(70 kg)	5	10	10	70	120

White Adipose Tissue

White adipose tissue (106) constitutes the most energy rich storage organ of the body. This energy is accumulated in the form of TG. In the postprandial state, the role of WAT is to store blood lipids: the NEFA transported by albumin and the NEFA generated by LPL action on chylomicrons and VLDL-TG. In the postabsorptive state, TG are broken down to NEFA (lipolysis) that are released into the blood and will be used as a fuel by peripheral organs. HSL has for decades been regarded as the main regulatory step in lipolysis in mammals. HSL is activated by catecholamines (the hormone adrenaline and the neurotransmitter noradrenaline) and inhibited by insulin (42). Recently, the discovery of a novel TG lipase termed adipose triglyceride lipase (ATGL) challenged the importance of the role of HSL in lipolysis (118). Stimulated and non-stimulated fat cell lipolysis was impaired in *in vitro* ATGL inhibition studies (118). Moreover catecholamine-stimulated lipolysis was markedly decreased in ATGL knockout mice (30). However, a recent study indicated that in humans HSL is of greater importance than ATGL in regulating catecholamine-induced lipolysis while both lipases are important for the regulation of basal lipolysis (89).

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Selectivity in the release of fatty acids from stored TG in adipose tissue has been reported. Under conditions of stimulated lipolysis, isolated rat fat cells did not mobilize fatty acids in direct proportion to their TG content, but according to fatty acid chain length and degree of unsaturation. Fatty acids were more readily mobilized when they were short and unsaturated (82). HSL was shown to be partially responsible for this selectivity when studying the hydrolysis capacity of recombinant rat or human HSL towards lipid emulsions with fatty acids of varying length and degree of saturation. However, polyunsaturated fatty acids were not found to be readily released by HSL compared with total fatty acids and vaccenic acid and oleic acid were among the most readily released fatty acids (83).

In addition to being energy substrates, NEFA play other important roles. They influence insulin action and glucose metabolism in muscle and liver, regulate insulin secretion by endocrine pancreas and act as signaling molecules regulating gene transcription. An example of gene expression regulation by fatty acids that was encountered during this work is given by ABCA1. Unsaturated fatty acids have been reported to regulate ABCA1 expression. This occurs via suppression of ABCA1 transcription (100, 101) and via increased degradation of ABCA1 protein. The latter involves activation of protein kinase C δ by diacylglyceride subspecies enriched in unsaturated fatty acids, resulting in phosphorylation and degradation of the ABCA1 protein (104).

Besides its role as a reservoir for energy storage, WAT is now known to secrete a variety of proteins termed adipokines, which act at both the local and systemic level, thus establishing WAT as an endocrine organ. Among the secreted substances are leptin, which primarily serves as a metabolic signal of energy sufficiency; adiponectin, the plasma level of which is negatively correlated with insulin resistance; and tumor necrosis factor α (TNF α) (51). In the liver, adiponectin enhances insulin sensitivity, increases fatty acid oxidation and reduces hepatic glucose production (51). TNF α represses liver mRNA transcription of several genes involved in glucose uptake and metabolism and genes involved in fatty acid oxidation (88).

In conclusion, WAT through the release of adipokines and NEFA, has a mean to communicate with other tissues, including the liver.

Skeletal Muscle

Skeletal muscle is a diverse tissue with different properties based upon its fiber-type composition. Muscle fiber classification has been evolving with time from being based on the color of fibers to measuring fiber speed and oxidative capacity to relying on myosin heavy chain isoform expression (95). With gross simplification, muscle fibers can be divided into type I slow twitch oxidative and type II fast twitch glycolytic (95). In this thesis, two categories of muscle have been used, extensor digitorum longus (EDL), which is mainly composed of fast twitch fibers, and gastrocnemius, which is composed of a mixture of type I and type II fibers (8, 75).

Skeletal muscle represents a highly metabolically active tissue. In humans under euglycemic hyperinsulinemic clamp experiments, 75% of whole body insulin-mediated glucose disposal was accounted for by skeletal muscle (3). The muscle can indifferently use glucose or NEFA as energy source and it is substrate availability through the coordinated control of metabolism at the whole body level that decides which substrate is used. After a meal when the plasma glucose concentration is elevated, the muscle will tend to switch to the use of glucose rather than NEFA. Indeed, elevated glucose concentrations stimulate insulin secretion, which then suppresses NEFA release from the WAT. Thus, plasma NEFA concentration will fall, reducing the uptake of NEFA since the rate of uptake is closely related to plasma NEFA concentration. In addition, muscle glucose uptake will be stimulated by insulin through upregulation of the number of glucose transporters 4 (GLUT4) in the plasma membrane and through increased disposal of glucose within the cell since insulin will activate glycogen synthase thus leading to the conversion of the up taken glucose to glycogen (20). Moreover, glucose oxidation will inhibit NEFA oxidation via the inhibiting action of malonyl-CoA on carnitine-palmitoyl transferase-1 (69).

In the postabsorptive state, the plasma glucose concentration falls a little, insulin secretion decreases, and the plasma NEFA concentration rises. More NEFA will be available for muscle oxidation. Since the oxidation of NEFA in muscle reduces the uptake and oxidation of glucose, the muscle will revert to NEFA utilization (84), thus sparing glucose for tissues such as the brain which are glucose dependent. This series of metabolic events coordinating glucose and fat metabolism was termed the glucose-fatty acid cycle by Randle and colleagues (84).

Fuel selection also occurs in skeletal muscle during exercise and is mainly determined by exercise intensity and duration. During high-intensity exercise, creatine phosphate degradation

Introduction

and glycogen breakdown are the major pathways activated in order to meet the increased need for energy. However, during prolonged submaximal exercise, oxidative metabolism of carbohydrates and lipids becomes the predominant mechanism to produce ATP (36).

The sources of carbohydrates for oxidation purpose in the working muscle are muscle glycogen and blood glucose (i.e. liver glycogen). Muscle blood glucose influx is increased during exercise due to the upregulation of the number of GLUT-4 in the plasma membrane which is triggered by muscle contraction, independently of insulin, and in response to the increased blood flow (40). Lipids destined for oxidation in the working muscle originate from four different sources: plasma NEFA, plasma TG (i.e. VLDL-TG), intramyocellular triglyceride (IMTG) stores and fatty acids liberated from adipocytes present in between muscle fibers (54). The magnitude of the contribution of IMTG to total energy expenditure during exercise has been debated, mostly because of methodological difficulties in measuring the IMTG pool (106). However, studies using ¹H-magnetic resonance spectroscopy or immunofluorescence microscopy have all supported the notion that the IMTG pool can function as an important source of energy during exercise (102). Consequently factors that control IMTG stores mobilization are especially important during exercise. HSL, which is expressed in skeletal muscle (Holm, 1987 #25), is likely to play a key role in the regulation of IMTG breakdown since HSL has been demonstrated to be activated in rat skeletal muscle in response to both adrenaline (59) and contraction (58). In addition, HSL translocation to IMTG droplets has been shown in rat skeletal muscle in response to both these stimuli (79).

The role of fat oxidation during prolonged exercise is to spare carbohydrate utilization in skeletal muscle and liver (86), thus improving endurance capacity since hypoglycemia and muscle fatigue will develop when liver and muscle glycogen stores, respectively, are depleted (41).

Beta-cells

Beta-cells (β -cells) through the production of insulin ensure the coordination between the various organs involved in maintaining energy homeostasis.

Insulin secretion is a two-step event comprising a triggering and an amplifying pathway. During the triggering pathway of glucose-induced insulin secretion, glucose that enters the β -cell by facilitated diffusion is metabolized by oxidative glycolysis leading to the formation of ATP and an increase in the ATP/ADP ratio. Consequently, the closure of ATP-sensitive K^+ (K_{ATP}) channels occurs, resulting in the depolarization of the β -cell plasma membrane and the opening of voltage-operated Ca^{2+} -channels. Cytosolic Ca^{2+} concentration increases triggering the activation of the exocytotic machinery and thus insulin secretion (38). The finding that glucose can control insulin release independently from changes in K_{ATP} -channels activity led to the concept of an amplifying pathway of glucose-induced insulin secretion (23). The amplifying pathway is dependent upon the elevated cytosolic Ca^{2+} concentration produced by the triggering pathway but is not mediated by any further rise in cytosolic Ca^{2+} concentration. It amplifies the secretory response to the triggering signal induced by glucose through mechanisms that are currently unknown (23).

One of the possible mechanisms underlying the amplifying pathway are signals derived from anaplerosis of the β -cell tricarboxylic acid (TCA) cycle (66). Anaplerosis describes metabolic pathways which replenish a metabolic cycle. In the case of the TCA-cycle, pyruvate can enter the cycle in two ways, via decarboxylation to acetyl-CoA by pyruvate dehydrogenase, or by anaplerosis via carboxylation to oxaloacetate by pyruvate carboxylase. Anaplerosis is in equilibrium with cataplerosis, i.e. exit of TCA-cycle intermediates, thus allowing metabolites to leave the TCA-cycle and act as metabolic coupling factors for insulin secretion. The importance of anaplerosis in the β -cell for insulin secretion is reflected by the expression of pyruvate carboxylase to levels that almost equal those of the gluconeogenic organs, liver and kidney. In addition, half of the glucose-derived pyruvate enters mitochondrial metabolism by carboxylation; and the rate of pyruvate carboxylation correlates better than the rate of its decarboxylation with the rate of insulin secretion (66).

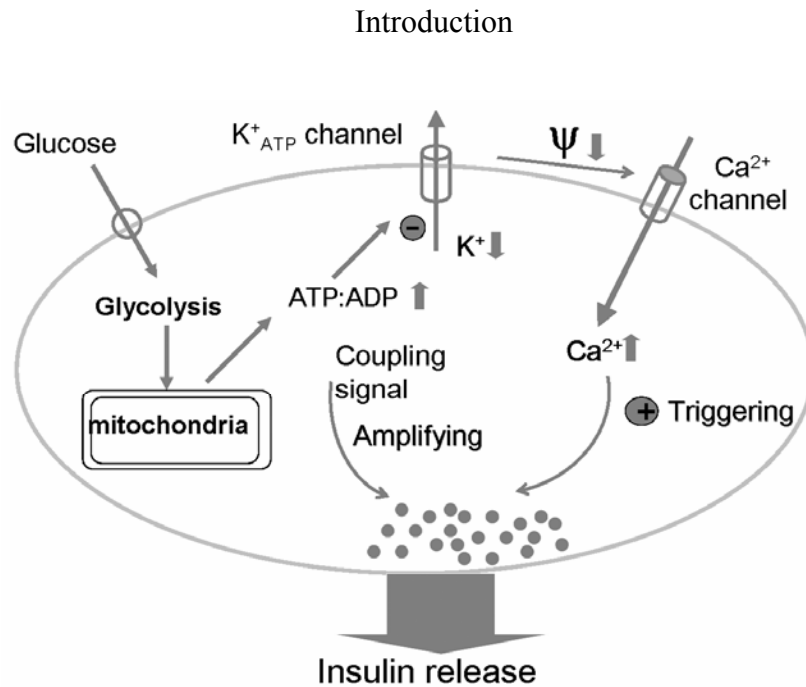


Figure 3. Glucose-induced insulin secretion in the pancreatic β-cell.

Defect in Energy Homeostasis

Diabetes Mellitus

Diabetes mellitus is characterized by increased blood glucose levels. Diabetes can be divided into two main types: type 1 and type 2. Type 1 diabetes results from the auto-immune destruction of the pancreatic β-cells. Type 2 diabetes is characterized by disorders of both insulin action and insulin secretion (13) and is tightly associated with obesity (68).

Insulin Resistance

Insulin resistance may be defined as a condition in which higher than normal insulin concentrations are needed to achieve normal metabolic responses or normal insulin concentrations fail to achieve a normal metabolic response (81). Classically, insulin resistance in muscle, liver and adipose tissue has been viewed as central to the pathogenesis of type 2 diabetes. Organ-specific deposition of fat is a strong predictor of insulin resistance (81). Insulin resistance manifests itself by a reduced ability of insulin to inhibit hepatic glucose output and to

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stimulate glucose uptake and metabolism in skeletal muscle and adipose tissue. The accelerated rate of hepatic glucose output is due entirely to an augmented gluconeogenesis and it plays an important role in maintaining the diabetic state (13). In the insulin resistant state, one factor responsible for the reduced glucose uptake in muscle is the decrease in muscle GLUT4 content. It was shown that GLUT4 content of whole muscle homogenates was decreased by approximately 50% in muscle of diabetic rats (40). Besides resistance to the glucoregulatory action of insulin, resistance to the antilipolytic action of insulin in adipose tissue usually occurs, resulting in elevated plasma NEFA levels (11, 68). In addition, the uptake and reesterification of NEFA in adipose tissue is reduced, further contributing to the increase in plasma NEFA, which in turn leads to an increased flux of NEFA into liver and muscle (1). Enhanced NEFA flux into the liver gives rise to corresponding changes in the size of the cytosolic pool of TG, which contributes to the overproduction of VLDL particles associated with insulin-resistant states (24). Liver insulin resistance will also contribute to the high rates of VLDL particles output due to the reduced capacity of insulin to inhibit VLDL assembly (24). Enhanced NEFA delivery to skeletal muscle will lead to increased fatty acid oxidation and could be responsible for skeletal muscle insulin resistance via the glucose-fatty acid cycle (Randle cycle). However, several studies have challenged this view (90). Healthy subjects that were submitted to a euglycemic-hyperinsulinemic clamp together with the infusion of high levels of NEFA displayed reduced insulin sensitivity at the level of skeletal muscle. However, intracellular glucose-6-phosphate levels in skeletal muscle were reduced and not increased as predicted by the Randle cycle. Savage and colleagues hypothesized that the infused fatty acids could have direct effects on GLUT4 activity or that it could alter insulin-regulated GLUT4 trafficking between intracellular compartments and the plasma membrane (90).

Beta-cell Dysfunction

The physiological response to insulin resistance is an increase in insulin secretion (85). As long as the pancreas sustains a sufficiently high insulin secretory response to counterbalance the insulin resistance, glucose tolerance remains normal or is only mildly impaired. However, once the β -cell begins to fail, glucose tolerance deteriorates rapidly and overt diabetes mellitus ensues (13). It is so far unknown why eventually the β -cells fail. However, the vast majority of insulin-

resistant individuals are able to compensate for this defect by continuing to secrete large amounts of insulin (85).

Hormone-Sensitive Lipase

General

Substrate Specificity

Compared with other lipases, HSL has a uniquely broad substrate specificity. It hydrolyzes acylglycerides (22), cholesteryl esters (22), retinyl esters (108), steroid fatty acid esters (61), and water-soluble esters such as p-nitrophenyl esters (99). The activity against diglycerides is higher than the activity against triglycerides and monoglycerides, by 10- to 12-fold and 5- to 10-fold, respectively (46). The cholesteryl ester hydrolase activity of HSL is twice as high as the activity towards triglycerides (46).

Tissue Expression

Expression of HSL has been demonstrated in various tissues, including WAT (43), brown adipose tissue (44), skeletal muscle (43), pancreatic β -cells (71), liver (72, 111) macrophages (10, 52, 94), steroidogenic tissues including testis (43, 47), intestinal mucosa (29), with the highest level in WAT. The expression of HSL in skeletal muscle is correlated to fiber type, being higher in oxidative than in glycolytic fibers (46).

Acute Regulation

The activity of HSL is hormonally regulated through reversible phosphorylation. Catecholamines increase the activity of HSL through a protein kinase A (PKA)-mediated phosphorylation. This effect is exerted by the binding of catecholamines to beta-adrenergic receptors which are coupled to adenylate cyclase via the stimulatory G-protein which leads to an increased production of cAMP and activation of PKA. Conversely, insulin prevents this phosphorylation mainly via reduction of the cAMP levels and therefore of PKA activity (42). In adipocytes, the decrease of

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cAMP is mainly the result of an insulin-mediated activation of phosphodiesterase 3B (14). The other main target for PKA-mediated phosphorylation in the adipocyte are perilipins, which are proteins covering the lipid droplets. Perilipins act as a barrier, blocking access of HSL to its lipid substrate in the basal state (114). Upon stimulation of adipocytes with catecholamines, phosphorylation of HSL triggers its translocation from the cytoplasmic compartment to the surface of the lipid droplets whereas phosphorylation of perilipins alleviates its barrier function (114).

As previously mentioned, HSL has been demonstrated to be activated in rat skeletal muscle in response to both adrenaline (59) and contraction (58). Adrenaline acts by beta-adrenergic stimulation of PKA whereas the effect of contraction appear to be mediated by PKC, partly via the extracellular signal regulated kinase pathway (57). In addition, HSL translocation to IMTG droplets has been shown in rat skeletal muscle in response to both adrenaline and contraction (79). Skeletal muscle, in contrast to adipose tissue, lacks perilipins (64). Instead two other lipid-binding proteins, adipose differentiation-related protein (ADRP) and tail-interacting protein of 47 kDa (TIP47), have been demonstrated to be associated with lipid droplets in skeletal muscle (79). Whether ADRP and TIP47 in skeletal muscle could play a similar role to that of perilipins in adipocytes, remains to be elucidated.

HSL-null Mouse Models

In recent years several HSL-null mouse models have been created (31, 72, 77, 103). One feature shared among all characterized HSL-null models is lack of obesity. HSL-null mice have in fact been shown to be resistant to diet-induced obesity (35). Other shared attributes are male sterility due to oligospermia and decreased plasma levels of NEFA in the fasted state resulting from impaired adipocyte lipolysis (19, 32, 35).

In the model established by Haemmerle et al., the decreased fasted plasma levels of NEFA have been shown to result in decreased hepatic synthesis of VLDL particles (32), which in turn presumably is the main cause of the low plasma triglyceride levels observed in fasted HSL-null mice (19, 32, 35, 112). Plasma total cholesterol is elevated in fasted HSL-null mice (19, 32, 103) and is accompanied by increased HDL-cholesterol in both the fed and fasted states (32). In addition, hepatic cholesterol content is increased in HSL-null mice (35). Noteworthy,

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neutral cholesteryl ester hydrolase activity is impaired in several HSL-expressing tissues: it is abolished in white adipose tissue, brown adipose tissue, testis, islets and small intestine; dramatically reduced in soleus muscle and liver, whereas it is only slightly reduced in macrophages (9, 18, 29, 72, 77). Diglyceride lipase activity, on the other hand, is intact or much less reduced in non-adipose tissues of HSL-null mice (18, 29, 72). This suggests that HSL acts as a cholesteryl ester hydrolase in many tissues, whereas its function as a lipase may be confined to adipose tissue. However, HSL also seems to play an important role as a lipase in skeletal muscle to hydrolyze IMTG stores since an increased amount of lipid droplets has been observed by transmission electron microscopy in the soleus muscle of HSL-null mice. Moreover, *ex vivo* incubated soleus muscle of HSL-null mice increased their utilization of glycogen, which suggests a metabolic switch from lipid to carbohydrate metabolism in soleus muscle of HSL-null mice (33).

Apart from the lack of obesity an inflammatory response characterized by macrophage infiltration and increased necrotic-like cell death has been observed in white adipose tissue of HSL-null mice (7, 34). Adipose tissue inflammation is a condition frequently observed in obese individuals and a possible trigger of obesity-associated insulin resistance. The HSL-null mouse line established in our laboratory is indeed characterized by insulin resistance observed at the level of skeletal muscle, adipose tissue and liver (72). The latter was demonstrated by an impaired insulin-mediated suppression of hepatic glucose production during a euglycaemic hyperinsulinemic clamp. However, conflicting data have been reported for other HSL-null mouse strains which show increased hepatic insulin sensitivity (78, 112). The discrepancy between these studies could be explained by differences in the conditions used to perform the euglycaemic hyperinsulinemic clamp. The infusion rate of insulin varied from low (i.e., 2.5 mU/kg/min) (Park, 2005 #93); 3.5 mU/kg/min (112) to high (i.e., 20 mU/kg/min) (Mulder, 2003 #63) between the clamp experiments. Consequently, the extent of insulin-mediated suppression of hepatic glucose production in the control mice varied from below 45% in the Voshol et al. and the Park et al. studies to approximately 90% of inhibition in the Mulder et al. clamp experiment. In addition, the nutritional status of the animals varied between the studies from over-night fasted (78, 112) to fed (72). Other explanations for this discrepancy that cannot be ruled out are differences in the genetic background of the mice or in intestinal microflora.

Introduction

Another discrepancy between the HSL-null mouse models concerns the role of HSL in β -cells. In one strain, the glucose responsiveness of insulin secretion was impaired both *in vivo* and *in vitro* thus arguing for a role of HSL in glucose-stimulated insulin secretion (87). However, in another strain of HSL-null mice no defect in insulin secretion was observed, which causes a disagreement on the importance of HSL for glucose-stimulated insulin secretion (18). Again, differences in the genetic background or in the intestinal microflora of the mice could explain the differences between the two models.

Models and Methods

Models

Animal Model

The HSL-null mice used in this thesis were generated by targeted homologous recombination of the HSL gene in 129SV-derived embryonic stem cells (29). HSL-null mice and wildtype mice were littermates and had a mixed genetic background from the inbred strains C57BL/6J and SV129.

In Papers I and III, HSL-null mice and wildtype mice were fed *ad libitum* a normal chow diet (ND) containing 4.8% fat and 0.005% cholesterol or a high-fat diet (HFD) containing 35.9% fat and 0.03% cholesterol.

In Paper I several nutritional states were investigated: fed, re-fed (over-night fasting for 12 hours followed by 2 hours of free access to food) and fasted for 16 hours over-night. The re-fed scheme was used in order to synchronize the mice and to ensure that a true postprandial state was analyzed. Fasting for 16 hours was used as a nutritional state that should maximize the impact of HSL deficiency.

Cell Model

Rat INS-1 832/13 pancreatic β -cells were used in Paper III. These cells belong to an insulin-secreting cell line (INS-1) derived from rat insulinoma cells that has been stably transfected with a plasmid containing the human insulin gene. Several cell clones were generated after transfection and the clone 832/13 has been found to be the one secreting the largest amount of insulin during glucose stimulation with a secretory response of 8- to 11-fold at 15 mM glucose relative to secretion at 3 mM glucose. This fold response is in range with what is obtained in freshly isolated rat islets thus making these cells a model for normal β -cells (39).

Methods

Short History

In this thesis several “-omics” techniques have been used. The Greek suffix “-om-” means a collection of things. The term genome, a collection of genes, was coined in 1920 by Hans Winkler, a German botanist, by combining the words “gene” and “chromosome” (70). In 1987, a new journal, dubbed Genomics, was founded by Victor McKusik and Frank Ruddle on the suggestion of Tom Roderick. Genomics was conceived as a new discipline “born from a marriage of molecular and cell biology with classical genetics and (...) fostered by computational science” (70). Later on genomics was subdivided into structural genomics and functional genomics. Structural genomics deals with obtaining the complete nucleotide sequence of an organism, whereas functional genomics deals with understanding genome-scale function and regulation. Techniques of functional genomics include methods for gene expression profiling at the transcript (transcriptomics), protein (proteomics) and metabolite (metabolomics) levels. The strategy in a functional genomics approach is to study all genes or proteins or metabolites of a system at once, irrespective of any hypotheses, in a systematic fashion instead of the classical approach of studying single genes or proteins or metabolites. Functional genomics has thus changed the way of doing research from the traditional hypothesis-driven approach to the hypothesis-generating approach where there is no real hypothesis to start with but the strategy is to generate an hypothesis from the data.

Transcriptomics

The term transcriptome, or set of expressed genes as well as their level of expression for a defined population of cells, was defined by Velculescu in 1997 (109). DNA microarray methodology is a powerful technique to obtain a global view of changes in gene expression patterns in a system exposed to different conditions. There are two types of DNA microarrays: cDNA microarrays and oligonucleotide microarrays. Oligonucleotides-based arrays, also called GeneChip, were developed by Affymetrix and were used in this thesis (Papers I and IV).

Proteomics

The term proteome was used for the first time at the 1994 international electrophoresis society meeting in Siena by Marc Wilkins to describe the collection of all proteins encoded by a genome

(5) and was defined first in an article in 1995 (105). Recently, the proteome term has been broadened to include the set of proteins expressed both in time and space (6). It has been established that mRNA levels do not necessarily correlate with protein levels (28). Post-transcriptional mechanisms, including protein translation, post-translational modification and degradation, may indeed influence the level of a protein present in a given system. Therefore, analysis of the proteome is necessary to confirm and extend the data obtained through analysis of the transcriptome.

2-D gel electrophoresis has so far been the most efficient way to separate large numbers of proteins. The method, which was established in 1975 (55, 74), separates the proteins according to their isoelectric point (pI) in the first dimension and according to their molecular weight in the second dimension. Isoelectric focusing during the first dimension is now performed using immobilized pH gradients. The second dimension is based on the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (27). Once the proteins have been separated they are stained for visualization followed by identification using mass spectrometry (MS). Peptide mass fingerprinting (PMF) is one approach to identify proteins. It is based on the finding that a set of peptide masses obtained by MS analysis of a protein digest provides a characteristic mass fingerprint of the protein in question that can be compared to theoretical peptide masses generated *in silico* for identification. Typically, PMF is performed using matrix-assisted laser desorption/ionization (MALDI) combined with a time-of-flight mass analyzer (2). Another approach to identify proteins is to couple two or more mass analyzers and is known as tandem MS (MS/MS). In tandem mass spectrometers a peptide ion is detected in the first mass analyzer and fragmented after which the resulting fragment ion masses are recorded in the second mass analyzer (2). The quadrupole TOF (QTOF), which was used in this thesis, is an example of such an instrument (Papers III and IV).

Metabolomics

The metabolome represents the complete set of all metabolites formed by the cell in association with its metabolism (73). It represents the next logic step after large-scale analysis of mRNA and proteins in the quest to understand the functional phenotype of an organism. The results obtained from a metabolomics analysis are usually difficult to interpret because the concentrations of metabolites are determined by the activities of many enzymes (part of the proteome). Currently

Models and Methods

there is no single method that enables the analysis of the complete set of all metabolites in an organism because of the wide range of metabolite chemical classes and the large dynamic range of metabolite concentration. Nonetheless, techniques exist to analyze many metabolites such as the combination of gas chromatography and MS (GC-MS) used in this thesis (Paper III). A major limitation of GC-MS is that polar metabolites have to be derivatized and its inability to handle high-molecular weight compounds (107). In Paper III, rather than doing an analysis of all metabolites present in a cell, we performed a so called metabolite profiling which is the analysis of a group of selected metabolites (73).

Present Investigations

Aims

In the present work, global analysis techniques such as microarrays, 2-D gel electrophoresis and GC-MS have been used to study models of type 2 Diabetes: the HSL-null mouse model and the rat INS-1 832/13 pancreatic β -cell model. More specifically, the liver phenotype of HSL-null mice was investigated at the transcriptome (Paper I and IV) and proteome (Paper IV) levels. In addition, the physiological response of HSL-null mice to aerobic treadmill exercise was studied in Paper II. Proteome and metabolome analyses were performed in paper III to study glucose-stimulated insulin secretion in a β -cell line.

The Role of HSL in Mouse Liver

In paper I, the liver phenotype of HSL-null mice was investigated. The decreased hepatic insulin sensitivity observed in our strain of HSL-null mice (72), together with the reported elevation of plasma total cholesterol in HSL-null mice (19, 32, 103), prompted us to perform a transcriptome analysis of the liver in order to gain insight into the mechanisms underlying these features of the HSL-null mouse model. The most striking finding upon this analysis was the downregulation of eight genes encoding enzymes catalyzing successive steps in *de novo* synthesis of cholesterol in HSL-null mice on HFD. This result drove a further investigation of cholesterol homeostasis, revealing increased hepatic levels of esterified cholesterol in HSL-null mice. An increase in esterified cholesterol is consistent with our previous demonstration that cholesteryl ester hydrolase activity is reduced in the liver of HSL-null mice (72). Whether HSL is expressed and enzymatically active in the liver has been debated. In rat liver, HSL was detected neither by Northern blot nor by Western blot and hepatic diglyceride lipase activity was unaffected when measured in the presence of an anti-HSL antibody (43, 45). However, in mouse liver, HSL was detected by Western blot (111) and was also found in our microarray analysis although at very low level. The data presented in Paper I together with those in the study by Mulder et al. imply that HSL indeed functions as a cytosolic cholesteryl ester hydrolase in mouse liver. Whether it is also the case in rats remains to be elucidated since neutral cholesteryl ester hydrolase activity has not yet been measured in rat liver homogenates in presence of an anti-HSL antibody. In addition,

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it could be interesting to feed the HSL-null mice a high cholesterol diet to see if hepatic cholesteryl esters content would be even more elevated.

Plasma cholesterol levels are elevated in HSL-null mice due to cholesterol enrichment of HDL and VLDL. One mechanism that could contribute to the elevation of HDL-cholesterol in HSL-null mice is the previously mentioned build-up of esterified cholesterol in the hepatocytes. SR-BI-mediated uptake of HDL-derived cholesteryl esters may indeed be impaired due to poor clearance of the cholesteryl esters on the cytosolic side, despite the fact that SR-BI expression is upregulated (HFD fasted) or unchanged (HFD fed and ND fasted) in HSL-null mice. Another mechanism likely to contribute to the elevation of HDL-cholesterol in HSL-null mice is the increased expression of hepatic ABCA1 in HSL-null mice described in paper I, since a role for hepatic ABCA1 in modulating plasma HDL-cholesterol level has been established (4, 98).

In conclusion, paper I demonstrates that HSL is a necessary cholesteryl ester hydrolase in the liver that influences overall cholesterol homeostasis by indirectly controlling HDL-cholesterol clearance.

The Role of WAT-Non-Adipose Tissues Axis in Metabolism

In paper I an increased expression of hepatic ABCA1 at the mRNA and protein levels is described in HSL-null mice. Expression of ABCA1 has been reported to be regulated by the LXR/RXR heterodimer with cholesterol-derived oxysterols as the physiological ligand (12). Levels of free cholesterol were unchanged in the liver of HSL-null mice, making it unlikely that increased activity of LXR/RXR is the cause of increased ABCA1 expression. Unsaturated fatty acids have also been reported to regulate ABCA1 expression. This occurs via suppression of ABCA1 transcription (100, 101) and via increased degradation of ABCA1 protein (104). The fatty acid composition of hepatic triglycerides was found to be significantly altered in HSL-null mice in the fasted state with several unsaturated fatty acids being less represented in HSL-null mice. The altered fatty acid profile may be at least one mechanism whereby hepatic ABCA1 expression is increased in HSL-null mice. Since the hepatic fatty acid profile in the fasted state is mainly the result of import of plasma-derived fatty acids generated in the adipose tissue through the action of HSL (21), this indicates a decreased transfer of unsaturated fatty acids from the WAT to the liver in HSL-null mice. Moreover, selective mobilization of fatty acids from a lipid

Present Investigations

emulsion by HSL according to chain length and degree of unsaturation has previously been demonstrated (83). The results in Paper I thus suggest that there is a cross-talk between WAT and the liver exerted by the type of fatty acids that are released. This cross-talk is most pronounced in the fasted state when HSL is maximally active and it determines the cholesterol content of the lipoproteins secreted by the liver, via regulation of ABCA1, and thus cholesterol homeostasis.

In Paper II the metabolic response of HSL-null mice challenged with exercise was investigated to examine if other lipases are able to fully compensate for the lack of HSL. Our hypothesis was that in the absence of HSL, the mice would not be able to respond to an aerobic exercise challenge with increased mobilization of the lipid stores in WAT, *i.e.* with liberation of NEFAs and glycerol to the circulation. As a consequence the impact of the described lipid sparing effect (86) on liver glycogen stores would be reduced, leading to faster depletion of this energy source and a decreased total running time in the HSL-null mouse strain. As anticipated, HSL-null mice reached exhaustion sooner – by 7.5 minutes - than wildtype mice when submitted to aerobic treadmill running. Moreover, after 25 minutes the content of liver glycogen was lower in HSL-null mice than in wildtype mice, whereas the degree of depletion was equal at the time of exhaustion. In line with the disturbed response of the WAT in HSL-null mice was the observed accumulation of diglyceride in WAT that occurred in exercised mice, leading to significantly reduced levels of both plasma NEFA and glycerol concentrations in HSL-null mice compared with wildtype mice after exercise. However, the role of IMTG during aerobic exercise cannot be ignored. Indeed, diglyceride accumulation was observed in HSL-null mice gastrocnemius muscle after exercise, which indicates a possible inhibition in the full mobilization of energy in the form of fatty acids from the HSL-null mice IMTG stores. Estimations made in humans indicate that IMTG stores contribute approximately 10% of the total energy expenditure during fasting exercise in males and to 10 – 25% in females (54). Thus, a perturbation of skeletal muscle metabolism is likely to have only a limited effect on the capacity of HSL-null mice to sustain sub-maximal exercise. In conclusion, paper II reveals that HSL plays an important role during aerobic exercise via controlling the mobilization of lipid stores from WAT and that other acylglyceride lipases cannot fully compensate for the lack of HSL.

A limitation in both paper I and II is that we have been using a general HSL knockout mouse model. The ultimate proof of a cross-talk between liver and WAT or skeletal muscle and

WAT would be to study liver-specific and muscle-specific mouse knockout models as well as a general HSL knockout model in which HSL expression has been rescued in WAT.

Metabolite Profiling in Relation to Glucose-Stimulated Insulin Secretion

In paper III, the goal was to identify the metabolites generated from glucose metabolism in pancreatic β -cells that control exocytosis of insulin. To this end, polar metabolites, extracted from the clonal 832/13 β -cell line cultured at 2.8 and 16.7 mM glucose, respectively, for 48 h were derivatized, followed by identification and relative quantification using GC-MS. Concurrent changes in protein expression were analysed by 2-D gel electrophoresis. After growing at 16.7 mM glucose for 48 h, 832/13 β -cells exhibited a glucotoxic phenotype with decreased insulin content, by approximately 80 %, and impaired insulin secretion in response to glucose under both K_{ATP} -dependent and K_{ATP} -independent conditions. Overall, changes in the level of metabolites pointed towards an increase in the glycolytic flux in 832/13 β -cells cultured at 16.7 mM glucose: increased content of glucose-6-phosphate and pyruvate, the first and last metabolite in glycolysis, and increased content of several intermediates of the tricarboxylic acid cycle. This is in agreement with what would be expected when there is an increased availability of glucose for cells to metabolize. Despite the increased turn-over rate of glucose in cells cultured at 16.7 mM glucose, glucose-stimulated insulin secretion was not enhanced. This demonstrates that it is the precise manner in which glucose is metabolized in the β -cell, not the extent of turn-over rate, which determines insulin secretion. The metabolite data aggregated in two clusters in a principal component analysis, allowing for the discrimination of the various samples according to the cell culture condition, thus indicating that the metabolite changes correlated with β -cell dysfunction.

Clearly, several important metabolites were not detected by our metabolomic approach. Some metabolites may be unstable and thus degraded; others may not be amenable to derivatization. Moreover, our approach was not to identify all the metabolites present in β -cells, but focused towards the identification of a set of metabolites belonging to the glycolysis pathway and the TCA cycle or involved in TCA cycle anaplerosis

Correlating Protein and Metabolite Data or mRNA and Protein Data

In paper III, combined analysis of polar metabolites and of protein expression by GC-MS and 2-D gel electrophoresis, respectively, was performed in cell extracts from the clonal 832/13 β -cell line cultured at 2.8 and 16.7 mM glucose for 48 h. Sixteen metabolites were identified of which ten were significantly differentially expressed between cells cultured at 2.8 mM glucose versus those cultured at 16.7 mM glucose and two metabolites were only present at high glucose ($P \leq 0.05$). With the proteome analysis, 305 spots were found to be significantly differentially expressed on the gels derived from 832/13 β -cells cultured in the presence of 2.8 mM glucose versus those cultured in 16.7 mM glucose ($P = 0.2$). Out of those, 187 were submitted to analysis by LC-MS/MS allowing the identification of 86 spots corresponding to 75 unique proteins. Only five of the identified proteins were metabolic enzymes that could be involved in the metabolic alterations observed. Only three of those were regulated in a fashion agreeing with the metabolites measurements: glycerol-3-phosphate dehydrogenase, α -enolase and transaldolase were up-regulated in β -cells cultured at high glucose in conformity with the observed increase in levels of glycolytic intermediates and ribose-5-phosphate. Thus, there appears to be a poor correlation between levels of metabolites and expression of the corresponding regulatory enzymes. This suggests that substrate availability is a more important determinant of metabolite levels than enzyme expression at the protein level. Additionally, post-translational modifications and allosteric regulation of the enzymes are likely to play a role. However, a bias in our approach could also explain the poor correlation between metabolite levels and expression of the corresponding regulatory enzymes. Indeed, only the proteins that were significantly expressed between low and high glucose gels were submitted to identification. Instead to be unbiased, one should have ensured that the enzyme regulating a given metabolite was indeed present on the 2-D gel and thus have submitted all the spots on the preparative gel to LC-MS/MS.

In Paper IV, a combined analysis of the transcriptome and of the proteome of mouse liver was performed using an oligonucleotide microarray and 2-D gel electrophoresis, respectively. Gene and protein expression changes were investigated in HSL-null mice and compared to wildtype mice, disregarding the diet, and in mice on HFD versus mice on ND, independently of the genotype. The oligonucleotide microarray analysis revealed changes in the expression of genes involved in lipid metabolism in HSL-null mice compared with wildtype mice and in the regulation of the immune system in mice on HFD compared to mice on ND. Changes observed

Present Investigations

at the protein level were mainly related to metabolism. Overall, only 8 of the proteins that were expressed differentially in response to the genotype and 6 of the proteins that were expressed differentially in response to the diet exhibited a positive correlation with the corresponding mRNA. This indicates that for many genes the measurement of the mRNA response is not reflected in the protein response. However, as previously mentioned in connection with Paper III, a bias exists in our experimental set-up since only proteins that were expressed differentially were selected for identification. Thus, while one can analyze the expression at the transcript level of differentially expressed proteins because the mRNA representing most of the regulated proteins are present on the microarray chip, finding the corresponding protein on a 2-D gel for a differentially expressed transcript would require the systematic identification of all the spots present on the 2-D gel.

Major Conclusions

- HSL plays an important role as a cholesteryl ester hydrolase in the liver and influences overall cholesterol homeostasis by indirectly controlling hepatic HDL-cholesterol clearance.
- There exists a cross-talk between WAT and liver that regulates cholesterol homeostasis via the type of released NEFA
- We have established the importance of the WAT-skeletal muscle axis to provide fuel during aerobic exercise and the central role played by HSL during this process
- We have established that it is the precise manner in which glucose is metabolized in the β -cell and not the extent of glucose turn-over rate which determines insulin secretion
- There is a poor correlation between metabolite levels and expression of the regulatory enzymes as well as between mRNA levels and the corresponding protein.

Popular Scientific Summary

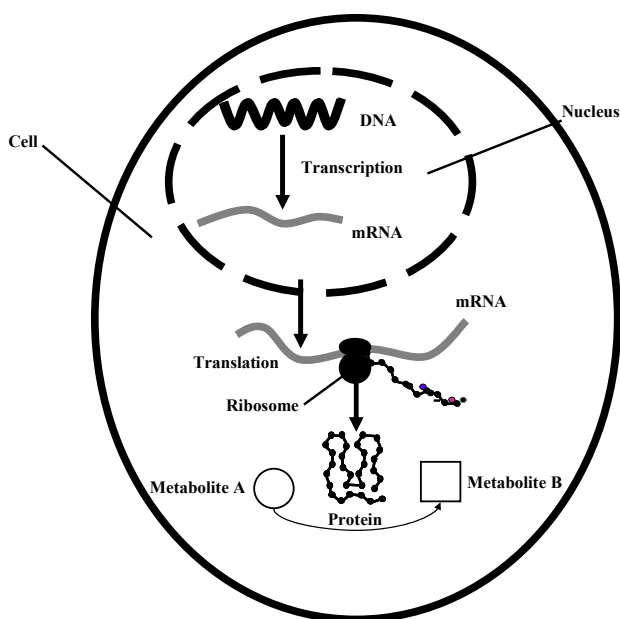
Diabetes mellitus is a disease characterized by increased blood glucose levels. For overt type 2 diabetes to develop, disturbance of both action and secretion of the hormone insulin must occur at the same time. In the present thesis, I describe the analysis of a mouse model where the gene encoding the fat-degrading enzyme hormone-sensitive lipase (HSL) was completely removed from the mouse by genetic manipulation. Those mice are referred to as HSL-null mice. Also described is the use of cultured cells which mimic the cells in the pancreas that are responsible for insulin secretion. Those two models were used to study the mechanisms underlying insulin insensitivity and insulin secretion perturbations, respectively. To this end, techniques allowing an extensive analysis of relative contents of proteins, messenger RNA (mRNA, the cell's signal to start the production of proteins) and metabolites (small-molecule products and substrates of the metabolism) were used (Figure 1).

HSL is an enzyme that breaks down fat stored in adipose tissue to deliver energy for the body in period of fasting, for example. But HSL has the capacity to break down other types of compounds besides fat. Moreover, HSL is present in many tissues besides adipose tissue, including the liver. The role of HSL in non-adipose tissues is not completely understood, but studies of HSL-null mice suggest that HSL has several functions in addition to its role in breaking down fat. The role of HSL in mouse liver was studied in this thesis (Papers I and IV). To achieve this, we used a chip to which mRNAs that are present in liver will bind, and give rise to a signal which allows for their relative quantification. We also used polyacrylamide gels to separate the proteins present in the liver according to charge and weight (Paper IV). The results obtained show that HSL plays an important role in the liver breaking down stored cholesterol and that HSL influences overall body cholesterol content in the mice by indirectly controlling the elimination by the liver of the good cholesterol present in blood. We also demonstrated the importance of a cross-talk between fat tissue and liver that regulates overall body cholesterol via the type of fat-derived compounds released from the fat tissue. Moreover, changes in the relative content of proteins involved in polyamines metabolism were observed in the liver of HSL-null mice and could be responsible for the increased liver weight characterizing HSL-null mice. The physiological response of HSL-null mice to aerobic physical activity was also investigated (Paper II). Aerobic in this context means that the mice burn completely the available nutrients to

generate energy. HSL was shown to play an important role during aerobic exercise by controlling the mobilization of fat stores present in the adipose tissue, a role which cannot be fully compensated for by any other enzymes.

Protein and metabolite analyses were performed in Paper III in a cell line which mimics the cells present in the pancreas that are responsible for insulin secretion. The cells were cultured in presence of normal or toxic glucose concentrations. A pattern of metabolites specific for the cells cultured at high glucose concentration was identified.

Figure 1.



Vulgarisation Scientifique

Le diabète est une maladie caractérisée par une élévation anormale de la concentration sanguine de glucose. Pour que la maladie se déclare, des perturbations au niveau de l'action de l'hormone insuline sur les tissus et au niveau de la sécrétion de l'insuline par les cellules pancréatiques doivent avoir lieu simultanément. Durant ma thèse, j'ai étudié un modèle de souris dans lequel la lipase hormono-sensible (LHS) a été totalement éliminée de la souris par manipulation génétique. Ces souris sont appelées ko-LHS. Par ailleurs, j'ai aussi utilisé une lignée cellulaire qui est similaire aux cellules du pancréas responsables de la sécrétion de l'insuline. Ces deux

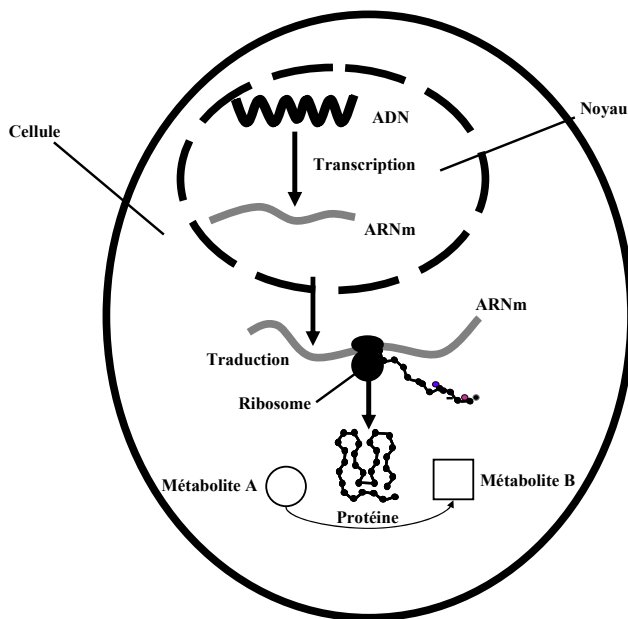
modèles ont été utilisés pour étudier les mécanismes responsables de la perte de réponse à l'insuline des tissus ainsi que les mécanismes responsables du développement de perturbations au niveau de la sécrétion de l'insuline, respectivement. Des techniques permettant une analyse extensive des protéines, ARN messagers (ARNm, le signal cellulaire pour commencer la synthèse des protéines) et métabolites (petites molécules qui peuvent être le produit ou le substrat du métabolisme) présents dans un tissu ont été utilisées (Figure1).

La LHS est une enzyme (protéine avec des propriétés spéciales) qui dégrade les graisses présentes dans le tissu adipeux pour libérer de l'énergie utilisable par l'organisme en période de jeûne par exemple. La LHS est capable d'agir sur d'autres composés que les graisses et est présente dans de nombreux tissus en plus du tissu adipeux. Le rôle de la LHS dans les tissus non adipeux n'est pas totalement élucidé, mais certaines études suggèrent que la LHS a une fonction autre que la mobilisation des graisses dans ces tissus. Dans cette thèse j'ai étudié le rôle de la LHS dans le foie, indirectement, en analysant le foie des souris ko-LHS (Articles I et IV). Dans cet objectif, une puce à laquelle les ARNm présents dans le foie des souris ko-LHS vont s'associer, produisant un signal permettant une quantification relative des ARNm contenus dans le foie, a été utilisée. Nous avons aussi utilisé des gels de polyacrylamide pour séparer les protéines présentes dans le foie en fonction de la charge et du poids moléculaire (Article IV). Les résultats obtenus montrent que la LHS présente dans le foie joue un rôle important dans la mobilisation des stocks de cholestérol hépatiques et agit indirectement sur le métabolisme du cholestérol dans la souris en contrôlant l'élimination par le foie du « bon » cholestérol présent dans le sang. Les résultats obtenus démontrent aussi une voie de signalisation allant du tissu adipeux au foie qui contrôle le métabolisme du cholestérol chez la souris au travers du type de produit dérivé des graisses délivré par le tissu adipeux. De plus une modification du contenu relatif en polyamines (protéines ayant la propriété de réguler la croissance cellulaire) a été mesuré dans les souris ko-LHS et pourrait être la cause de l'augmentation du poids du foie chez ces souris. La capacité des souris ko-LHS à pratiquer une activité physique en conditions aérobies a aussi été étudiée en faisant courir les souris sur un tapis-roulant miniature. Dans ce contexte, des conditions aérobies indiquent que les souris vont brûler totalement les nutriments disponibles pour produire de l'énergie. A travers cette étude l'importance du rôle de la LHS lors de la performance d'une activité physique en condition aérobie a été démontrée. La LHS en

effectuant la dégradation des graisses présentes dans le tissu adipeux est en effet nécessaire pour fournir l'énergie indispensable aux souris pour réaliser cet exercice.

Une analyse des protéines et des métabolites présents dans une lignée cellulaire ressemblant aux cellules du pancréas qui sécrètent l'insuline a été effectuée dans l'article III. Les cellules, avant étude, ont été maintenues dans un environnement contenant une quantité physiologique ou toxique de glucose. Les cellules maintenues dans des conditions toxiques de glucose contiennent une composition en métabolites caractéristique qui permet leur identification seulement sur la base de ce contenu en métabolites.

Figure 1



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