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EIJA PIRINEN

Involvement of Polyamine Catabolism in the Regulation of Glucose, Energy and Cholesterol Metabolism in Mice

Doctoral dissertation

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ABSTRACT

Polyamines (putrescine, spermidine and spermine) are ubiquitous low molecular weight amines that are positively charged under physiological conditions. Homeostatic control of intracellular polyamine levels is achieved by regulating the synthesis, catabolism and transport of these molecules. Spermidine/spermine N^1-acetyltransferase (SSAT) is the key enzyme in the catabolism of polyamines. The overexpression or loss of this enzyme leads to the corresponding activation or deficiency of polyamine catabolism. Polyamines are involved in many cellular processes such as cell growth, signal transduction, stabilization of negative charged macromolecules, modulation of potassium and calcium channels and stimulation of transcription and translation. Since transgenic mice overexpressing SSAT (SSAT mice) surprisingly exhibited reduced subcutaneous white adipose tissue (WAT) mass, the role of polyamine catabolism in the regulation of glucose, energy and lipid metabolism was investigated using transgenic mice overexpressing or deficient for SSAT.

The characterization of SSAT mice revealed that these mice exhibited severely reduced whole body WAT mass, elevated energy expenditure, high insulin sensitivity, a low tissue triglyceride content, increased number of mitochondria and overexpression of the oxidative phosphorylation genes coordinated by increased levels of peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) in WAT. The induction of PGC-1α was attributable to activated polyamine catabolism-mediated depletion in the cellular ATP pool which activated the cellular energy sensor, 5'-AMP-activated protein kinase (AMPK) and PGC-1α. These results suggest that the enhancement of cellular ATP consumption is an efficient way to reduce body WAT mass and improve glucose metabolism.

Furthermore, activated polyamine catabolism caused a significant reduction in plasma total cholesterol levels in SSAT mice by increasing the elimination of cholesterol from the body via bile acid synthesis. The hepatic expression of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting gene in bile acid synthesis, was induced by PGC-1α, the protein stability and activity of which was increased most likely due to the reduction in the protein levels of Akt evoked by activated polyamine catabolism. Therefore, activation polyamine catabolism is a novel way of inducing bile acid synthesis and lowering circulating total cholesterol levels.

The deficiency in polyamine catabolism resulted in insulin resistance in aged SSAT knockout (KO) mice. Thus, the maintenance of normal glucose homeostasis seems to require functional polyamine catabolism. However, the deficiency of polyamine catabolism did not evoke any major changes in body WAT mass or lipid metabolism in SSAT-KO mice.

Our results demonstrate that polyamine catabolism is involved in the regulation of energy balance, and glucose and cholesterol metabolism in mice.

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To the Memory of Professor Juhani Jänne

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The aim of this study has been finally achieved. Let’s party!

Kuopio, November 2009

Eija Pirinen

ABBREVIATIONS

ABCA1	ATP binding cassette protein A1
ABCG5	ATP binding cassette protein G5
ABCG8	ATP binding cassette protein G8
ACAT2	Acyl CoA:cholesterol acyltransferase 2
ACC	Acetyl-CoA carboxylase
AdoMetDC	S-adenosylmethionine decarboxylase
dcAdoMet	Decarboxylated S-adenosylmethionine
AMPK	5'-AMP-activated protein kinase
ARC	Arcuate nucleus
AS160	Akt substrate of 160 kDa
AZ	Antizyme
BAT	Brown adipose tissue
cAMP	Cyclic AMP
CE	Cholesteryl ester
CEBP/α	CCAAT/enhancer binding protein α
CETP	Cholesteryl ester transfer protein
CPT-I	Carnitine palmitoyltransferase I
CREB	cAMP response element binding protein
CYP7A1	Cholesterol 7α-hydroxylase
CYP27A1	Sterol 27-hydroxylase
DENSPM	N^1,N^{11}-diethylnorspermine
DFMO	Difluoromethylornithine
ERRα	Estrogen-related receptor α
FFA	Free fatty acid
FXR	Farnesoid X receptor
G6Pase	Glucose-6-phosphatase
GLUT	Glucose transporter
GLUT4	Glucose transporter 4
GSK-3β	Glycogen synthase kinase 3β
HDL	High density lipoprotein
HK	Hexokinase
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HNF	Hepatocyte nuclear factor
HNF-4	Hepatocyte nuclear factor 4
HSL	Hormone sensitive lipase
IDL	Intermediate-density lipoprotein
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LXRα	Liver X receptor α
MAPK	Mitogen activated protein kinase
MEF-2	Myocyte enhancement factor 2
NPC1L1	Niemann–Pick C1-like 1 protein
NRF	Nuclear respiratory factor
NRF-1	Nuclear respiratory factor 1
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP1	Na^+-independent organic anion transporting polypeptide
ODC	Ornithine decarboxylase

OXPHOS	Oxidative phosphorylation
PAO	Polyamine oxidase
PGC-1	Peroxisome proliferator activated receptor γ co-activator 1
PGC-1α	Peroxisome proliferator activated receptor γ co-activator 1α
PEPCK	Phosphoenolpyruvate carboxykinase
PDH	Pyruvate dehydrogenase
PKC	Protein kinase C
PI3K	Phosphatidylinositol 3 -kinase
PPAR	Peroxisome proliferator activated receptor
PPARγ	Peroxisome proliferator activated receptor γ
PRMT1	Protein arginine methyltransferase I
RS	Arginine-serine
RQ	Respiratory quotient
SIRT1	Sirtuin 1
SMO	Spermine oxidase
SPDSy	Spermidine synthase
SPMSy	Spermine synthase
SR-BI	Class B type 1 scavenger receptor
SREBP	Sterol regulatory element binding protein
SSAT	Spermidine/spermine N^1-acetyltransferase
SSAT-KO	SSAT knockout
SSAT mice	Transgenic mice having ubiquitous overexpression of SSAT
TG	Triglyceride
UCP	Uncoupling protein
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to by their Roman numerals:

I Pirinen E., Kuulasmaa T., Pietilä M., Heikkinen S., Tusa M., Itkonen P., Boman S., Skommer J., Virkamäki A., Hohtola E., Kettunen M., Fatrai S., Kansanen E., Koota S., Niiranen K., Parkkinen J., Levonen A-L., Ylä-Herttuala S., Hiltunen J.K., Alhonen L., Smith U., Jänne J., and Laakso M. (2007) Enhanced polyamine catabolism alters homeostatic control of white adipose tissue mass, energy expenditure, and glucose metabolism. *Mol. Cell. Biol.* **27**(13):4953-4967.

II Pirinen E., Gylling H., Itkonen P., Yaluri N., Heikkinen S., Pietilä M., Kuulasmaa T., Tusa M., Cerrada-Gimenez M., Pihlajamäki J., Alhonen J., Jänne J., Miettinen T.A. and Laakso M. (2009) Activated polyamine catabolism leads to low cholesterol levels by enhancing bile acid synthesis. *Amino Acids,* in press

III Niiranen K., Keinänen T.A., Pirinen E., Heikkinen S., Tusa M., Fatrai S., Suppola S., Pietilä M., Uimari A., Laakso M., Alhonen L., and Jänne J. (2006) Mice with targeted disruption of spermidine/spermine N^1-acetyltransferase gene maintain nearly normal tissue polyamine homeostasis but shows signs of insulin resistance upon aging. *J. Cell Mol. Med.* **10**(4):933-945.

Some unpublished results are also presented.

CONTENTS

1 INTRODUCTION

Energy is required for all cellular processes (Alberts et al., 1994). Energy homeostasis occurs when energy intake is matched to energy expenditure through complex peripheral and central regulatory pathways. Energy obtained from the chemical bonds present in nutrients is released during cellular respiration and this energy is used to form the cellular energy currency, ATP, through oxidative phosphorylation (OXPHOS) in mitochondria.

Glucose is a vital source of energy in the cell (Murray et al., 1993; Aronoff et al., 2004). In particular, the brain is entirely dependent upon glucose as a fuel. The circulating glucose level is under the homeostatic control in order to maintain a near-constant glucose level to protect the brain from fuel deprivation. Several hormones, particularly insulin and glucagon, act to control glucose levels. An increase in the glucose concentration stimulates the pancreas to release insulin, and insulin in turn promotes glucose uptake in peripheral tissues. A low glucose level stimulates the pancreas to release glucagon which promotes the hepatic conversion of organic molecules such as lactate, pyruvate, glycerol and amino acids to glucose through gluconeogenesis. In addition, glucagon stimulates glycogen breakdown to glucose to normalize the blood glucose levels.

Cholesterol is an important membrane component serving as a precursor for the synthesis of bile acids, steroid hormones and D vitamin (Murray et al., 1993). Bile acids are known to be important for the solubilization of other lipids but they also act as regulatory molecules (Hylemon et al., 2009). Bile acids enhance energy expenditure via cyclic AMP (cAMP)-dependent thyroid hormone activation (Watanabe et al., 2006) and control glucose homeostasis by inducing insulin signaling in the liver (Dent et al., 2005). The intracellular cholesterol content is regulated via coordinated control of cholesterol synthesis, secretion and uptake (Dietschy et al., 1993). The balance of bile acid synthesis, biliary excretion and reabsorption from the small intestine plays a pivotal role in the regulation of cellular cholesterol homeostasis.

Peroxisome proliferator activated receptor γ co-activator 1 (PGC-1) α (PGC-1α) is a key factor regulating energy and glucose homeostasis by stimulating mitochondrial biogenesis, fatty acid oxidation, OXPHOS, uncoupling, thermogenesis, gluconeogenesis and glucose transport (Puigserver and Spiegelman 2003). Overexpression of PGC-1α in white adipose tissue (WAT) reduces WAT mass, increases metabolic rate, uncoupling and insulin sensitivity in mice (Cederberg et al., 2001; Tsukiyama-Kohara et al., 2001). In addition to being the key regulator of energy metabolism, PGC-1α also participates in the control of

cholesterol homeostasis because it stimulates the rate-limiting gene in hepatic bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1) (Shin et al., 2003).

The naturally occurring polyamines, putrescine, spermidine and spermine, are small cationic molecules that are found in all eukaryotic cells (Jänne et al., 2005). One well-known function of polyamines is their ability to promote cell growth and neoplastic transformation. The polyamine content in the cell is regulated through the complex control of synthesis, catabolism, uptake and excretion. The rate-limiting enzyme in polyamine catabolism is spermidine/spermine N^1-acetyltransferase (SSAT). Overexpression of this enzyme activates polyamine catabolism leading to the accumulation of putrescine and a reduction in the spermidine and/or spermine pools. Transgenic mice having ubiquitous overexpression of SSAT (SSAT mice) were originally generated for cancer research. Since SSAT mice had severely reduced subcutaneous WAT mass (Pietilä et al., 1997), we investigated the involvement of polyamine catabolism in the regulation of glucose, energy, and cholesterol metabolism and especially in the control of PGC-1α expression in WAT and liver using mice either overexpressing or deficient for SSAT.

2 REVIEW OF LITERATURE

2.1 Polyamine metabolism

2.1.1 Overview of polyamines

The polyamines, putrescine, spermidine and spermine, are small aliphatic molecules that exists in all eukaryotic cells (Cohen 1998). They have two or more primary amino groups and are positively charged at physiological pH. Due to their cationic nature, polyamines can interact very tightly via hydrogen bonding, ionic and covalent linkages, and hydrophobic interactions with negatively charged molecules such as DNA, RNA, proteins and phospholipids and this way they can change their structure and function. Polyamine metabolism can be more preferably viewed as a cycle rather than as two distinct linear pathways consisting of synthesis and catabolism (Fig. 1).

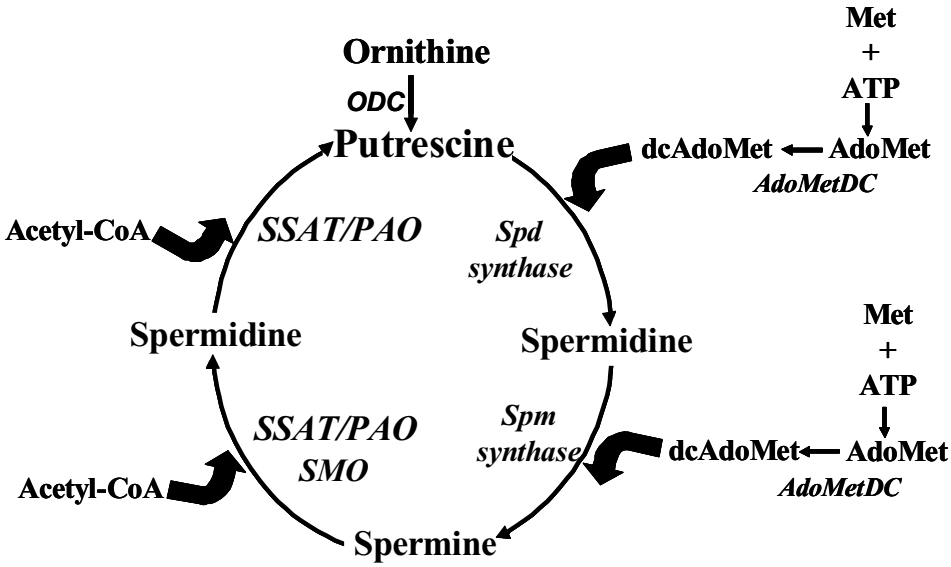

Figure 1. Polyamine cycle. Spd, spermidine; Spm, spermine; Met, methionine and AdoMet, S-adenosylmethionine. Modified from (Jänne et al., 2005).

2.1.2 Synthesis

2.1.2.1 Overview

The enzymes involved in the biosynthesis of polyamines are ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase (SPDSy) and

spermine synthase (SPMSy) (Fig. 1) (Cohen 1998). The first rate-limiting step in the synthesis of polyamines is the formation of putrescine by decarboxylation of L-ornithine, which is catalyzed by ODC (EC 4.1.1.17). This is followed by the conversion of S-adenosylmethionine to decarboxylated S-adenosylmethionine (dcAdoMet) by AdoMetDC (EC 4.1.1.50). The addition of dcAdoMet to putrescine leads to the synthesis of spermidine via the action of SPDSy (EC 2.5.1.16). The addition of dcAdoMet to spermidine then leads to synthesis of spermine catalyzed by SPMSy (EC 2.5.1.22). In mammals, ornithine is an exclusive precursor for *de novo* synthesis of polyamines. It can be derived from the circulation or it can be synthesized within the cell from arginine by the enzyme arginase.

2.1.2.2 Ornithine decarboxylase

Eukaryotic ODC is a pyridoxal phosphate-dependent enzyme which acts as a homodimer (Coleman et al., 1994). ODC has a very low half-life (~10-30 min) (Davis et al., 1992). The stability of ODC is regulated by a protein termed antizyme (AZ) which binds to ODC monomer and directs ODC to degradation by the 26S proteosome (Elias et al., 1995). A decline in polyamine concentrations triggers a decrease in the transcription and translation of AZ while high levels of polyamines increase AZ content by increasing synthesis and reducing degradation (Nilsson et al., 1997). The effect of AZ on ODC can be blocked by antizyme inhibitor which binds AZ more tightly than ODC and thereby releases ODC from the ODC-AZ complex (Murakami et al., 1996; Nilsson et al., 2000). Another level of the control of the ODC protein amount is translational regulation by polyamines through cap-dependent and –independent mechanisms (Shantz and Pegg 1999; Pyronnet et al., 2000). Third level regulation of ODC occurs in transcription which is affected by many factors e.g. hormones, growth factors and oncogene *c*-myc (Pegg 2006). A specific inhibitor of ODC, difluoromethylornithine (DFMO) has been evaluated as a cancer chemopreventive agent due to its capability to deplete intracellular polyamine content (Gerner and Meyskens 2004).

2.1.2.3 S-adenosylmethionine decarboxylase

The second rate-limiting enzyme in the biosynthesis of polyamines is AdoMetDc which has a moderately rapid turnover (a half-life about 1-3 h) (Stjernborg and Persson 1993). AdoMetDC is a pyruvoyl enzyme which is synthesized as a proenzyme that undergoes an intramolecular cleavage reaction forming α- and β-subunits (Pegg et al., 1998). In mammals, AdoMetDC exists as a tetramer consisting of two pairs of α- and β-subunits. Growth factors

and hormones like insulin increase the transcription of AdoMetDC (Soininen et al., 1996). Putrescine increases the activity of AdoMetDC and accelerates the cleavage of proenzyme (Pegg et al., 1988; Stanley et al., 1994). High levels of spermidine and spermine suppress the transcription and mRNA translation of AdoMetDC whereas depletion of these higher polyamines increases the mRNA and protein synthesis (Shirahata and Pegg 1986; White et al., 1990; Shantz et al., 1992).

2.1.2.4 Spermidine synthase and spermine synthase

SPDSy and SPMSy share similar properties. Both consist of two identical subunits, they are constitutively expressed and the proteins are substantially more stable than ODC and AdoMetDC (Pegg 1986; Kajander et al., 1989; Seiler 1990). These enzymes have been less extensively studied since they do not play a rate-limiting role in polyamine biosynthesis.

2.1.3 Catabolism

2.1.3.1 Overview

Intracellular polyamine concentrations are also controlled by catabolism, allowing the conversion of spermine and spermidine back to putrescine (Cohen 1998). The polyamine catabolism involves acetylation of spermidine and spermine by the rate-limiting enzyme SSAT (Fig. 1) (EC 2.3.1.57). Acetyl-CoA is the acetyl group donor for this reaction in which SSAT transfers the acetyl group from acetyl-CoA to the N^1-positions of spermidine and spermine. Thereafter, polyamine oxidase (PAO) (EC 1.5.3.11) converts acetylated polyamines into spermidine and putrescine, respectively (Fig. 1). In addition, spermine can be converted directly to spermidine without acetylation by spermine oxidase (SMO) (Fig. 1.) (EC 1.5.3.3) (Vujcic et al., 2003).

2.1.3.2 Spermidine/spermine N^1-acetyltransferase

Human SSAT gene is located on chromosome Xp22.1 (Xiao et al., 1992) and it encodes a 20 kDa protein consisting of 171 amino acids (Casero et al., 1991). The SSAT gene contains six exons which encode mRNAs of about 1.3 and 1.5 kb (including polyA) (Fogel-Petrovic et al., 1993). An alternative splice variant including an additional 110 bp exon between exons 3 and 4 has also been identified (Hyvönen et al., 2006). The promoter region of SSAT lacks a TATA box but contains putative recognition sequence(s) for polyamines and transcription

factors such as Sp1, AP1, nuclear factor κB, CCAT/enhancer binding protein β, cAMP response element binding protein (CREB) and peroxisome proliferator activated receptor (PPAR) γ (PPARγ) (Wang et al., 1998; Tomitori et al., 2002; Ignatenko et al., 2004; Pegg 2008).

SSAT is considered to be predominantly a cytosolic enzyme, but recent studies have revealed that SSAT also localizes into nucleus and mitochondria (Holst et al., 2008; Uimari et al., 2009). SSAT acts as a homodimer (Bewley et al., 2006) and has a rapid turnover rate having a half-life less than 30 min (Matsui and Pegg 1981; Persson and Pegg 1984). Under basal conditions, the SSAT activity is very low but SSAT shows high inducibility in response to several factors including polyamines and polyamine analogues (Pegg 2008), stress, fasting, hormones, growth factors, toxic agents, nonsteroidal anti-inflammatory drugs (Babbar et al., 2006) and resveratrol (Ulrich et al., 2006). The induction of SSAT activity by polyamines and polyamine analogues occurs through several mechanisms. Gene transcription is increased by polyamines via nuclear factor erythroid 2-related factor 2 which binds to the polyamine response element in the promoter of SSAT gene (Wang et al., 1998). Polyamines and polyamine analogues increase the stability of SSAT mRNA (Fogel-Petrovic et al., 1993; Fogel-Petrovic et al., 1996; Hyvönen et al., 2006), mRNA translational efficiency (Parry et al., 1995) and stabilize SSAT protein by preventing its ubiquitination and targeting to proteosomal degradation (Coleman and Pegg 2001). The other inducers of SSAT differs from polyamines and polyamine analogues in that they may increase SSAT activity by elevating the cellular content of polyamines either by increasing polyamine synthesis or by releasing polyamines from bound sites but also by increasing the transcription through the multiple binding sites for the transcription factors (Pegg 2008). N^1,N^{11}-diethylnorspermine (DENSPM), an N-alkylated polyamine analog, is the most potent and widely used SSAT activator which has been demonstrated to cause growth inhibition e.g. by depleting polyamine pools, inhibiting mitochondrial protein synthesis and production of reactive oxygen species (Snyder et al., 1994; Casero et al., 2005).

The activation of polyamine catabolism by overexpression of SSAT *in vivo* causes a massive putrescine accumulation, the appearance of N^1-acetylspermidine and a reduction in spermidine and/or spermine pools (Pietilä et al., 1997). The initial fall in the spermidine and spermine pools compensatorily increases polyamine biosynthesis, leading to a continuous supply of polyamines for acetylation by SSAT. This accelerates the turnover rate of polyamine cycle and the overall flux of polyamines (Jänne et al., 2006). As a consequence of the enhanced polyamine flux, the consumption of the SSAT co-factor acetyl-CoA, a central

metabolic intermediate, increases. Indeed, markedly reduced acetyl-CoA pools have been detected in prostate and WAT of SSAT mice (Kee et al., 2004; Jell et al., 2007).

2.1.3.3 Polyamine oxidase and spermine oxidase

PAO is a flavin adenine dinucleotide-dependent amino oxidase which uses mainly acetylated polyamines as its preferred substrates (Seiler 1987). PAO is a constitutively expressed protein having a long half-life (seven days) (Seiler et al., 1980) but N-alkylated polyamine analogues have been shown to induce the enzyme slightly (Vujcic et al., 2003). A recently discovered enzyme, SMO, is a flavoenzyme which can use only spermine as a substrate (Vujcic et al., 2003). Its expression is induced by polyamine analogues mainly at the mRNA level (Wang et al., 2001). Both PAO and SMO produce cytotoxic H_2O_2 as a by-product (Seiler 1987; Vujcic et al., 2002). It has been noted that the release of H_2O_2 is mainly derived from SMO, not PAO, in polyamine analogue –treated breast cancer cells (Pledgie et al., 2005). Other products of PAO and SMO are reactive aldehydes, acetamidopropanal and aminopropanal, respectively (Seiler 1987; Vujcic et al., 2002).

2.1.4 Transport

Polyamine transport (uptake and export) is also one of the main ways to control the intracellular content of polyamines. However, the transport mechanisms in mammalian cells are not well characterized. In general, the uptake of polyamines is stimulated when the intracellular polyamine content decreases e.g. due to the inhibition of ODC, whereas the accumulation of polyamines stimulates export of polyamines out of cells (Seiler et al., 1996). Extracellular source of polyamines is diet and polyamines can be also released from intestinal microbes and other cells. The uptake is mediated through an energy-requiring transport system but transporter gene(s) have not been cloned yet in mammals. However, a protein that downregulates polyamine uptake has been identified. AZ, in addition to inhibiting of ODC, reduces polyamine uptake in order to prevent the accumulation of polyamines to toxic levels (Sakata et al., 2000). The function of polyamine exporter system is not fully understood. It appears to be carrier-mediated and presumably not facilitated by the transporter involved in the uptake (Seiler et al., 1996). However, AZ has been also shown to stimulate polyamine excretion (Sakata et al., 2000). The main polyamines exported from the cell are acetylated polyamines and putrescine whereas the predominant intracellular polyamine pool consists of spermidine and spermine (Wallace and Mackarel 1998). Thus, polyamine export seems to be

a selective and regulated process. Since polyamine catabolism produces acetylated polyamines, it does appear that polyamine catabolism and export are regulated by the same factors.

2.1.5 Functions of polyamines and polyamine metabolism

The most apparent function of polyamines is their requirement for cell growth (Tabor and Tabor 1984; Jänne et al., 2005). Elevated activity of ODC and the subsequent increase in polyamine concentrations stimulate cell proliferation (Auvinen et al., 1992). In addition, polyamines seem to regulate expression of p53, the key factor controlling the cell cycle, (Li et al., 1999) and polyamines are required for cell cycle progression (Oredsson 2003). Polyamines have also been shown to play an important role in the regulation of differentiation though this effect seems to be cell-type specific. Differentiation is inhibited by polyamines in many tumor cells (Teti et al., 2002) but in other situations polyamines are essential e.g. during adipogenesis in 3T3-L1 cells (Bethell and Pegg 1981; Vuohelainen et al., 2009). A very recent study revealed that spermidine is needed to support expression of the key regulators of adipocyte differentiation, PPARγ and CCAAT/enhancer binding protein α (CEBP/α) (Vuohelainen et al., 2009). In addition to cell proliferation and differentiation, polyamines modulate many types of ion channels such as inward-rectifier potassium channels (Oliver et al., 2000; Phillips and Nichols 2003), voltage dependent Ca^{2+} channels, and N-methyl-D-aspartic acid receptors (Williams 1997) Furthermore, polyamines stimulate transcription and translation (Coffino 2000), stabilize DNA (Tabor 1962) and act as signaling molecules (Bachrach et al., 2001).

The role of polyamines in glucose and lipid metabolism has been largely unexplored. Spermidine and spermine can mimic insulin in isolated rat adipocytes (Lockwood and East 1974) and increase the stability of insulin mRNA (Welsh 1990) and stimulate proinsulin biosynthesis (Sjöholm 1993). Furthermore, polyamines, especially spermidine and spermine, possibly increase the activities of 1,2-diacylglycerol acyltransferase (Jamdar and Osborne 1983) and lipoprotein lipase (LPL) (Giudicelli et al., 1976) but inhibit lipolysis induced by epinephrine (Lockwood and East 1974). In contrast, a recent study demonstrated that spermidine is essential for the expression of hormone sensitive lipase (HSL), the rate-limiting enzyme for lipolysis, and fatty acid synthase (Vuohelainen et al., 2009). The role of putrescine in glucose metabolism has remained unclear due to controversial results regarding its effect on glucose transport (Lockwood et al., 1971; Shelepov et al., 1990). Putrescine

together with spermine has been shown be important for the maintenance of mitochondrial respiratory chain activity in tumor-bearing mice (Ushmorov et al., 1999).

2.1.5.1 Physiological effects of SSAT overexpression

In humans, SSAT overexpression has been claimed to be involved in some disease states. Keratosis follicularis spinulosa decalvans is a rare X-linked syndrome, which is caused by the duplication of X-chromosomal region containing the SSAT gene (Fig. 2) (Gimelli et al., 2002). This disease causes follicular hyperkeratosis, alopecia and alterations that affect vision. Isolated fibroblasts from these patients display increased SSAT activity and putrescine accumulation.

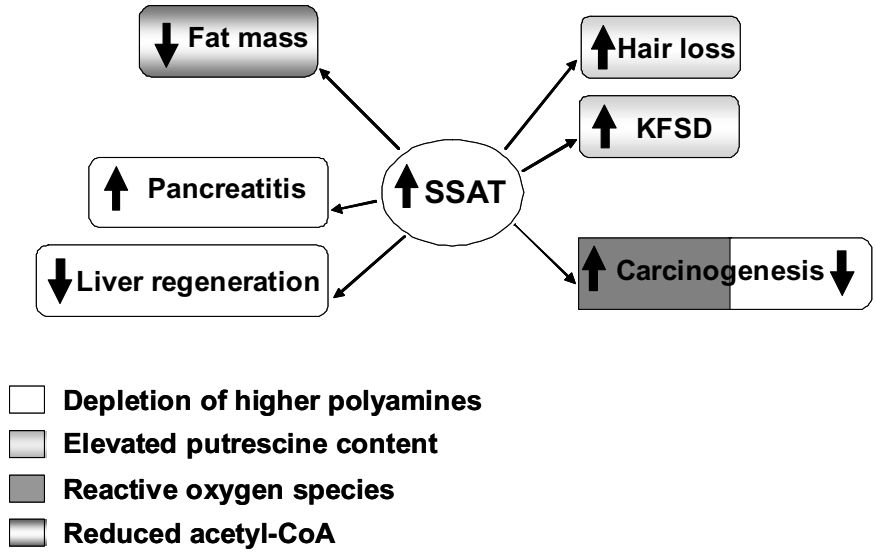

Figure 2. Effects of SSAT overexpression. KFSD, keratosis follicularis spinulosa decalvans. (Modified from (Pegg 2008). The shading of the boxes presents the most likely cause for the physiological change.

The characterization of transgenic mice ubiquitously overexpressing SSAT has revealed a variety of effects of increased SSAT activity on normal physiology. SSAT mice have a permanent hair loss at the age of 3 to 4 weeks, reduced subcutaneous fat mass and female infertility due to the lack of a corpus luteum (Fig .2) (Pietilä et al., 1997). Furthermore, behaviour and neurological changes like hypoactivity and spatial learning impairment have been observed (Kaasinen et al., 2004). Most of these changes were proposed to be related to

putrescine accumulation. Indeed, elevated putrescine accumulation has been shown to disturb keratinocyte differentiation *in vitro* and a reduction in the putrescine levels by inhibition of putrescine biosynthesis was sufficient to stimulate hair regrowth in SSAT mice (Pietilä et al., 2005). Transgenic rats overexpressing SSAT under the control of the metallothionein promoter have markedly elevated SSAT activity and thus exhibit a subsequent depletion of spermidine and spermine pools in the pancreas and liver and these animals have been noted to experience acute pancreatitis and a blockage of liver regeneration (Fig. 2) (Alhonen et al., 2000; Alhonen et al., 2002). Since the treatment with stable spermidine analogues was able to protect them from the onset of pancreatitis and to restore liver regeneration, it was concluded that spermidine seemed to be the critical polyamine needed for the maintenance of normal tissue integrity and growth in pancreas and liver, respectively (Räsänen et al., 2002; Hyvönen et al., 2007).

It has been hypothesized that increased SSAT activity and a subsequent reduction in spermidine and spermine pools would impair tumorigenesis. However, there is evidence for and against this hypothesis (Fig. 2). For example, SSAT mice have decreased tumor incidence in the skin when challenged in a two-stage tumorigenesis test (Pietilä et al., 2001). Opposite results were obtained with a mouse line in which SSAT cDNA was driven by keratin 6 promoter (Coleman et al., 2002). In addition, SSAT overexpression has been reported to enhance tumorigenesis in mice susceptible to intestinal cancer, $APC^{MIN/+}$ mice (Debruyne et al., 2001; Tucker et al., 2005). It has been proposed that the increased number of tumors in mice overexpressing SSAT maybe be attributable to a compensatory increase in the biosynthesis of polyamines. Another possibility is that the generation of reactive oxygen species from by-products of polyamine catabolism, H_2O_2 and reactive aldehydes, contribute to tumor formation.

Recent studies have shown that altered SSAT expression can change WAT metabolism in mice (Jell et al., 2007). SSAT overexpression leads to severely reduced whole body WAT mass with a reduction in acetyl-CoA and malonyl-CoA in WAT (Fig. 2). In SSAT knockout (SSAT-KO) mice, an opposite relationship between the metabolic intermediates and WAT mass was observed. Malonyl-CoA is formed from acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase (ACC). Malonyl-CoA is considered to be a key regulator of energy metabolism because it is an intermediate used for fatty acids synthesis (Wakil et al., 1983) and it inhibits the rate-limiting enzyme in fatty acid oxidation, carnitine palmitoyltransferase I (CPT-I) (McGarry and Brown 1997). Jell and coworkers suggested that the accelerated polyamine flux would result in a diversion of acetyl-CoA to polyamine acetylation instead of

the formation of malonyl-CoA (Jell et al., 2007). Consequently, the low malonyl-CoA levels then activate fatty acid oxidation and inhibit fatty acid synthesis as observed in ACC2 knock-out mice (Oh et al., 2005). Conversely, a SSAT deficiency increases the availability of acetyl-CoA for the formation of malonyl-CoA which causes increased fatty acid synthesis and reduced fatty acid oxidation with a concomitant increase in WAT accumulation. Therefore, polyamine catabolism does seem to be an important regulator of lipid and energy metabolism.

2.2 Regulation of glucose homeostasis

2.2.1 Overview

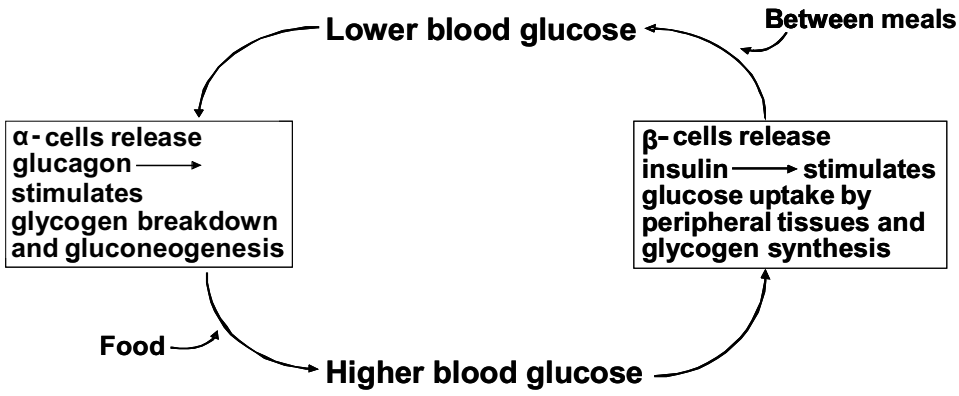

Figure 3. The maintenance of glucose homeostasis.

The maintenance of blood glucose concentration at a near-constant level is crucial to ensure a continuous supply of glucose to the brain (Murray et al., 1993). Glucose homeostasis is controlled very similarly in humans and mice through insulin secretion by the pancreas, hepatic glucose output, and glucose uptake by liver and peripheral tissues (skeletal muscle and WAT) (Aronoff et al., 2004). The main hormones regulating glucose homeostasis are insulin and glucagon, and the balance between these two hormones is the key factor in the control of glucose homeostasis. Other important hormones are e.g. epinephrine and glucocorticoids.

Glucose can enter the circulation from three sources: intestinal absorption after the digestion of food, glycogen breakdown and gluconeogenesis (Aronoff et al., 2004). When the blood glucose concentration raises, pancreatic β-cells release insulin into the circulation (Fig. 3.). The result is a lowering of blood glucose concentration. When blood glucose is low,

pancreatic α-cells release glucagon into the circulation thereby elevating the blood-glucose concentration to the desired level (Fig. 3).

2.2.2 Insulin

Insulin is the key anabolic hormone which reduces glucose levels by stimulating glucose uptake in peripheral tissues, increasing glycogen synthesis and inhibiting hepatic glucose production (Murray et al., 1993; Aronoff et al., 2004). Insulin promotes the storage of lipids by increasing triglyceride (TG) synthesis, inhibiting lipolysis, enhancing hydrolysis of TGs from either hepatic very-low-density lipoproteins (VLDLs) or dietary chylomicrons and inhiting the conversion of diet-originated free fatty acids (FFAs) to TG-rich VLDL particles. In addition, insulin promotes the storage of proteins by increasing the synthesis and enhancing the cellular uptake of amino acids in liver and skeletal muscle.

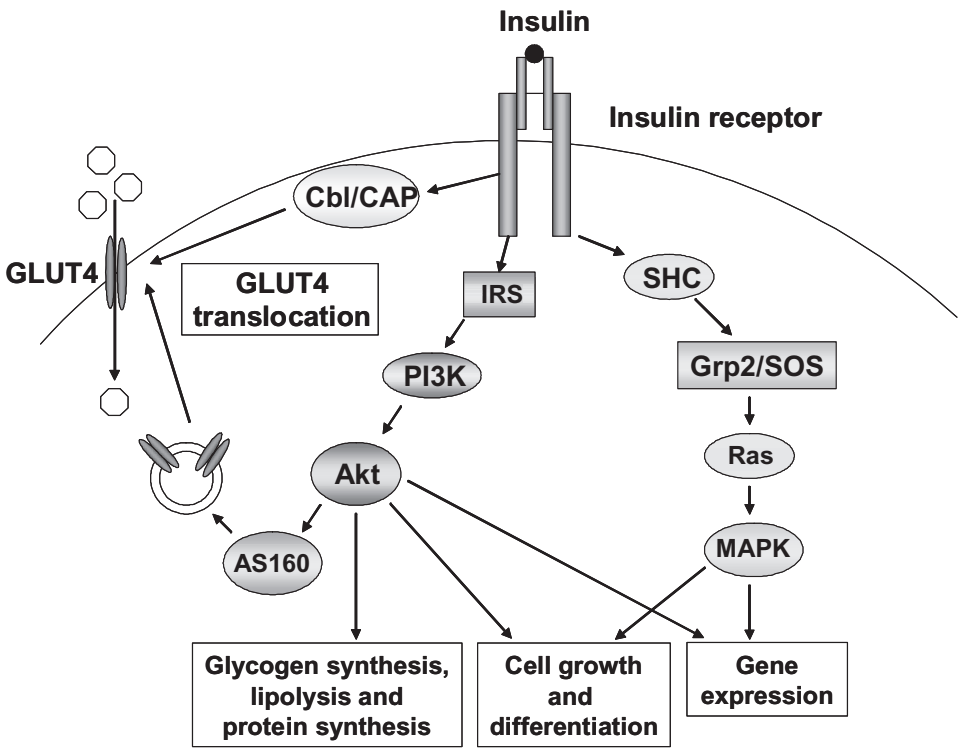

Figure 4. Simplified overview of insulin signalling pathways. Grp2, growth receptor binding protein 2; SOS, Son of sevenless; IRS, insulin receptor substrate proteins; CAP, Cbl-associated protein and Cbl, c-Cbl proto-oncogene.

The effects of insulin are mediated through the integrated network of insulin signalling molecules (Fig. 4). The initial step in the signal transduction cascade is the binding of insulin to its receptor which undergoes autophosphorylation of tyrosine residues (Taniguchi et al., 2006). Increase in insulin receptor tyrosine kinase activity results in the phosphorylation of insulin receptor substrate proteins which mediate the signal via either the phosphatidylinositol 3 –kinase (PI3K)–Akt–Akt substrate of 160 kDa (AS160) pathway or down the Ras-mitogen activated protein kinase (MAPK) pathway. The PI3K–Akt-AS160 pathway regulates the metabolic actions of insulin such as glucose transporter (GLUT) 4 (GLUT4) translocation and synthesis of glycogen, lipids and proteins. The Ras–MAPK pathway regulates gene expression and stimulates cell growth and differentiation cooperatively with PI3K-Akt pathway. In addition, the insulin receptor tyrosine kinase phosphorylates c-Cbl proto-oncogene which forms a complex with Cbl-associated protein, resulting in the activation of GTPase TC10 and an increase in GLUT4 translocation. However, the importance of c-Cbl proto-oncogene/Cbl-associated protein pathway in insulin signal transduction is not clear.

2.2.3 Glucagon

Glucagon is the key catabolic hormone consisting of 29 amino acids (Aronoff et al., 2004). It is secreted from pancreatic α-cells, and its secretion is stimulated by low and inhibited by high concentrations of glucose. The metabolic actions of glucagon are the opposite to those of insulin. Glucagon stimulates hepatic endogenous production of glucose and ketogenesis and inhibits hepatic glycogen synthesis and glycolysis (Aronoff et al., 2004; Agius 2007).

2.2.4 Glucose uptake

After an oral glucose load, approximately one-third of the total glucose amount is equally taken up by peripheral, splanchnic (liver and intestine) and non-insulin-dependent (brain and kidney) tissues (DeFronzo 2004; Meyer et al., 2002). Skeletal muscle is the major site for insulin-stimulated glucose disposal whereas only 5-10% is accomplished by WAT (DeFronzo 2004). Glucose is a hydrophilic molecule and therefore specific transport proteins are required to allow it to enter cells (Bouche et al., 2004). In mammals, energy-independent transport of glucose is mainly accomplished by 13 different isoforms of facilitative glucose transporters (GLUT1-13) (Uldry and Thorens 2004). GLUT1 is a widely expressed isoform which facilitates glucose transport in many cells under basal conditions. GLUT2 is present in pancreatic β-cells and in tissues exposed high glucose flux like intestine and liver. GLUT4

mediates insulin-stimulated glucose uptake in skeletal and cardiac muscle, WAT and brown adipose tissue (BAT) (Uldry and Thorens 2004). GLUT4 is found in the intracellular storage vesicles and insulin increases the rate of GLUT4 translocation from its storage site to the plasma membrane through the mechanisms shown in Figure 4. Exercise also stimulates the movement of GLUT4 to the cell surface through insulin-independent mechanisms including the action of 5'-AMP-activated protein kinase (AMPK), calcium/calmodulin-dependent protein kinase and protein kinase C (PKC) (Rose and Richter 2005). After transport to the cell, glucose is phosphorylated to glucose-6-phosphate by different isoforms of hexokinase (HK) (HKI-III, glucokinase) (Wilson 1995). HKII is the main isoform in insulin-sensitive tissues whereas glucokinase is expressed in the liver and pancreas.

2.2.5 Glycolysis and glucose oxidation

Glycolysis is the main pathway for glucose utilization (Murray et al., 1993; Bouche et al., 2004). It occurs in the cytosol of all mammalian cells and it represents the formation of pyruvate from glucose. The first phase, conversion of glucose to fructose 1,6-bisphosphate, is the energy requiring phase and the second phase, degradation of fructose 1,6-bisphosphate to pyruvate, is considered as an energy-yielding phase. Under anaerobic conditions (e.g. in exercising muscle), pyruvate is metabolized to lactate in a reaction catalyzed by lactate dehydrogenase. In the presence of oxygen, pyruvate goes down two mitochondrial steps, citric acid cycle and respiratory chain, to produce ATP (glucose oxidation). Three irreversible reactions catalyzed by hexokinase (or glucokinase), phosphofructokinase and pyruvate kinase are the major sites for the regulation of glycolysis. These enzymes are under allosteric control and their rate of transcription is regulated by insulin and glucagon. The pyruvate dehydrogenase (PDH) complex determines the conversion of pyruvate to acetyl-CoA in mitochondria and is the major indicator of the rate of glucose oxidation. The regulation of PDH activity is complex, involving allosteric, post-translational and transcriptional control mechanisms (Patel and Korotchkina 2006).

2.2.6 Endogenous production of glucose

Gluconeogenesis means the formation of glucose from noncarbohydrate sources, such as the breakdown of muscle proteins (alanine), anaerobic glycolysis (lactate) and the glycerol portion of fats (Murray et al., 1993; Bouche et al., 2004). The liver is the main tissue responsible for gluconeogenesis but the kidneys can also participate to some exent in times of

extreme starvation. Glucose-6-phosphatase (G6Pase), the enzyme necessary for the release of glucose into the circulation, catalyzes the rate-limiting enzyme step but phosphoenolpyruvate carboxykinase (PEPCK) plays also an important role in this process. Transcription of G6Pase and PEPCK is stimulated by glucagon, epinephrine and glucocorticoids whereas it is suppressed by insulin (Barthel and Schmoll 2003). Glucose storage occurs in skeletal muscle and in liver where glucose is converted to glycogen through glycogenesis (Murray et al., 1993; Bouche et al., 2004). Insulin stimulates the rate-limiting enzyme in glycogenesis, glycogen synthase, by promoting its dephosphorylation while glucagon and epinephrine induce glycogen synthase phosphorylation and inhibit its activity. Complete breakdown of glycogen to glucose is accomplished through glycogenolysis by the concomitant action of glycogen phosphorylase and glycogen debranching enzyme. Glucagon and epinephrine promote glycogenolysis by increasing phosphorylation and activation of glycogen phosphorylase whereas insulin has an opposite effect on the phosphorylation status of glycogen phosphorylase, leading to inactivation of this enzyme (Jiang and Zhang 2003; Bouche et al., 2004). The liver can release the newly formed glucose into the circulation for uptake by other cells (Murray et al., 1993; Bouche et al., 2004). In contrast, skeletal muscle uses glycogen as a source of energy only within muscle tissue itself due to the lack of G6Pase enzyme. Both gluconeogenesis and glycogenolysis represent sources of glucose during fasting and exercise.

2.2.7 Insulin secretion

Insulin is a peptide hormone consisting of 51 amino acids, and it is secreted from pancreatic β-cells when the glucose concentration rises above a stimulatory level (~5 mM) in the circulation (Barg 2003). Glucose is transported into the cell via GLUT2 where it is phosphorylated to glucose 6-phosphate by glucokinase. The increased availability of glucose-6-phosphate increases the rate of glycolysis and the subsequent increase in ATP/ADP ratio closes ATP-sensitive potassium channels in the plasma membrane. Elevated potassium levels depolarize the cell, causing opening of calcium channels and an influx of calcium through voltage dependent L-type Ca^{2+} channels. Finally, an increase in the intracellular free calcium concentration triggers the release of insulin.

Insulin secretion after glucose stimulation is biphasic. The first phase of insulin secretion is a rapid release (5–10 min) of preformed insulin while the second, prolonged, phase is exocytosis of newly synthesized insulin. While glucose is the most potent stimulator, insulin secretion can also be triggered by increased concentration of some amino acids, such

as leucine, arginine, and lysine, and gastrointestinal hormones e.g. glucose-dependent insulinotropic peptide, and glucagon-like peptide-1 (Vilsboll and Holst 2004).

2.3 Regulation of energy homeostasis

2.3.1 Overview

The maintenance of energy homeostasis is critical for the survival of all species (Alberts et al., 1994). In humans and mice, several complex but nevertheless very similar mechanisms regulate energy intake and energy expenditure. ATP is the major energy currency molecule in the cell. All living cells need to maintain a relatively high concentration of ATP and this is achieved by adjusting the rate of ATP production to match the rate of ATP utilization. In eukaryotes, mitochondria are responsible for the ATP synthesis through OXPHOS (Fig. 5.). ATP production is controlled via the action of several key regulators e.g. AMPK, PGC-1α and sirtuin 1 (SIRT1).

2.3.2 Regulation of food intake

The food intake is controlled by signals from the periphery that influence the central nervous system. The chief brain area involved in the control food intake is the arcuate nucleus (ARC) of hypothalamus (Cone et al., 2001). Satiation signals such as cholecystokinin, glucagon-like peptide-1, peptide tyrosine-tyrosine and ghrelin (Strader and Woods 2005; Valassi et al., 2008) which are secreted from the gastrointestinal tract during the meal reach the nucleus of the solitary tract in the caudal brainstem via the vagus nerve. From the nucleus of the solitary tract, afferent fibers project to ARC where satiety signals interact with adiposity signals. These adiposity signals are mediated by leptin and insulin which enter the brain across the blood-brain barrier. Neurons in ARC synthesize proopiomelanocortin, neuropeptide Y and agouti-related peptide. Proopiomelanocortin is cleaved to α-melanocyte-stimulating hormone which binds to melanocortin 3 and 4 receptors in other hypothalamic areas to reduce food intake. Agouti-related peptide is an antagonist of melanocortin 3 and 4 receptors and therefore counteracts the effects of melanocyte-stimulating hormone. In addition, neuropeptide Y binds to so-called Y receptors to stimulate food intake. ARC neurons are indirectly or directly sensitive to the action of satiation signals. The effects of adiposity signals, ghrelin and nutritional state on food intake are mediated by hypothalamic AMPK which modulates the expression of neuropeptides (Minokoshi et al., 2008). For example both

fasting and ghrelin activate AMPK which increases food intake (Andersson et al., 2004; Minokoshi et al., 2004; Minokoshi et al., 2008) while feeding, leptin and insulin inhibit AMPK, leading to suppression of food intake.

2.3.3 Cellular respiration

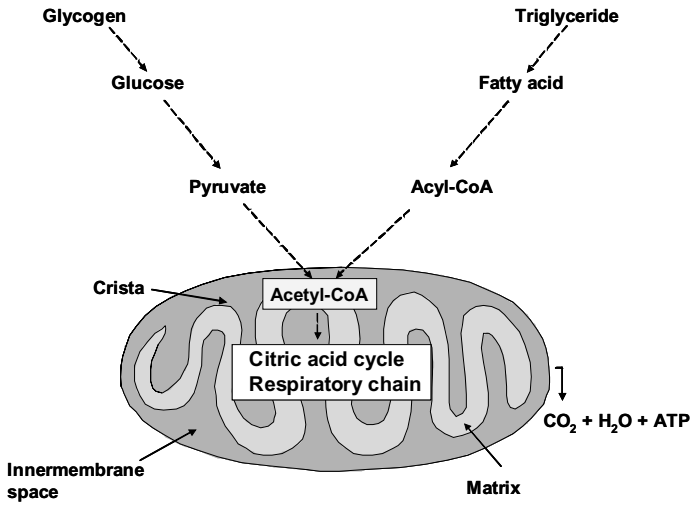

Figure 5. Cellular respiration in the mitochondrion.

Cellular respiration is a mitochondrial process in which the chemical energy of food is released and captured in the form of ATP in the presence of oxygen (Fig. 5) (Alberts et al., 1994). Glucose, amino acids and FFAs can all be used as fuels in cellular respiration. The breakdown of glucose and FFAs through glycolysis and β-oxidation, respectively, generates acetyl-CoA which is then oxidized to carbon dioxide in citric acid cycle in the mitochondrial matrix (also known as Krebs or tricarboxylic acid cycle). Amino acids can also enter the citric acid cycle after they have been converted to various intermediates of the citric acid cycle. The energy gathered in oxidation reactions in the citric acid cycle is conserved as the reducing equivalents NADH and $FADH_2$ which are oxidized in the mitochondrial respiratory chain to form ATP via OXPHOS. The mitochondrial respiratory chain is composed of five enzyme complexes (I-V) embedded in the inner mitochondrial membrane and of two electron carriers, ubiquinone and cytochrome c. The reducing equivalents, NADH and $FADH_2$, enter into the mitochondrial respiratory chain by complex I and complex II, respectively. From there, the electrons are transferred downhill to ubiquinone, complex III, cytochrome *c*, complex IV through sequential reduction-oxidation reactions. The ultimate destination for the

electrons is molecular oxygen which is reduced to two molecules of water. The free energy liberated in this process is used to pump protons from mitochondrial matrix to inner mitochondrial membrane by complexes I, III and IV. The influx of the protons back to mitochondrial matrix through complex V (ATP synthase) allows phosphorylation of ADP into ATP. However, not all of the energy liberated in the respiratory chain is coupled to ATP synthesis; some is consumed by proton leak reactions in which protons pumped out of the mitochondrial matrix are able to pass back into the mitochondria through proton conductance pathways in the inner membrane which bypass ATP synthase (Brand 1990). This means that some of the energy derived from the oxidation of food is dissipated and released as heat. The uncoupling proteins (UCPs) are mitochondrial inner membrane proteins that can dissipate the proton gradient before it can be used to provide the energy for OXPHOS (Echtay 2007).

2.3.4 The key regulators of energy expenditure

2.3.4.1 5'-AMP-activated protein kinase

AMPK is a cellular energy sensor that is found in all eukaryotic cells. AMPK is a heterotrimeric enzyme consisting of a catalytic α-subunit and regulatory β- and γ-subunits (Hardie et al., 1998; Winder 2001). In mammals, each subunit has two or three isoforms designated as α1, α2, β1, β2, γ1, γ2 and γ3. All possible combinations of these isoforms appear to be expressed. The α-subunits contain an N-terminal protein kinase catalytic domain, a central autoinhibitory region and a C-terminal domain involved in binding of regulatory subunits (Fig. 6). The β-subunits contain a glycogen-binding domain, the function of which is not clear, but it is involved in the association of AMPK with glycogen particles (Fig. 6). The β-subunits also contain a domain participating in the formation of complexes with α- and γ-subunits. The γ-subunits contain four tandem repeats of a motif known as a cystathionine beta synthase domain (Fig. 6). These domains represent the binding sites for regulatory nucleotides AMP and ATP.

AMPK is activated by an increase in the AMP/ATP ratio in mammals (Hardie et al., 2003). AMP activates AMPK by three mechanisms. AMP allosterically activates AMPK through the binding to its γ-subunit. In addition, AMP can stimulate phosphorylation of a critical threonine residue (Thr-172) in the activation loop of catalytic α-subunit by an upstream kinase LKB1 (Fig. 6). Furthermore, AMP inhibits dephosphosphorylation of Thr-172 by protein phosphatases. These effects of AMP are antagonized by the high concentrations of ATP. The activators of AMPK are glucose deprivation, ischemia, hypoxia

and oxidative stress that interfere with ATP production. In addition, exercise and muscle contraction activate AMPK by increasing ATP consumption (Winder and Hardie 1996; Hutber et al., 1997).

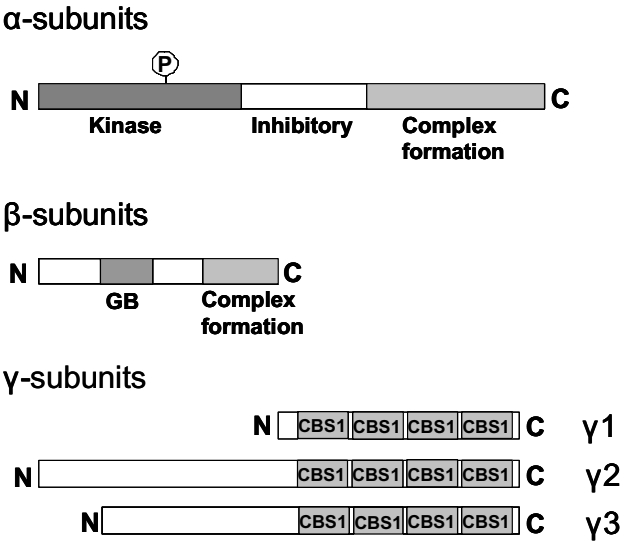

Figure 6. Structure of AMPK subunits. GB, Glycogen binding domain and CBS, cystathionine beta synthase domain.

AMPK controls energy homeostasis by increasing energy intake and energy expenditure. Once activated, AMPK initiates cellular responses aimed at restoring the ATP levels. AMPK enhances energy expenditure by activating pathways producing ATP, such as fatty acid oxidation, glucose uptake and glycolysis. One of the important functions of AMPK is the regulation of lipid metabolism through its ability to control the intracellular content of malonyl-CoA. AMPK phosphorylates and inhibits both isoforms of ACC (ACC1 and ACC2), which catalyze the formation of malonyl-CoA from acetyl-CoA (Abu-Elheiga et al., 1995), leading to reduced malonyl-CoA synthesis (Winder and Hardie 1996). In addition, AMPK stimulates malonyl-CoA degradation by phosphorylating and activating malonyl-CoA decarboxylase (Saha et al., 2000). A reduced level of malonyl-CoA decreases the rate of fatty acid synthesis and this relieves CPT-I from its inhibition resulting in increased fatty acid oxidation. In several tissues, AMPK is known to inhibit fatty acid synthesis also by reducing the expression of ACC1, fatty acid synthase and sn-glycerol-3-phosphate acyltransferase (Woods et al., 2000; Zhou et al., 2001). The mechanism by which AMPK exerts its actions on mitochondrial biogenesis in skeletal and cardiac muscle seems to depend on the activation

of PGC-1α and nuclear respiratory factor (NRF) 1 (NRF-1) (Bergeron et al., 2001; Zong et al., 2002). AMPK suppresses lipolysis by phosphorylating and inhibiting HSL (Daval et al., 2006). AMPK also regulates several aspects of glucose metabolism. It stimulates glucose uptake in skeletal muscle by activating GLUT4 translocation in resting muscle (Merrill et al., 1997; Koistinen et al., 2003). This effect seems to be mediated via inhibitory phosphorylation of AS160 (Sano et al., 2003). AMPK stimulates glucose utilization by activating the transcription of HKII (Stoppani et al., 2002), by phosphorylating and activating phosphofructokinase 2 (Marsin et al., 2000) and by increasing the activity of PDH (Smith et al., 2005). In line with the role of AMPK as an intracellular energy gauge, AMPK reduces energetically expensive processes such as gluconeogenesis and glycogen synthesis by downregulating the expression of the key gluconeogenic enzymes (G6Pase and PEPCK) (Lochhead et al., 2000) and phosphorylating the rate-limiting enzyme, glycogen synthase (Miyamoto et al., 2007), respectively.

2.3.4.2 Peroxisome proliferator activated receptor γ co-activator 1α

PGC-1α is a member of a small family of transcriptional coactivators including PGC-1α, PGC-1β and PGC-1-related coactivator which all share common functions in the regulation of mitochondrial biogenesis and oxidative metabolism (Andersson and Scarpulla 2001). Human PGC-1α is mapped to chromosome 4p15 (Esterbauer et al., 1999) and it encodes about 90 kDa protein containing an N-terminal transcriptional activation domain including a major nuclear hormone receptor interacting LXXLL motif, a central regulatory/repression domain and C-terminal RNA-binding and arginine-serine (RS) rich domains (Fig. 7) (Puigserver and Spiegelman 2003). Since it is a transcriptional coactivator, PGC-1α interacts directly with several transcription factors, such as nuclear receptors which bind to the DNA promoter regions in the nucleus.

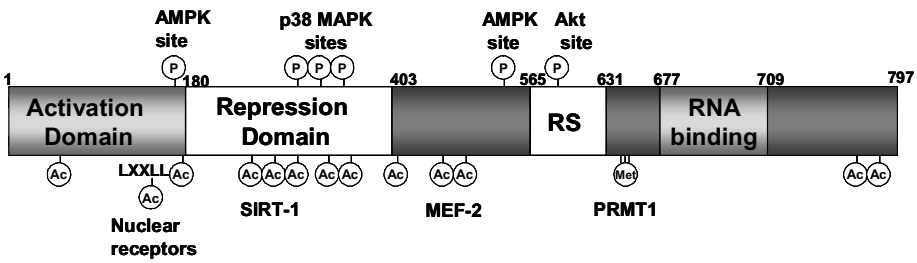

Figure 7. Architecture of PGC-1α protein.

Transcription of PGC-1α is induced by exercise (in muscle), cold exposure (in BAT and skeletal muscle), fasting (in liver) and energy demand. The effect of exercise on PGC-1α expression is mediated by CREB (Herzig et al., 2001), myocyte enhancement factor 2 (MEF-2) (Handschin et al., 2003) and AMPK (Fig. 8) (Terada et al., 2002). The induction of PGC-1α transcription by cold and fasting is accomplished by increased cAMP levels which activate CREB (Fig. 8) (Gomez-Ambrosi et al., 2001; Herzig et al., 2001).

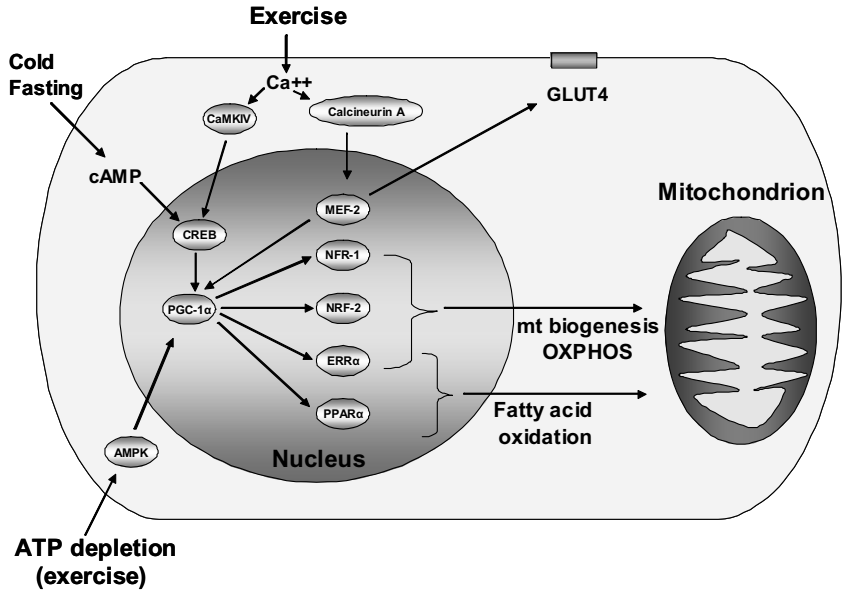

Figure 8. Function of PGC-1α in skeletal muscle (modified from (Scarpulla 2008)). CaMKIV, calcium/calmodulin-dependent protein kinase IV and mt, mitochondrial.

The activity and stability of PGC-1α are also affected by post-translational modifications. Phosphorylation of PGC-1α by stress-activated p38MAPK in the repression domain increases protein stability and the transcriptional activity of PGC-1α (Fig. 7). Fasting, cytokines and oxidative stress elicit their effect on PGC-1α through this mechanism (Puigserver et al., 2001; Cao et al., 2005; Kim et al., 2006). In contrast, phosphorylation of PGC-1α by glycogen synthase kinase 3 β (GSK-3β) targets PGC-1α protein to ubiquitin-mediated proteolysis iniated by E3 ubiquitin ligase SCF^{Cdc4} (Olson et al, 2008). AMPK also phosphorylates PGC-1α resulting in a more active protein (Fig. 7) (Jager et al., 2007). PPARδ increases PGC-1α protein levels in the absence of any increase in PGC-1α mRNA levels (Hancock et al, 2008) and fasting-induced SIRT1, class III histone deacetylase, deacetylates PGC-1α leading to an increase in gluconeogenic action of PGC-1α (Fig. 7) (Rodgers et al., 2005). In addition, methylation of PGC-1α by protein arginine methyltransferase I (PRMT1)

at C-terminus has been observed to be essential for the coactivator function of PGC-1α (Fig. 7) (Teyssier et al., 2005). In contrast, insulin inhibits PGC-1α through Akt which phosphorylates PGC-1α at the RS domain (Fig. 7) (Li et al., 2007; Rodgers et al., 2005).

Adaptations to exercise include induction of mitochondrial biogenesis (Scarpulla 2002), fatty acid oxidation (Vega et al., 2000), OXPHOS (Mootha et al., 2003)), glucose uptake and fiber type switching (Puigserver and Spiegelman 2003), processes which are all stimulated by PGC-1α (Fig. 8). The effect of PGC-1α on mitochondrial biogenesis is mainly mediated by NRF-1, NRF-2 and estrogen-related receptor α (ERRα) (Fig. 8) (Scarpulla 2002; Schreiber et al., 2004). PGC-1α stimulates fatty acid oxidation through ERRα and PPARα, both of which activate the key enzyme(s) in the β-oxidation of fatty acids (Fig. 8) (Vega et al., 2000; Huss and Kelly 2004). Exercise-induced PGC-1α stimulates the conversion of muscle fibers from type II (fast twitch) to type I (slow twitch) i.e. to fibers having a high oxidation capacity via MEF-2 (Handschin et al., 2003). PGC-1α increases the expression of GLUT4 mainly via MEF-2 in skeletal muscle and stimulates glucose uptake (Michael et al., 2001) (Fig. 8). In the liver, PGC-1α is induced in the fasted state. Once PGC-1α is induced, it binds and coactivates forkhead box O1, hepatocyte nuclear factor (HNF) 4 (HNF-4) and the glucocorticoid receptor to increase the expression of gluconeogenic genes (G6Pase and PEPCK) (Puigserver et al., 2003; Rhee et al., 2003). The stimulation of fatty acid oxidation is mediated via ERRα and PPARα coactivation similarly to the situation in skeletal muscle (Vega et al., 2000; Louet et al., 2002; Huss and Kelly 2004). PGC-1α also activates CYP7A1, the rate-limiting gene in bile acid synthesis (Shin et al., 2003) and represses low-density lipoprotein (LDL) receptor (LDLR) gene expression (Jeong et al., 2009). Therefore PGC-1α is also involved in the control of cholesterol homeostasis. In BAT in rodents, cold exposure strongly induces the expression of PGC-1α, with the consequence of the stimulation of heat production through UCP1 (Puigserver et al., 1998).

2.3.4.3 Sirtuin 1

Sirtuins are a class of NAD^+-dependent protein deacetylases which utilize the cofactor NAD^+ to deacetylate lysine residues of protein substrates (Blander and Guarente 2004). Of the seven mammalian sirtuins (sirtuin 1-7), SIRT1 has been most extensively studied. SIRT1 is induced by elevated concentrations of NAD and/or the ratio of NAD/NADH (Revollo et al., 2004). Fasting, exercise and oxidative stress induce SIRT1, most likely elevating the NAD/NADH ratio.

SIRT1 participates in the control of energy expenditure. It increases the activity of PGC-1α through deacetylation. In the liver, fasting-induced SIRT1 stimulates PGC-1α which evokes an increase in gluconeogenesis and a suppression of glycolysis (Rodgers et al., 2005). In WAT, SIRT1 suppresses adipogenesis and enhances the release of FFA through lipolysis by repressing PPARγ (Picard and Auwerx 2002; Picard et al., 2004). *In vitro* studies have revealed that SIRT1 has a possible regulatory role in the insulin signalling pathway since inhibition of SIRT1 reduces insulin-induced Akt and insulin receptor substrate-2 protein tyrosine phosphorylation (Zhang 2007). In mice, treatment with the SIRT1 activator, resveratrol, has been shown to result in beneficial changes in glucose and energy metabolism. Resveratrol increases mitochondrial number and activity in the liver, skeletal muscle and BAT, improves aerobic capacity, enhances energy expenditure and is protective against high-fat diet-induced insulin resistance through SIRT-mediated induction of PGC-1α and AMPK (Baur et al., 2006; Lagouge et al., 2006).

2.4 Regulation of cholesterol homeostasis

2.4.1 Overview

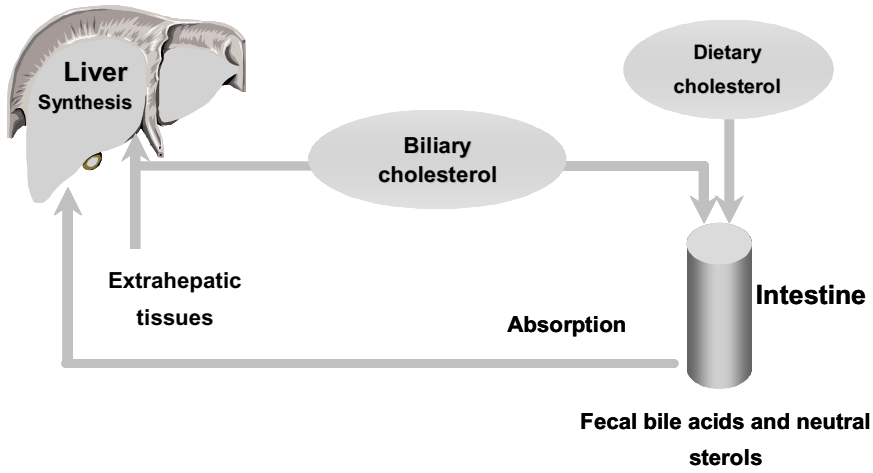

Figure 9. Cholesterol homeostasis.

Cholesterol is an essential constituent of mammalian cellular membranes and a precursor for the synthesis of steroid hormones, vitamin D and bile acids (Murray et al., 1993). Humans and mice exhibit major differences in cholesterol metabolism but cholesterol homeostasis is similarly achieved by controlling cholesterol biosynthesis, cholesterol absorption, cholesterol

conversion to bile acids and excretion of bile acids in both species (Fig. 9) (Dietschy et al., 1993). Cholesterol in the body is derived from two sources, hepatic *de novo* synthesis and the diet. Cholesterol is ultimately eliminated from the body by the conversion to bile acids and secretion into bile.

Cholesterol is transported in the circulation by lipoproteins which are classified according to their density and composition: 1) chylomicrons, 2) VLDL, 3) intermediate-density lipopoteins (IDL), 4) LDL and 5) high-density lipoprotein (HDL) (Murray et al., 1993). In humans, the proportion of HDL cholesterol from total cholesterol is 20% of total cholesterol whereas LDL cholesterol represents more than 50% of total cholesterol (Argmann et al., 2006). In contrast, mouse HDL cholesterol varies between 65 to 75% of total cholesterol and correspondingly the LDL cholesterol fraction is only between 10 to 20 %. Lipoproteins contain a variety of lipids such as TGs, phospholipids, cholesterol and cholesteryl esters (CEs) (Murray et al., 1993). Chylomicrons and VLDL particles are TG-rich particles whereas LDL and HDL particles contain predominantly cholesterol and phospholipids, respectively. IDL particles have equal amounts of CEs, TGs and phospholipids.

Chylomicrons transport dietary cholesterol and TG absorbed from the small intestine to the liver (Murray et al., 1993). In the circulation, TGs of chylomicrons are hydrolyzed by LPL leading to the formation of chylomicron-remnants which are cleared by the liver through the LDLR related protein. The cholesterol entering the liver can then be used for lipoprotein formation, bile acid synthesis, secretion to bile, or storage as CEs.

The assembly of VLDL is a two step lipidation process (Olofsson et al., 2000; Adiels et al., 2008)]. In the first step, apoliporotein B100 is lipidated by microsomal transfer protein which leads to the formation of pre-VLDL. In the second step, pre-VLDL is converted to VLDL by ADP ribosylation factor-1. In the circulation, TG-rich VLDLs are hydrolyzed by LPL and converted to IDL. The subsuquent hydrolysis of IDL particles by hepatic lipase produces LDL particles which deliver cholesterol to the extrahepatic tissues where the uptake of cholesterol is facilitated by LDLRs. LDL particles are catabolized mainly by hepatic uptake via LDLRs. The rate of hepatic LDL clearance is significantly greater in mice than in humans (Dietschy and Turley 2002).

The biosynthesis of HDL begins in the liver where apolipoprotein A-I interacts with ATP binding cassette protein A1 (ABCA1) and apolipoprotein A-I is then secreted into the circulation as a lipid-poor particle (Rader 2006). These particles then recruit more phospholipids and free cholesterol from peripheral tissues through the action of ABCA1,

which is expressed on the surfaces of macrophages in the arterial wall, forming nascent HDL_2 particles. Lecithin:cholesterol acyltransferase within nascent-HDL particles esterifies free cholesterol resulting in the formation of mature small HDL_3-particles. The fusion of two small HDL_3 particles by phospholipid transfer protein generates one larger size HDL_2 particle. In mice, the expression of phospholipid transfer protein is significantly higher than in humans. Hepatic lipase and endothelial lipase degrade HDL_2 lipoproteins to HDL remnant particles which are cleared from the circulation by hepatic class B type 1 scavenger receptor (SR-BI). The cholesterol acquired from peripheral tissues by HDLs can be also transferred to VLDLs and LDLs via the action of HDL-associated enzyme, cholesteryl ester transfer protein (CETP), in humans but not in mice which do not express CETP (Hogarth et al., 2003). The function of CETP allows peripheral cholesterol to be returned to the liver as LDL particles via LDLRs.

2.4.2 Cholesterol synthesis

The major part of cholesterol is synthesized in the liver. The murine liver is relatively more important as a site for cholesterol synthesis than its human counterpart (Dietschy and Turley 2002). Cholesterol biosynthesis can be divided into five steps (Fig. 10): 1) synthesis of mevalonate from acetyl-CoA, 2) formation of isoprenoid units, 3) formation of squalene from six isoprenoid units 4) conversion of squalene to lanosterol, and 5) conversion of lanosterol to cholesterol either through desmosterol or demethyl- or methylsterols (Murray et al., 1993). The rate-limiting step is the formation of mevalonate from 3-hydroxy-3-methylglutaryl-CoA, catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (Goldstein and Brown 1990). After the synthesis, acyl CoA:cholesterol acyltransferase 2 (ACAT2) esterifies cholesterol to CEs which can be stored or used in the formation of lipoproteins (Chang et al., 2001).

The rate of cholesterol formation is highly responsive to changes in the intracellular cholesterol level in humans and mice. This feedback regulation is mediated primarily by changes in the amount and activity of HMGCR which is controlled at four levels. First, degradation of HMGCR is stimulated by cholesterol whose binding to a transmembrane sterol-sensing domain activates the degradation of the enzyme via the 26S proteasome (DeBose-Boyd 2008). Second, sterol regulatory element binding proteins (SREBPs) activate the transcription of HMGCR and other genes involved in cholesterol biosynthesis (Brown and Goldstein 1997). When cholesterol levels are low, SREBPs induce HMGCR but cholesterol accumulation decreases the expression of SREBPs. Third, the rate of translation

of HMGCR mRNA is inhibited by sterol and nonsterol end-products of mevalonate metabolism and dietary cholesterol (DeBose-Boyd 2008). In addition to feedback regulation, HMGCR activity is inhibited by phosphorylation through the action of AMPK when the cellular ATP content is low (Clarke and Hardie 1990). Hepatic *de novo* synthesis and intestinal absorption are reciprocally regulated, e.g. when cholesterol absorption decreases, cholesterol synthesis increases and vice versa (Grundy et al., 1969).

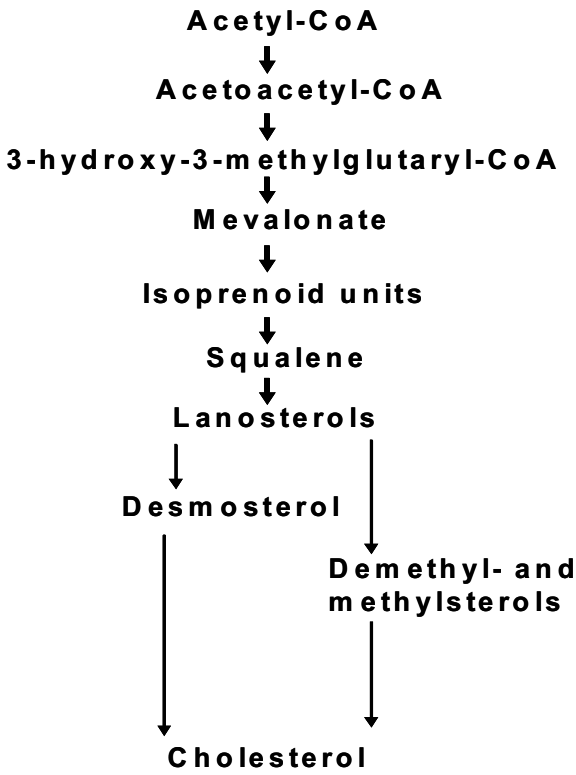

Figure 10. Cholesterol biosynthesis in mammals.

2.4.3 Cholesterol absorption

Cholesterol absorbed from the intestinal lumen originates from three sources, diet, bile and turnover of intestinal epithelium (Vuoristo and Miettinen 2000). The amount of dietary cholesterol absorbed varies between 30-70% in humans and mice (Dietschy and Turley 2002). Only free cholesterol can be effectively absorbed and thus, CEs are de-esterified by the pancreatic cholesterol esterase (Vuoristo and Miettinen 2000). Cholesterol, which is

insoluble in an aqueous environment, needs to be incorporated into bile acid micelles prior to absorption. The micelles are then transported to the brush border membrane of enterocytes where cholesterol passes through a diffusion barrier that is located at intestinal lumen-enterocyte membrane interface. Cholesterol absorption was previously thought to be simple passive diffusion process. However, cholesterol uptake transporter protein, Niemann–Pick C1-like 1 protein (NPC1L1), was recently identified in the jejunum (Altmann et al., 2004). SR-BI and CD36 are the two other transporters that facilitate cholesterol transport (Nauli et al., 2006; Labonte et al., 2007). In addition to cholesterol uptake transporters, there are three cholesterol efflux transporters in the brush border membrane, ABCA1, ATP binding cassette protein G5 (ABCG5) and ATP binding cassette protein G8 (ABCG8) which excrete cholesterol back into the intestinal lumen and limit cholesterol absorption (Vuoristo and Miettinen 2000). After absorption, cholesterol is esterified by ACAT2 to CEs, incorporated into chylomicron particles through the action of the microsomal tranfer protein and transported via the lymph to the circulation.

Any factor that can change cholesterol transport from intestinal lumen to the enterocyte can influence intestinal cholesterol absorption (Vuoristo and Miettinen 2000). The genetic control of cholesterol absorption is clearly evident. For example mouse strains display significant differences in absorption rates when fed a high cholesterol diet (Carter et al., 1997), and human and mouse studies have also shown a high variation in cholesterol absorption efficiency between different human individuals (25 to 75%) and mice (22 to 66%) (Miettinen and Kesaniemi 1989; Schwarz et al., 2001). Dietary fiber, especially viscous fiber, inhibits cholesterol absorption by disturbing micellar solubilization and/or increasing the diffusion barrier (Vuoristo and Miettinen 2000). In addition, dietary plant sterols such as those found in vegetable oils, inhibit cholesterol absorption by displacing cholesterol from micelles and inducing cholesterol efflux (Plat and Mensink 2002). Changes in hepatic output and the size of bile acid pool markedly influence cholesterol absorption. For example, the expansion of the total bile acid pool inhibits cholesterol absorption by decreasing the expression of NPC1L1 in mice (Ratliff et al., 2006).

2.4.4 Cholesterol elimination through bile acid synthesis

In mammals, liver controls the elimination of cholesterol from the body. The hepatic formation of bile acids is mainly accomplished through the neutral/classic pathway in humans and mice. The rate-limiting enzyme responsible in this pathway is hepatic CYP7A1 (Russell and Setchell 1992; Fuchs 2003). The second pathway termed as the acidic (or alternative)

pathway is initiated by sterol 27-hydroxylase (CYP27A1). The major bile acids in humans are cholic acid and chenodeoxycholic acid. Since murine chenodeoxycholic acid is converted to muricholic acid, cholic acid and muricholic acid are the main bile acids found in rodents (Houten et al., 2006). After synthesis, bile acids are conjugated with taurine or glycine in humans but only taurine conjugates exist in mice. Bile acids are exported from hepatocytes to biliary canaliculi by a bile salt export pump, secreted into bile and stored in the gallbladder (Russell and Setchell 1992; Fuchs 2003). After the meal, the gallbladder secretes bile into the intestine where bile acids are converted to secondary and tertiary bile acids by intestinal bacteria. Most bile acids are reabsorbed in the ileum by the action of apical Na^+-dependent bile acid transporter or ileal bile acid binding protein and transported back to the liver via the portal circulation. Hepatic uptake of bile acids is mainly facilitated by Na^+-taurocholate cotransporting polypeptide (NTCP) and Na^+-independent organic anion transporting polypeptide (OATP1) (Meier and Stieger 2002). The function of bile acids is to emulsify lipids in the intestine and to act as signalling molecules (Hylemon et al., 2009).

The conversion of cholesterol into bile acids is controlled by CYP7A1 which is mainly regulated at the gene transcriptional level by bile acids, nutrients, cytokines and hormones (Jelinek et al., 1990; Fuchs 2003). In mice, dietary cholesterol activates CYP7A1 transcription through liver X receptor α (LXRα) whereas the human CYP7A1 promoter lacks the LXRα binding site (Chiang et al., 2001). Important activators of CYP7A1 transcription are HNF-4α, COUP-TFII, α-fetoprotein transcription factor, liver receptor homolog 1 and PGC-1α in humans and rodents (Galarneau et al., 1996; Nitta et al., 1999; Hayhurst et al., 2001; Shin et al., 2003). PGC-1α activates CYP7A1 transcription by increasing HNF-4α-mediated transactivation of CYP7A1 (De Fabiani et al., 2003; Shin et al., 2003). One well-conversed repression mechanism between species is the activation of small heterodimer partner by bile-acid induced farnesoid X receptor (FXR). Small heterodimer partner inhibits HNF-4-, α-fetoprotein transcription factor- or liver receptor homolog 1-mediated transactivation of CYP7A1 transcription (Makishima et al., 1999; Lu et al., 2000). The transcription of CYP27A1 is also suppressed by bile acids but this effect is less potent than that occuring at the CYP7A1 gene (Chiang et al., 2001).

3 AIMS OF THE STUDY

The main purpose of this study was to investigate the effects of activated and inactivated polyamine catabolism on glucose, energy and lipid metabolism in mice.

The specific aims were:

1. To characterize the metabolic phenotype of SSAT mice and to elucidate the molecular mechanism explaining the phenotype **(I)**

2. To investigate the effect of activated polyamine catabolism on cholesterol and bile acid metabolism in SSAT mice and to determine the molecular mechanism leading to these changes **(II)**

3. To characterize the metabolic phenotype of SSAT-KO mice **(III)**

4 MATERIALS AND METHODS

4.1 Animals (I-III)

The mice used in these studies were as follows (the mouse strain is shown in parentheses): a) transgenic mice overexpressing SSAT under endogenous SSAT promoter (DBA/2 x Balb/c) (Pietilä et al., 1997), b) Hairless (hr/hr) or normally haired (hr/+) (HsdOla) mice purchased from Harlan, UK and c) SSAT-KO mice (C57BL/6J) generated in collaboration with Karolinska Institute, Sweden. The animals were housed under standard conditions and were fed a regular laboratory chow (R3, Lactamin AB, Stockholm, Sweden). The study protocols were approved by the Animal Care and Use Committee of the University of Kuopio and the Provincial government. Generation of SSAT-KO mice was performed as followed. The SSAT targeting vector (Niiranen et al., 2002) was electroporated into the mouse RW-4 embryonic stem cell line. The correctly targeted SSAT-KO embryonic stem cell clone was injected into C57Bl/6J blastocysts which were transplanted into pseudopregnant females. The SSAT-KO mice were backcrossed in the C57BL/6J mouse strain for at least six generations to dilute the 129/SvJ genetic background originating from embryonic stem cells.

4.2 Polyamine analogues, antibodies and primers (I-III)

DENSPM was synthesized in the University of Kuopio as previously published (Rehse et al., 1990). DFMO was a gift from Ilex Oncology Inc, USA. The antibodies used in these studies were as follows: PGC-1α (Millipore, Billerica, MA, USA), HNF-4α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), LXRα (Abcam, UK), FXR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phosphorylated α-subunit of AMPK (Thr172) (Cell Signaling Technology Inc, Danvers, MA, USA), SIRT1 (Millipore, Billerica, MA, USA), dually phosphorylated p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology Inc, Danvers, MA, USA), PRMT1 (Cell Signaling Technology Inc, Danvers, MA, USA), phosphorylated Akt (Ser473) (Cell Signaling Technology Inc, Danvers, MA, USA), Akt (Cell Signaling Technology Inc, Danvers, MA, USA), PI3K (Cell Signaling Technology Inc, Danvers, MA, USA), phosphorylated GSK-3β (Ser21/9) (Cell Signaling Technology Inc, Danvers, MA, USA), GSK-3β (Cell Signaling Technology Inc, Danvers, MA, USA), PPARδ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Quantitative RT-PCR primers and probes were designed using Assay-by-Design system from Applied Biosystems, USA.

4.3 Data and statistical analyses (I-III)

Affymetrix data was analyzed using Affimetrix Microarray Suite 5.0, GenMAPP (Dahlquist et al., 2002) and MAPPFinder (Doniger et al., 2003) software. Statistical analysis was performed with Student's two-tailed t-test when applicable. In the multiple comparisons, two-way ANOVA was used. A p value less than 0.05 was considered significant.

4.4 Analytical methods, and in vitro and in vivo experiments (I-III)

Table 1 shows methods used to measure plasma, serum, fecal or tissue metabolite concentrations in the original publications I-III. Plasma or serum samples were taken from the saphenous or tail vein in the fed or fasted state (12-18 h fasting). Fecal samples and tissues were collected in the fed state. Table 2 summarizes methods or kits used in the different analyses in the original publications I-III. Tables 3 and 4 summarize *in vivo* and *in vitro* studies performed in original publications I-III, respectively. A detailed description of all methods is provided in the original publications I-III given in Tables 1-4 and in the references provided in Table 2.

Table 1. Analysis of plasma, serum, fecal or tissue metabolites in publications (Publ) I-III.

Metabolite	Method	Equipment/Kit	Publ
Plasma TG	Enzymatic	Microlab 200 analyzer	**I, III**
Serum FFA	Enzymatic	TG detection kit (Wako)	**I**
Plasma alkaline phosphatase	Colorimetric	Microlab 200 analyzer	**II**
Plasma alanine aminotransferase	Kinetic	Microlab 200 analyzer	**II**
Plasma glucose	Enzymatic	Microfluorometry	**I, III**
Plasma insulin	ELISA	Rat insulin kit (Chrystal Chem)	**I, III**
Plasma leptin	ELISA	Mouse leptin kit (Chrystal Chem)	**I**
Serum β-hydroxybutyrate	Enzymatic	Hitachi 717 analyzer	**I**
Plasma glycerol	Enzymatic	Microfluorometry	**I**
Plasma total cholesterol	Enzymatic	Microlab 200 analyzer	**III**
Plasma, liver and fecal total cholesterol	GCL	Gas chromatography	**II**
Serum HDL cholesterol	Enzymatic	Hitachi 717 analyzer	**II**
Plasma cholesterol precursors	GCL	Gas chromatography	**II**
Plasma cholesterol absorption markers	GCL	Gas chromatography	**II**
Serum total bile acids	Enzymatic	Spectrophotometry	**II**
Fecal bile acids	GCL	Gas chromatography	**II**
Tissue polyamine content	HPLC	HPLC	**I, III**
Adipocyte ATP content	Chemiluminesence	ATPlite one step kit (Perkin Elmer)	**I**
Tissue DENSPM content	HPLC	HPLC	**III**

ELISA, Enzyme-linked immunosorbent assay; GLC, gas-liquid chromatography and HPLC, high pressure liquid chromatography.

Table 2. Methods used in the original publications (Publ) I-III.

Method	Reference/Kit	Publ
Magnetic resonance imaging	(Harrington et al., 2002)	**I**
Tissue TG content using glycerol assa	(Wieland 1974)1]	**I**
Indirect calorimetry	(McLean and Tobin 1990)	**I**
Telemetric core temperature and activity measurement	(Hohtola et al., 1991)	**I**
Fatty acid oxidation	(Mannaerts et al., 1979; Osmundsen 1981)	**I**
Isolation of mitochondria	(Pallotti and Lenaz 2001)	**I**
Histology	(Rantala and Lounatmaa)	**I-III**
Macrophage staining	(Leppanen et al., 1998)	**I**
Adipocyte isolation	(Rodbell 1964)	**I**
Adipocyte cell sizing	(Krotkiewski et al., 1983)	**I**
Electron microscopy	(Lounatmaa and Rantala 1991)	**I**
Affymetrix MG-U74A-v2 chip analys	Affymetrix protocols	**I**
RNA isolation	RNAeasy kit (Qiagen), (Chomczynski and Sacchi 1987)	**I, II**
Mitochondrial DNA isolation	(Straus 1998)	**I**
DNAse treatment	DNA free^{TM} kit (Ambion)	**I, II**
cDNA synthesis	High capacity archive kit (Applied Biosystem	**I, II**
Sodium dodecyl sulfate (SDS) page	(Laemmli 1970)	**I, II**
Immunoblotting and -detection	(Gallgher et al., 2008)	**I, II**
SSAT activity	(Bernacki et al., 1995)	**I, III**
Protein concentration	Bio Rad Protein Assay	**I-III**
Double-stranded DNA amount	(Giles and Myers 1964)	**I**

Table 3. Tests or treatments performed in mice in the original publications (Publ) I-III.

	Mice	Test or treatment	Fast hours	Treatment	Time point(s)	Measurements
I	SSAT, wt	GTT	12	i.p. 2 mg/g D-glucose	0, 15, 30, 60 and 120 min	Plasma Glc and Ins
III	SSAT-KO, wt	GTT	16	i.p. 2 mg/g D-glucose	0, 15, 30, 60 and 120 min	Plasma Glc and Ins
I	SSAT, wt	ITT	12	i.p. 0.25 mU/g insulin	0, 20, 40 and 80 min	Plasma Glc
I	SSAT, wt	ITT	5	i.p. 0.15 mU/g insulin + 0.4 mg/g D-glucose	0, 20, 40 and 80 min	Plasma Glc
III	SSAT-KO, wt	ITT	16	i.p. 0.25 mU/g insulin	0, 20, 40 and 80 min	Plasma Glc
II	SSAT, wt	Chol and BA absorption	0	Oral gavage 1 μCi [^{14}C]cholesterol, 1 μCi [^{3}H]taurocholate and 2 μCi [^{3}H]sitostanol	4 d	Fecal Chol and BA content
I	SSAT, wt	DFMO treatment	0	0.5% DFMO in drinking water	2, 4 or 5 wk	ATP and PA content, gene expression
I	Wt	Putrescine injections	0	i.p. 1000 mg/kg putrescine dihydrochloride	2 h	PA content, gene expression, western blot
III	SSAT-KO, wt	DENSPM treatment	0	i.p. 125 mg/kg DENSPM per day	3 d	SSAT activity, PA and DENSPM content
III	SSAT-KO, wt	DENSPM treatment	0	i.p. 125 mg/kg DENSPM per fay	10 d	Survival, SSAT activity, PA and DENSPM content
III	SSAT-KO, wt	DENSPM treatment	0	i.p. 0.1 ml/kg CCl_4 per day	24 h	SSAT activity, PA content

Wt, wild-type; i.p., intraperitoneal injection; GTT, glucose tolerance test; ITT, insulin tolerance test; Glc, glucose; Ins, insulin; Chol, cholesterol; BA, bile acid and PA, polyamine.

Table 4. Cell culture studies performed in the original publications (Publ) I-II.

Publ	Cells	Origin	Culture medium	Treatment	Time points	Measurements
I	3T3-L1	ATCC	DMEM	1 mM putrescine dihydrochloride with and without 1 mM aminoguanidine	16 h	2-deoxyglucose uptake (Traxinger and Marshall 1990)
I	3T3-L1	ATCC	DMEM	0, 5 or 10 mU/ml of glucose oxidase	16 h	Gene expression
I	Mouse fetal fibroblats	Isolated from wt mice (Alhonen et al., 1998)	Glucose- and sodium pyruvate-free DMEM	10 μM DENSPM	24 h and 48 h	SSAT activity, ATP content
II	HepG2	ATCC	DMEM	10 μM DENSPM	48 h	SSAT activity, western blot

ATCC, Am erican Type Culture Collection; wt, wild-type and DMEM, Dulbecco's modified Eagle's medium.

5 RESULTS

5.1 Effect of activated polyamine catabolism on glucose and energy metabolism in mice (I)

Characteristics of body composition and white adipose tissue. After birth, female and male SSAT mice were slightly lighter than their littermates until they were 8 weeks old while female SSAT mice tended to have a higher body weight at the age of 3- to 4-months. Activated polyamine catabolism severely reduced WAT mass (Table 5) and this was already evident at the age of 4 weeks. Furthermore, the tissue TG content was significantly reduced in SSAT mice. Adipocytes in SSAT mice were smaller than in wild-type mice but otherwise the morphology of the adipocytes was normal showing typical unilocular cells (Fig. 11). Transmission electron microscopic analysis of WAT revealed that SSAT mice had an increased size and number of mitochondria in WAT as compared with wild-type mice (Fig. 11). The expression of the key factors regulating adipocyte differentiation, PPARγ, CEBP/α and SREBP1c were increased in WAT of SSAT mice. Surprisingly, activated polyamine catabolism caused enlargement of internal organs in female SSAT mice while adult males exhibited only an enlarged spleens. Furthermore, lean body mass was significantly higher in female SSAT mice as compared with female wild-type mice (Table 5). In contrast, the weight of skin was increased both in female and male SSAT mice (Table 5).

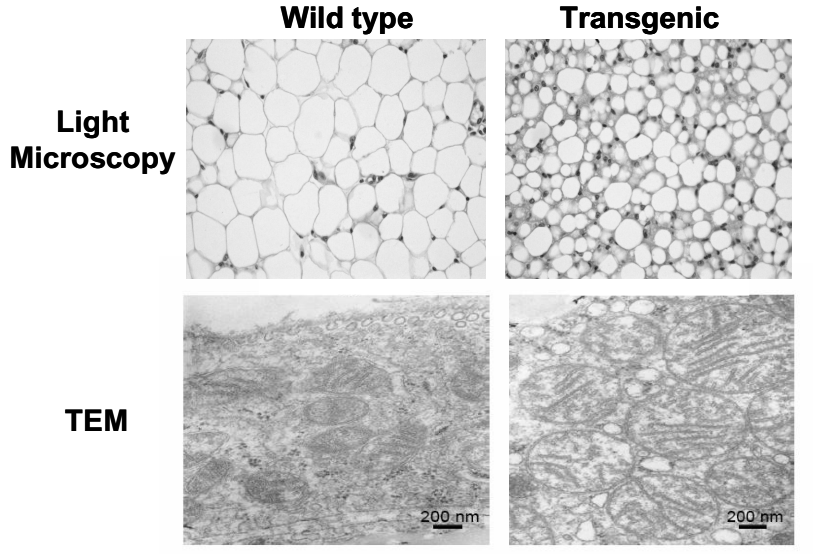

Figure 11. Morphology of WAT in SSAT and wild-type mice. TEM, transmission electron microscopy.

Table 5. Characteristics of female SSAT mice as compared to wild-type mice.

Parameter	Wild type	SSAT
Whole body WAT mass (% of bw)	26.5 ± 0.5	9.4 ± 2.8**
Lean body mass (% of bw)	60.3 ± 0.5	69.8 ± 2.8*
Skin mass (% of bw)	13.2 ± 0.3	20.8 ± 0.6*
Fasting Glc (mM)	10.3 ± 0.5	8.5 ± 0.2***
Fasting Ins (ng/ml)	0.6 ± 0.2	0.3 ± 0.1*
Glucose AUC during GTT	1913 ± 191	1320 ± 95*
Insulin sensitivity index, ITT	0.13 ± 0.02	0.22 ± 0.03*
Fasting plasma TG (mM)	1.4 ± 0.1	1.1 ± 0.1*
Fasting plasma glycerol (mM)	1.16 ± 0.12	0.88 ± 0.05*
Fasting serum 3-hydroxybutyrate (mM)	2.45 ± 0.42	0.58 ± 0.43***
Fasting plasma leptin (ng/ml)	2.9 ± 0.8	0.3 ± 0.1*
Fasting serum FFA (mM)	0.67 ± 0.05	0.72 ± 0.06
$\dot{V}O_2$ (ml/min/kg), young haired	34.2 ± 0.1	40.6 ± 0.1**
$\dot{V}O_2$ (ml/min/kg), adult hairless	22.8 ± 0.4	32.2 ± 0.4***
Food intake (g/day/mouse)	3.03 ± 0.08	4.67 ± 0.24*
RQ, active and fasting state	0.86 ± 0.01	0.82 ± 0.01*
Palmitate oxidation in WAT (pmol/μgDNA), pooled samples	75.7	144.9

WAT, white adipose tissue; bw, body weight; Glc, glucose; Ins, insulin; AUC, area under the curve; GTT, glucose tolerance test, ITT, insulin tolerance test; RQ, respiratory quotient. Results are means ± SEM of 5-19 mice. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

Glucose and lipid metabolism. SSAT mice had significantly reduced fasting glucose and insulin levels (Table 5). Furthermore, SSAT mice displayed improved glucose tolerance and increased insulin sensitivity based on glucose and insulin tolerance tests (Table 5). The protein and mRNA levels of two important factors increasing glucose transport, PGC-1α and AMPK, were upregulated in WAT of SSAT mice. In addition, expressions of the genes involved in glucose phosphorylation and glucose oxidation (HKII and PDH E1 component α-subunit) were significantly increased in WAT of SSAT mice. In the fasting state, TG levels, glycerol, 3-hydroxybutyrate and leptin levels were significantly lower in SSAT mice as compared to wild-type mice whereas FFA levels were unaltered (Table 5).

Energy homeostasis. Activated polyamine catabolism increased oxygen consumption ($\dot{V}O_2$) in young haired and adult hairless SSAT mice (Table 5). Consistent with the increased energy expenditure, food intake was higher in SSAT mice than in wild-type mice (Table 5) whereas locomotor activity was lower in SSAT mice, especially during the night-time. The respiratory quotient (RQ) was significantly lower in the fasting and active state in SSAT mice

as compared with wild-type mice (Table 5) indicating higher fatty acid oxidation in SSAT mice. Indeed, the rate of palmitate oxidation was enhanced in isolated adipocytes of SSAT mice (Table 5) but not in skeletal muscle and liver. Correspondingly, the expressions of the genes involved in β-oxidation of fatty acids (acyl-CoA dehydrogenases) and fatty acid uptake (CD36, fatty acid transport protein 1 and adipocyte-specific fatty acid-binding protein 4) were elevated in WAT of SSAT mice. With respect to the key factors regulating fatty acid oxidation, PGC-1α, ERRα and PPARδ, were unregulated in WAT of SSAT mice. The genes governing fatty acid synthesis (e.g. ACC1 and fatty acid synthase) and lipolysis (HSL) were upregulated in WAT of SSAT mice.

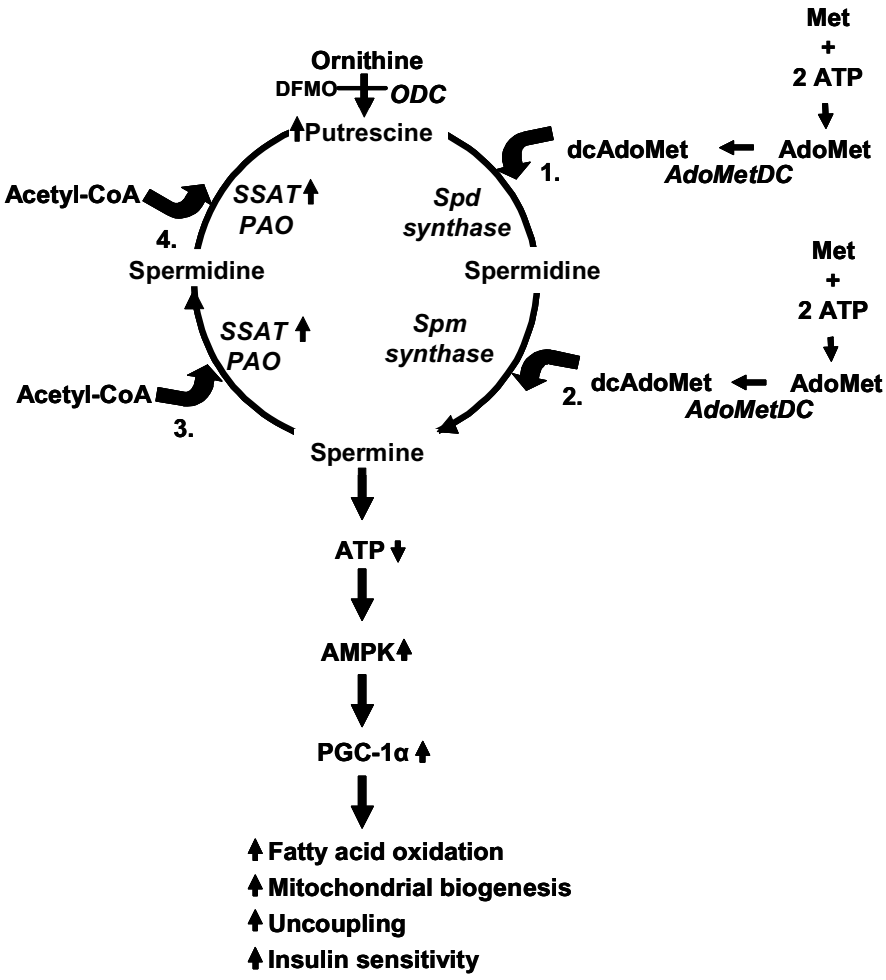

Figure 12. Proposed molecular mechanism causing the phenotype of SSAT mice. Spd, spermidine, Spm, spermine; Met, methionine and AdoMet, S-adenosylmethionine.

The mechanism leading to the induction of peroxisome proliferator activated receptor γ co-activator 1α. Our mechanistic studies demonstrated that the factor connecting activated polyamine catabolism and PGC-1α is AMPK, i.e. both isoform expression and protein amount of this enzyme were significantly elevated in WAT of SSAT mice. Since AMPK is activated by the increase in the AMP/ATP ratio, we hypothesized that the enhanced polyamine catabolism may have evoked a depletion of the ATP pool in WAT of SSAT mice because each polyamine cycle theoretically consumes 4 ATP equivalents (2 ATP and 2 acetyl-CoA) (Fig. 1 and Fig. 12). Indeed, SSAT mice had approximately ~50 % lower ATP concentrations in isolated adipocytes than wild-type mice. In addition, DENSPM treatment reduced significantly ATP concentrations in isolated mouse fetal fibroblasts during nutrient deprivation. Therefore, it is hypothesized that the depletion of ATP levels activates AMPK which in turn induces PGC-1α in WAT of SSAT mice. (Fig. 12)

Since SSAT overexpression accelerates the rate of polyamine cycle and the overall flux of polyamines, we tested whether a reduction in the rate of polyamine flux will increase ATP concentrations and reverse the phenotype of SSAT mice by blocking ODC using DFMO (Fig. 12). Indeed, the lowered rate of polyamine cycle, detected by significantly decreased putrescine concentrations in WAT of SSAT mice, elevated ATP concentrations in the adipocytes with a concomitant increase in perigonadal WAT mass. DFMO treatment also restored the changes in gene expression in WAT of SSAT mice to the levels resembling those observed in wild-type mice.

5.2 Effect of activated polyamine catabolism on cholesterol homeostasis in mice (II)

Cholesterol metabolism. During characterization of the metabolic phenotype of SSAT mice, it was observed that mice with SSAT overexpression exhibited low plasma total and HDL cholesterol levels but increased serum total bile acid concentrations. In contrast, fasting plasma alkaline phosphatase and alanine aminotransferase were similar compared with wild-type values indicating normal liver function. To determine the reasons behind these changes, we analysed variables of cholesterol metabolism and expression of the genes involved in the regulation of cholesterol homeostasis. Plasma cholesterol precursors were significantly elevated in SSAT mice interpreted as evidence of increased cholesterol synthesis. This result was verified by analyzing expression of cholesterol biosynthetic genes. Indeed, HMGCR, squalene synthase and 7-dehydrocholesterol reductase were upregulated in the livers of SSAT mice (Fig. 13). Since the plasma cholesterol absorption markers were lowered and expression

of cholesterol transporters, NPC1L1 and SR-BI, were decreased in jejunum in SSAT mice (Fig. 13), it seemed that cholesterol absorption was reduced in SSAT mice. This concept was verified by measuring cholesterol absorption efficiency which proved to be significantly lower in SSAT mice. Unaltered hepatic expression of LDLR and SR-BI in SSAT mice excluded the possibility that enhanced hepatic clearance of cholesterol led to lowered cholesterol levels in SSAT mice (Fig.13). Interestingly, we observed that hepatic expression of ACAT2 was significantly reduced in the fed state, indicating that VLDL assembly may have been disturbed. Moreover, expression of ABCA1, the key regulator of HDL lipidation, was similar in SSAT mice as compared with wild-type.

Bile acid metabolism. When bile acid metabolism was investigated, it was noticed that SSAT mice had enhanced bile acid synthesis since there were elevated expressions of the rate-limiting genes in the neutral and acidic bile acids synthesis pathways, CYP7A1 (Fig. 13) and CYP27A1. In contrast, hepatic bile acid transporters responsible for bile acid uptake, NTCP and OATP1, were significantly downregulated in the fed state (Fig. 13). Intestinal bile acid absorption was comparable to that of wild-type mice since bile acid absorption efficiency and expression of apical Na^+-dependent bile acid transporter and ileal bile acid binding protein were unchanged (Fig. 13). Since the fecal bile acid content was increased in SSAT mice, it appears very likely that bile acid secretion was increased. However, gene expression studies did not reveal increase in the expression of bile salt export pump. Cholesterol secretion to bile was not presumably increased since mRNA levels of biliary cholesterol exporters, ABCG5 and ABCG8, remained unchanged.

The mechanism leading to increased bile acid synthesis. In order to investigate the mechanism for the activation of main bile acid synthesis pathway, we analyzed hepatic expression of factors activating or repressing CYP7A1 transcription (PGC-1α, HNF-4α, LXRα, FXR, pregnane X receptor, constitutive androstane receptor, SREBP1c, PPARα, histone deacetylase 7 and HNF-6). Gene expression analyses did not reveal any marked alterations in the expression of these factors. Therefore, the next step was to analyze the protein levels of these factors. The results showed that the protein levels of the key activator of CYP7A1, PGC-1α, were significantly elevated in SSAT mice in the fasting and fed state (Fig. 13). As mRNA levels of PGC-1α were unchanged in the livers of SSAT mice, it was concluded that post-translational modification of PGC-1α most likely was responsible for the elevated PGC-1α protein levels. The first candidate considered to cause this effect was AMPK because enhanced polyamine catabolism is known to induce AMPK in WAT of SSAT mice by depleting the ATP pool. Surprisingly, despite a slightly decreased ATP pool in

the liver (unpublished data), phosphorylated AMPK levels were reduced in the fasting state in the livers of SSAT mice while protein levels were unchanged in the fed state.

As more active and/or stable PGC-1α protein is achieved through deacetylation by SIRT1, phosphorylation by p38MAPK, inhibition of phosphorylation by GSK-3β or methylation PRMT1, the protein levels of these modulators were analyzed. PRMT1, SIRT1 and phosphorylated, i.e. active, p38MAPK and GSK-3β were unaltered in the fed state whereas the protein amounts of inhibitor of PGC-1α, phosphorylated and i.e. active Akt, were significantly reduced in the livers of SSAT mice in the fed state (Fig. 13). A reduction in the activity of Akt may lead to decreased PGC-1α protein phosphorylation resulting in a more stable protein as proposed by Rodgers and coworkers (Rodgers et al., 2005). The effect of activated polyamine catabolism on phosphorylation of Akt seems to be Akt protein degradation- and PI3K pathway-independent since protein levels of total Akt and an upstream regulator of Akt, PI3K, were not altered in SSAT mice. Since it is well-established that a SSAT activator, DENSPM, can reduce phosphorylation of Akt in glioblastoma (Jiang et al., 2007) and breast cancer cells (Nair et al., 2007), we investigated whether activated polyamine catabolism could cause this effect also in hepatocytes. Indeed, a reduction in the protein amount of phosphorylated Akt was detected in DENSPM-treated human hepatoma HepG2 cells at 48 h as compared with untreated cells.

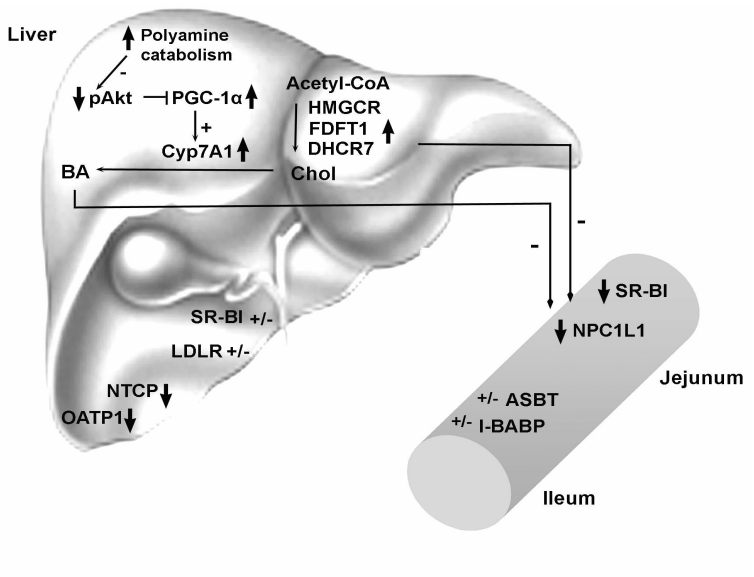

Figure 13. Main findings in cholesterol metabolism in SSAT mice. Chol, cholesterol; FDFT1, squalene synthase; DHCR7, 7-dehydrocholesterol reductase; BA, bile acid; pAkt, phosphorylated Akt; ASBT, Apical Na^+-dependent bile acid transporter and I-BABP, ileal bile acid binding protein.

5.3 Effect of deficiency of polyamine catabolism on the metabolic phenotype in mice (III)

Polyamine homeostasis. In order to investigate whether deficiency of polyamine catabolism has an opposite effect on metabolism as compared with activated polyamine catabolism, we characterized the metabolic phenotype of SSAT-KO mice. However, first the effect of polyamine catabolism deficiency on polyamine homeostasis was investigated. The absence of inducible SSAT activity was initially confirmed by carbon tetrachloride and DENSPM treatments and as anticipated, no inducible SSAT activity was observed in SSAT-KO mice whereas these treatments caused typical activated catabolism-caused changes in tissue polyamine content in wild-type mice. Under basal conditions, SSAT activity was close to the wild-type mice level in SSAT-KO mice and a small amount of N^1-acetylspermidine was also detected. The SSAT deficiency caused only minor changes in the polyamine pool since only a slight increase in spermidine concentrations was manifested during aging in all analyzed tissues, with the exception of the pancreas. Since activated polyamine catabolism has been shown to cause enhanced sensitivity to the toxicity of DENSPM in SSAT mice (Alhonen et al., 1999), it was hypothesized that SSAT deficiency confers resistance against the DENSPM-induced harmful effects. Therefore, SSAT-KO and wild-type mice were subjected to daily injections of DENSPM. Surprisingly, SSAT-deficient mice (median=5 day) died approximately 3 days earlier than wild-type mice (median=8 day). The DENSPM treatment significantly induced SSAT activity and evoked the typical changes of activated polyamine catabolism in the polyamine concentrations in several tissues of wild-type mice. In contrast, DENSPM did not have any effect on the tissue polyamine content in SSAT-KO mice although the extent of DENSPM accumulation was similar in both genotypes. Histological examination of tissues did not reveal any differences between DENSPM-treated SSAT-KO and wild-type mice.

Body composition and energy homeostasis. SSAT deficiency did not affect body weight when the average body weights of SSAT-KO mice were compared to those of wild-type mice. However, a subgroup of animals having a higher body weight was observed. Furthermore, no change in whole body WAT mass was detected in the magnetic resonance imaging or weight of the perigonadal WAT pads. Consistent with these results, plasma leptin levels did not differ from wild-type mice (unpublished data). Adipocyte morphology and organ weights were similar as those encounted in wild-type mice (unpublished data) while histological examination of kidneys of aged SSAT-KO mice revealed dilation of Bowman's space and tubules of inner medulla, atrophy of glomeruli and associated cells, and

inflammation and necrosis of renal papillae. Oxygen consumption and other parameters related to energy expenditure were not analyzed in SSAT-KO mice. However, the food intake of these mice was observed to be rather similar to that of wild-type counterparts.

Glucose and lipid metabolism. SSAT deficiency led to the development of increased fasting blood glucose levels after the age of 12 months. A glucose tolerance test in 16-month-old SSAT-KO mice showed similar glucose disposal from the circulation but significantly elevated insulin levels in SSAT-KO mice (Fig. 14). The presence of peripheral insulin resistance was confirmed using an insulin tolerance test which displayed significantly higher glucose levels in response to insulin at all time points in SSAT-KO mice (Fig. 14). The deficiency of SSAT did not cause alterations in plasma total cholesterol and TG levels in young or aged in SSAT-KO mice. Plasma HDL and LDL cholesterol or other parameters involved lipid metabolism were not analyzed in SSAT-KO mice.

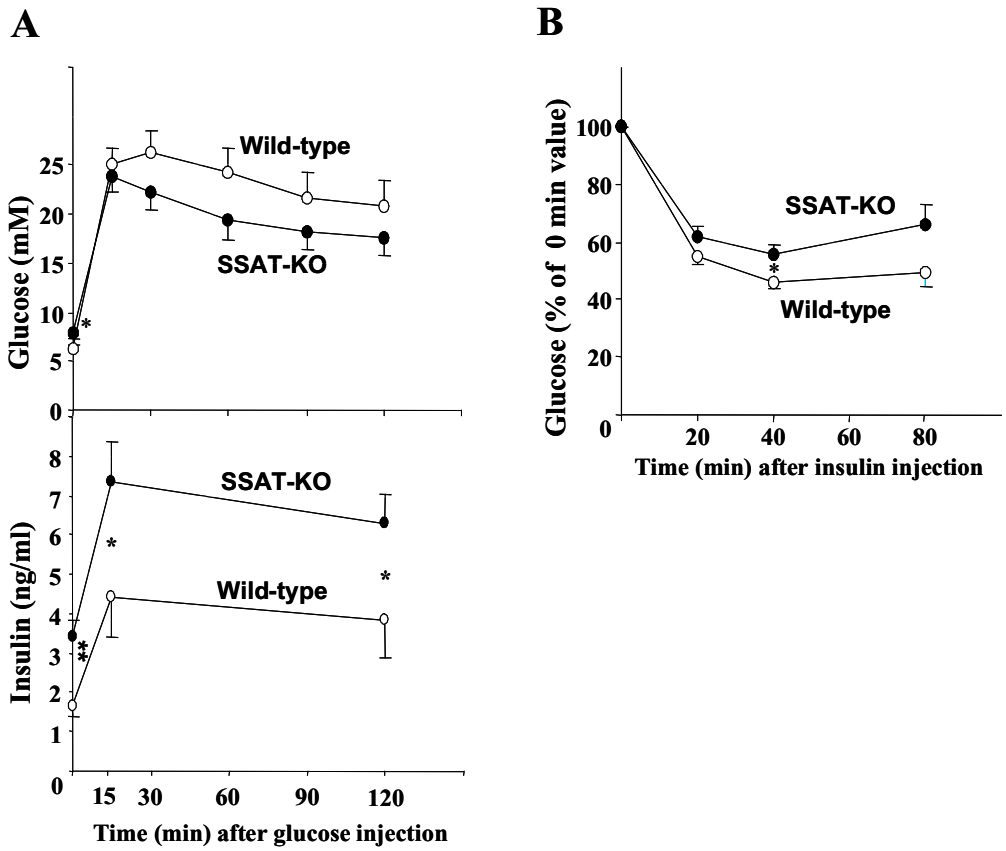

Figure 14. Glucose and insulin tolerance tests in SSAT-KO and wild-type mice. Fasted 16-month-old SSAT-KO and wild-type male mice were subjected to A) intraperitoneal glucose tolerance (2 mg/g D-glucose) and B) insulin tolerance (0.25 mU/g insulin) tests. Results are means ± SEM of 10-12 mice. $p<0.05$ and **, $p<0.01$.

6 DISCUSSION

SSAT is the rate-controlling enzyme in the catabolism of polyamines (Jänne et al., 2004). The overexpression or loss of SSAT is sufficient to cause the corresponding activation or deficiency of polyamine catabolism. We determined the role of polyamines and polyamine metabolism in the regulation of glucose, energy and lipid metabolism.

Activated polyamine catabolism, white adipose tissue and body composition (Study I). A novel finding in our study was that the endogenous SSAT promoter targeted the highest SSAT activity into WAT. This resulted in severely reduced whole body WAT mass in young and adult mice, a phenomenon also demonstrated by Jell and coworkers (Jell et al., 2007). Since the adipocytes of SSAT mice were smaller but their number was unchanged, the reduction in WAT mass seemed to be due to diminished TG accumulation and not due to impaired differentiation of the adipocytes. This concept was supported by the finding that the expressions of key factors regulating adipogenesis, PPARγ, CEBP/α and SREBP1c, were upregulated in WAT of SSAT mice. Furthermore, the levels of spermidine, which has been observed to be necessary for adipogenesis (Bethell and Pegg 1981; Vuohelainen et al., 2009), were not reduced in WAT of SSAT mice. The reduced TG accumulation in the adipocytes of SSAT mice was attributable to the increased expression of PGC-1α, the critical regulator of energy metabolism which is also involved in the differentiation of preadipocytes to brown adipocytes (Puigserver and Spiegelman 2003). In WAT, the expression of PGC-1α is normally low (Puigserver and Spiegelman 2003) but ectopic expression of PGC-1α in adipocytes can induce a conversion of white adipocytes into brown-like adipocytes leading to activation of UCP1, OXPHOS and fatty acid oxidation (Tiraby and Langin 2003). Indeed, the adipocytes of SSAT mice were transformed from fat-storing white adipocytes towards fat-burning brown adipocytes as mitochondria number, palmitate oxidation and expression of genes involved in OXPHOS were elevated in the adipocytes. No induction of UCP1 was observed in WAT of SSAT mice whereas UCP3 was elevated. However, it has been suggested that UCP3 does not participate in the regulation of thermogenesis in the same way as UCP1 but it promotes fatty acid oxidation (MacLellan et al., 2005). In line with our results that the adipocytes of SSAT mice were converted to fat-burning cells, Jell and coworkers have demonstrated that the content of malonyl-CoA levels were significantly decreased in WAT of SSAT mice leading to increased fatty acid oxidation and reduced fatty acid synthesis (Jell et al., 2007) as occurs in mice lacking ACC2 (Oh et al., 2005). In disagreement with the results of Jell and coworkers, it was noticed that expression of fatty acid synthase was

elevated in WAT of SSAT mice but this may be a compensatory increase in response to the reduced activity of fatty acid synthase. Based on gene expression studies, it seemed that FFA uptake was enhanced in the adipocytes of SSAT mice because all of the studied FFA transporters were upregulated. Furthermore, release of FFAs through lipolysis seemed to be diminished in WAT of SSAT mice because circulating glycerol levels were significantly reduced in SSAT mice during fasting and serum FFA levels were unaltered. Moreover, AMPK, the inhibitor of HSL, was upregulated in WAT of SSAT mice. The expression of HSL was enhanced in WAT of SSAT mice but again this may represent compensating for the decreased activity of HSL. Taken together, circulating FFAs seems to be channelled into WAT of SSAT mice for combustion, leading to severely reduced WAT mass.

Although activated polyamine catabolism resulted in reduction in WAT depots, this led to the development of organomegaly and an increase in lean body mass, especially in female SSAT mice. An increase in tissue size is typically caused by accumulation of substances e.g. fat, glycogen and iron, hypertrophy or hyperplasia of the cells or congestion from heart failure. In SSAT mice, the TG contents of internal organs and skeletal muscle were significantly reduced and glycogen accumulation was only evident in the heart (unpublished data) thereby excluding the possibility that the accumulation of these substances could account for the organomegaly in SSAT mice. Histological examination demonstrated that the cell size of internal organs was unaltered and there was no sign of malignant transformation, suggesting that benign hyperplasia contributes to the development of the organomegaly. Since polyamine depletion caused by activated polyamine catabolism has been shown to reduce cell growth (Casero and Pegg 1993; Hughes et al., 2003), other factors than polyamines are enhancing the proliferation of cells in the internal organs of SSAT mice. The underlying mechanisms need to be investigated in future studies.

Activated polyamine catabolism slightly affected the body weights of SSAT mice because they were smaller after birth until the age of 2 months but otherwise body weights remained unchanged or were slightly higher. The reduction in body weights in young SSAT mice is explained by the loss of WAT mass which was observed already after weaning. In adult mice, the development of organomegaly and the heavier skin most likely even up the body weights of SSAT mice. In disagreement with our results, Jell and coworkers reported that body weights of SSAT mice were lowered at the age of 30 weeks (Jell et al., 2007). One reason to account for these inconsistencies may be the fact that Jell and coworkers had their SSAT mice in a C57Bl/6 mouse strain. However, in agreement with our unpublished results,

Jell and coworkers demonstrated that SSAT mice are protected from gaining weight if fed a high-fat diet (Jell et al., 2007).

Activated polyamine catabolism and energy metabolism (Study I). The involvement of polyamines in the regulation of energy metabolism has previously been poorly understood. Our studies showed that activated polyamine catabolism enhances energy expenditure since oxygen consumption was increased in SSAT mice. In line with the enhanced energy expenditure, SSAT mice exhibited increased food intake. This may be at least partially related to low leptin levels which stimulate food intake in hypothalamus. Hairlessness *per se* did not seem to increase energy expenditure in SSAT mice because oxygen consumption was increased before and after hair loss in SSAT mice. Organomegaly may increase energy expenditure in SSAT mice because larger tissues consume more oxygen through metabolism but enhanced energy expenditure was also evident in young SSAT mice when they did not display organomegaly. In addition, an increase in locomotor activity accelerates energy expenditure but this present and previous studies (Kaasinen et al., 2004) have demonstrated that SSAT mice are hypoactive. The reduction in WAT mass may *per se* increase energy expenditure because the loss of a metabolically rather inactive organ elevates oxygen consumption. However, the enhanced rate of WAT mitochondrial oxidative capacity and fatty acid oxidation most likely contributed to enhanced energy expenditure in SSAT mice as has been observed in another mouse model overexpressing PGC-1α in WAT (Tsukiyama-Kohara et al., 2001). However, one unanswered question is whether enhanced fatty acid oxidation only in WAT was sufficient to increase the oxygen consumption and lower the RQ values so markedly in SSAT mice. Ketogenesis, which occurs mainly during high rates of fatty acid oxidation when large amounts of acetyl-CoA are generated, may reduce RQ values but it is likely that ketogenesis was reduced, not elevated, in the livers of SSAT mice as serum 3-hydroxybutyrate levels were decreased in SSAT mice in the fasting state. To clarify the impact of WAT on whole-body energy metabolism in SSAT mice, fatty acid metabolism needs to be investigated in more detail in skeletal muscle and liver. Furthermore, mice having WAT-specific overexpression of SSAT could also provide valuable information.

Activated polyamine catabolism and glucose metabolism (Study I). The impact of polyamines on the control of glucose homeostasis has remained largely unexplored. It was observed that SSAT mice exhibited increased insulin sensitivity as demonstrated by their increased insulin-stimulated peripheral glucose uptake in insulin tolerance test, improved glucose tolerance and lowered fasting insulin levels. These results are consistent with the leaner phenotype and reduced tissue TG content which are known to correlate with higher

insulin sensitivity (Krssak et al., 1999; Heilbronn et al., 2004). Corresponding with the enhanced peripheral glucose uptake, the protein amount of plasma membrane GLUT4 was greater in WAT and skeletal muscle in SSAT mice than in wild-type mice (unpublished data). In WAT, this was most likely related to the overexpression of AMPK and PGC-1α. In addition, the enhanced glucose oxidation in WAT demonstrated by Jell and coworkers (Jell et al., 2007) was apparently attributable to the AMPK-induced increase in glucose utilization since expressions of HKII and PDH E1 component α-subunit were elevated. Given the fact that AMPK and PGC-1α induce muscle glucose transport in response to exercise (McGee and Hargreaves 2006), it would be worthwhile to investigate whether increased plasma membrane GLUT4 content in skeletal muscle was also mediated by these two factors. Consistent with the increased insulin action in SSAT mice, gluconeogenesis was impaired in the livers of SSAT mice since they had significantly reduced fasting glucose levels. Interestingly, SSAT mice had elevated hepatic expression of PGC-1α which stimulates gluconeogenesis through the transcription factor forkhead box O1. The ability of PGC-1α to induce gluconeogenesis is largely regulated through deacetylation by SIRT1. Since hepatic SIRT1 was significantly reduced in the fasting state, acetylation of PGC-1α was most likely increased, leading to a lowered capability to induce gluconeogenesis. However, mechanisms leading to impaired gluconeogenesis and increased insulin sensitivity in WAT, skeletal muscle and liver need to be investigated in more detail. Our observations in SSAT mice indicate that enhanced energy expenditure is associated with increased insulin sensitivity which has also been observed e.g. in resvaratrol–treated mice (Baur et al., 2006; Lagouge et al., 2006). Moreover, our findings support the results of a recent report that the levels of circulating bile acid correlate with insulin sensitivity (Shaham et al., 2008) i.e. SSAT mice had both elevated serum total bile acids and increased insulin sensitivity.

Polyamines have been suggested to stabilize insulin mRNA (Welsh 1990) and to be necessary for proinsulin biosynthesis in pancreatic β-cells (Sjöholm 1993). However, polyamine depletion increases the insulin content and sensitivity to some secretory stimuli, but not to glucose, in DFMO-treated rat insulinoma cells (Sjöholm et al., 1993). In SSAT mice, it would be important to determine the islet polyamine content, and insulin biosynthesis and secretion because the levels of higher polyamines are significantly decreased in pancreas (Pietilä et al., 1997). In our study, circulating insulin levels were significantly reduced in SSAT mice but this was likely to be attributable to increased insulin sensitivity rather than impaired insulin secretion. However, since spermine is important for the function of inward rectifying potassium channels (Williams 1997; Phillips and Nichols 2003), it would be

interesting to determine the effect of activated polyamine catabolism on glucose-stimulated activation of potassium calcium channels in pancreatic β-cells.

Activated polyamine catabolism and cholesterol metabolism (Study II). It was observed that plasma total cholesterol levels were significantly lowered in SSAT mice in the fed and fasting states. Lowering of circulating cholesterol levels in the body can be achieved via reducing cholesterol synthesis, impairing intestinal cholesterol and bile acid absorption or enhancing bile acid synthesis. SSAT mice displayed enhanced bile acid synthesis and reduced cholesterol absorption while cholesterol synthesis was increased and intestinal bile acid absorption was not altered. Based on these findings, it is likely that the primary cause of the reduced cholesterol levels was CYP7A1-mediated enhanced conversion of cholesterol into bile acids. If increased cholesterol synthesis was the primary cause in SSAT mice, this would cause induced bile acid synthesis by elevating the hepatic cholesterol pool which augments CYP7A1 transcription via LXRα (Russell 1999). Given that the mRNA and protein levels of LXRα and hepatic cholesterol pool were unchanged in SSAT mice, increased cholesterol synthesis cannot be the primary cause for the alterations in cholesterol homeostasis in SSAT mice. Impaired cholesterol absorption is not likely the primary mechanism to account for the low cholesterol levels in SSAT mice as reduced cholesterol absorption would lead to the induction of hepatic *de novo* cholesterol synthesis and as discussed above, cholesterol synthesis was not influencing bile acid synthesis. The increased cholesterol synthesis observed in SSAT mice was most probably a mechanism to compensate for the increased bile acid synthesis in order to keep hepatic cholesterol pool size unchanged. Increased hepatic *de novo* cholesterol synthesis subsequently most likely reduced intestinal cholesterol absorption in SSAT mice due to reciprocal regulation of these pathways. Another possibility is that a CYP7A1-mediated expansion of the hepatic bile acid pool reduced cholesterol absorption by decreasing the expression of jejunal NPC1L1, as was previously demonstrated by Ratliff and coworkers (Ratliff et al., 2006). The conclusions presented above are supported by the observations in CYP7A1 overexpressing rodent models which also display lowered total plasma cholesterol values, compensatorily increased cholesterol synthesis and reduced cholesterol absorption (Spady et al., 1995, 1998; Miyake et al., 2001; Ratliff et al., 2006).

The reduction in total cholesterol levels observed in SSAT mice was due to declines in the LDL/VLDL/IDL and HDL fractions. Since hepatic cholesterol clearance was not enhanced in SSAT mice as evident from the results of the gene expression studies, the mechanism responsible for the reduction in both LDL/VLDL/IDL and HDL fractions was

apparently enhanced channelling of newly synthesized cholesterol to bile acid synthesis, leading to decreased formation of lipoproteins. At least, VLDL formation was likely reduced in SSAT mice since the ACAT2 expression was decreased in the fed state most likely impairing the formation of CEs and the assembly of VLDL particles. The second finding in support of the lowered VLDL formation was that fasting plasma TG levels were significantly reduced in SSAT mice. A measurement of the lipoprotein formation rate needs to be performed to verify these concepts.

Since most bile acids are cytotoxic, there are a variety of hepatoprotective mechanisms intended to prevent the accumulation of abnormally high levels of bile acids. Irrespective of the enhanced bile acid formation in the livers of SSAT mice, no liver damage was observed based on plasma alkaline phosphatase and alanine aminotransferase levels. Therefore, SSAT mice have most likely developed mechanisms to minimize hepatic bile acid accumulation. One preventive mechanism was reduced uptake of hepatic bile acids as the expressions of hepatic bile acid transporters NTCP and OATP1 were decreased. Another mechanism was increased biliary bile acid excretion demonstrated by higher bile acid content in feces. However, the latter mechanism together with unaltered intestinal bile acid absorption probably caused prolonged exposure of the intestine to elevated bile acid concentrations in SSAT mice. This provides a novel mechanism to explain why SSAT overexpression could enhance tumorigenesis in mice susceptible to intestinal cancer, $APC^{MIN/+}$ mice (Debruyne et al., 2001; Tucker et al., 2005).

The enhanced CYP7A1-mediated bile acid synthesis in SSAT mice was attributable to increased expression of PGC-1α in the livers of SSAT mice. PGC-1α has been demonstrated to be a critical activator of CYP7A1 in mice and humans (Shin et al., 2003). Nonetheless, the consequence of stable hepatic overexpression of PGC-1α on cholesterol homeostasis has not yet been demonstrated *in vivo*. However, transient adenoviral hepatic overexpression of PGC-1α has been shown to elevate total cholesterol levels by increasing VLDL production (Lin et al., 2005). Thus, this present study is the first to demonstrate that the activation of CYP7A1 through PGC-1α is an efficient way to reduce plasma total cholesterol levels *in vivo*.

Molecular mechanisms leading to the activation of peroxisome proliferator activated receptor γ co-activator 1α in white adipose tissue and liver (Study I-II). The metabolic phenotype of SSAT mice was attributable to the increased expression of PGC-1α both in WAT and liver. When we investigated the activator of PGC-1α in WAT, it was confirmed that the known and presumed consequences of activated polyamine catabolism such as hairlessness, putrescine accumulation, oxidative stress and elevated cytokines do not

have any influence on PGC-1α expression in SSAT mice. Our mRNA and protein analyses of WAT samples demonstrated that the effect of activated polyamine catabolism on PGC-1α was most likely mediated by AMPK (Fig. 15) which induces PGC-1α in response to exercise in skeletal muscle. This interaction of AMPK and PGC-1α in WAT has now been observed also in recent studies (Crowe et al., 2008; Sutherland et al., 2008; Gaidhu et al., 2009). In the liver, instead of AMPK, Akt was most likely the key modulator of PGC-1α protein stability and activity (Fig. 15).

It is postulated that SSAT overexpression accelerates the overall flux of polyamines in the polyamine cycle and the continuous supply of putrescine and dcAdoMet produced by ODC and AdoMetDC keeps the cycle running. The consequences of accelerated polyamine flux are ATP and acetyl-CoA depletion. Low ATP levels then induce the cellular energy sensor AMPK (Fig. 15) which inhibits the formation of malonyl-CoA. Furthermore, enhanced consumption of acetyl-CoA reduces the availability of acetyl-CoA for malonyl-CoA synthesis. This polyamine flux theory was tested in SSAT and wild-type mice by blocking the putrescine formation through ODC by administering DFMO. The DFMO treatment clearly reduced the rate of polyamine flux which led to a subsequent increase in ATP concentrations in the adipocytes of SSAT mice and to the reversal of the metabolic phenotype of SSAT mice. This confirmed our hypothesis that the polyamine cycle can be considered as a futile cycle and the shortage of ATP in WAT was the key contributing factor leading to the metabolic phenotype of SSAT mice.

Interestingly, accelerated polyamine catabolism did not activate AMPK in the liver although a slight reduction in ATP concentrations was observed (unpublished data). In contrast, polyamine catabolism caused a reduction in the phosphorylation of Akt in the livers of SSAT mice and also in human hepatoma HepG2 cells which has been previously noticed in glioblastoma (Jiang et al., 2007) and breast cancer cells (Nair et al., 2007). The interaction of activated polyamine catabolism with Akt in the liver cells is poorly understood but activated polyamine catabolism did not have any effect on the protein levels of total Akt and the upstream regulator of Akt, PI3K in SSAT mice. Further studies will be needed to investigate the mechanism leading to the reduction of Akt phosphorylation. Therefore, the mechanism causing the activation of PGC-1α in the liver seems to be different from that observed in WAT of SSAT mice. It is tempting to speculate that because SSAT activity is lower in the livers (4-fold) of SSAT mice as compared with that in WAT (22-fold), the depletion of ATP pool, as a consequence of activated polyamine catabolism, does not reach the threshold needed to activate AMPK in the livers of SSAT mice. Another possible

explanation is that the mechanism to account for why activated polyamine catabolism can induce PGC-1α is tissue-specific. This is not unexpected because the function of AMPK differs in liver and in WAT. AMPK induces fatty acid oxidation and inhibits the production of cholesterol, TG and glucose in the liver whereas in WAT, the main purpose of AMPK is to stimulate basal glucose transport and to inhibit lipolysis (Winder and Hardie 1999). Therefore, the induction of AMPK by activated polyamine catabolism in the liver could disrupt normal homeostasis. Instead, Akt is involved in controlling vital cellular functions such as apoptosis, cell cycle progression and glucose metabolism in several tissues (Franke 2008). Whether Akt is also involved in the signal transduction between activated polyamine catabolism and PGC-1α in WAT will require further investigation.

Deficiency of polyamine catabolism and glucose, energy and lipid metabolism (Study III). To study the effect of SSAT deficiency on glucose, energy and lipid metabolism, the metabolic phenotype of SSAT-KO mice was investigated. Under basal conditions, SSAT-KO mice had SSAT activity comparable to that of wild-type mice and exhibited traces of N^1-acetylspermidine which most likely reflects the presence of other acetylases than SSAT. The deficiency of polyamine catabolism did not have as dramatic effects on tissue polyamine homeostasis in mice as the activation of polyamine catabolism. In SSAT-KO mice, only slightly increased spermidine pools appeared in several tissues including WAT similar to those observed *in vitro* in SSAT-deficient embryonic stem cells (Niiranen et al., 2002). SSAT has been proven to be the key enzyme in the backconversion of spermidine to putrescine (Niiranen et al., 2002) whereas the catabolism of spermine is mainly achieved by the action of SMO (Vujcic et al., 2002). Thus, this slight increase in spermidine levels in SSAT-KO mice was best explained by the lowered rate of polyamine cycle due to the absence of SSAT with the concomitant compensatory increase in the conversion of spermine to spermidine by SMO.

Since SSAT-KO deficient embryonic stem cells are more resistant to the effect of DENSPM (Niiranen et al., 2002), it was a surprise that SSAT-KO mice were more sensitive to DENSPM-induced toxicity. The cytotoxic effect of DENSPM has been considered to be related to its ability to induce SSAT, depleting spermidine and spermine pools, increasing cellular oxidative stress through the generation of H_2O_2 and inhibiting mammalian target of rapamycin-mediated protein synthesis (Snyder et al., 1994; Casero et al., 2005; Jiang et al., 2007). As expected, DENSPM treatment had only a negligible effect on tissue polyamine content in SSAT-KO mice and the tissue accumulation of this SSAT inducer did not differ between the mouse lines. Therefore, the mechanism leading to cytotoxicity of DENSPM in

SSAT-KO mice is not attributable to the size of tissue polyamine pool. One possible explanation is that as SSAT is not only the target of DENSPM, the binding of DENSPM to SSAT may protect from harmful effects caused by other targets (Pegg 2008).

SSAT-KO mice did not have increased WAT mass when compared to their body-weight matched wild-type mice. However, Jell and coworkers observed that a ~20% portion of mice showed higher WAT mass than wild-type mice even if body weights were not elevated in SSAT-KO mice (Jell et al., 2007). Furthermore, they demonstrated that a SSAT deficiency led to increased susceptibility to high-fat diet-induced obesity which is in disagreement with our unpublished data. The reason for these differences between the studies is unknown but Jell and coworkers did not use littermates as controls and they may have had their mice in a different C57Bl/6 mouse strain which could account for these discrepancies between results. Taking into account the fact that WAT mass was not changed and there was no significant difference in food consumption between SSAT-KO and wild-type mice, the conclusion is that most likely energy expenditure is not altered in SSAT-KO mice. Indeed, SSAT-KO mice have been reported to have unaltered energy expenditure (Jell et al., 2007). Our study showed that glucose homeostasis was altered in aged SSAT-KO mice because they had increased fasting glucose levels. Insulin resistance with a concomitant reduction in peripheral glucose uptake was also evident as seen from the results of the insulin tolerance test (Fig. 15). Moreover, Jell and coworkers demonstrated that glucose oxidation was impaired in WAT of SSAT-KO mice (Jell et al., 2007). However, glucose tolerance was not impaired in SSAT-KO mice because insulin resistance was compensated by higher insulin secretion from the pancreas. Interestingly, histological examination of aged SSAT-KO mice revealed renal alterations resembling the characteristics of diabetic nephropathy (Fig. 15).

SSAT-KO mice were insulin resistant and therefore, one could expect that these mice show hypertriglyceridemia and hypercholesterolaemia as insulin resistance causes e.g. enhanced production of TG-rich VLDL and cholesterol-rich LDL particles (Taskinen 2003). In contrast to our expectations, no changes in plasma TG levels and total cholesterol were observed in SSAT-KO mice. As SSAT-KO mice were hyperinsulinemic, this may compensate for the hepatic insulin resistance and thus maintain lipoprotein production within the normal range.

Jell and coworkers have suggested that the obese phenotype of SSAT-KO mice is attributable to increased acetyl- and malonyl-CoA levels which enhance fatty acid synthesis and downregulate fatty acid oxidation (Jell et al., 2007). In addition, the deficiency of SSAT may decrease ATP consumption causing the subsequent reduction in the AMP/ATP ratio that

will reduce the activity of AMPK and possibly also PGC-1α. The mechanism responsible for insulin resistance will have to be investigated in SSAT-KO mice in future studies.

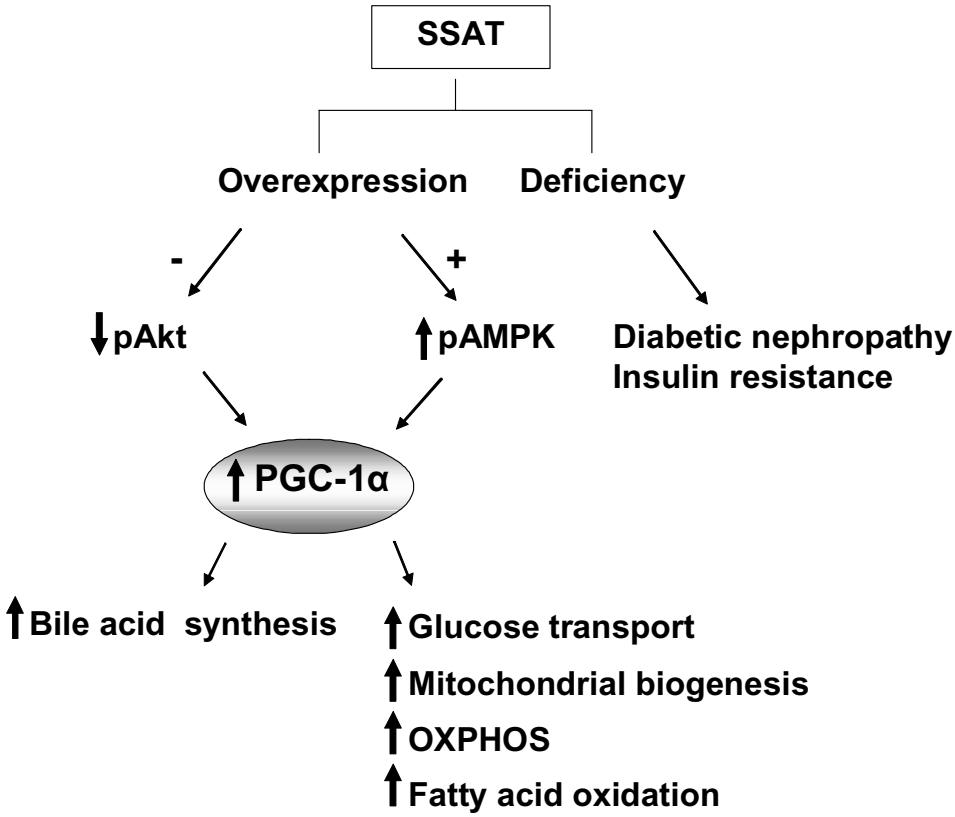

Figure 15. The main findings in SSAT and SSAT-KO mice.

7 CONCLUDING REMARKS

Our results summarized in Figure 16 provide new evidence that activated polyamine catabolism, but not the polyamines themselves, can enhance energy expenditure, increase insulin sensitivity, reduce circulating total cholesterol levels and cause organomegaly. Furthermore, we observed that the deficiency of polyamine catabolism results in insulin resistance.

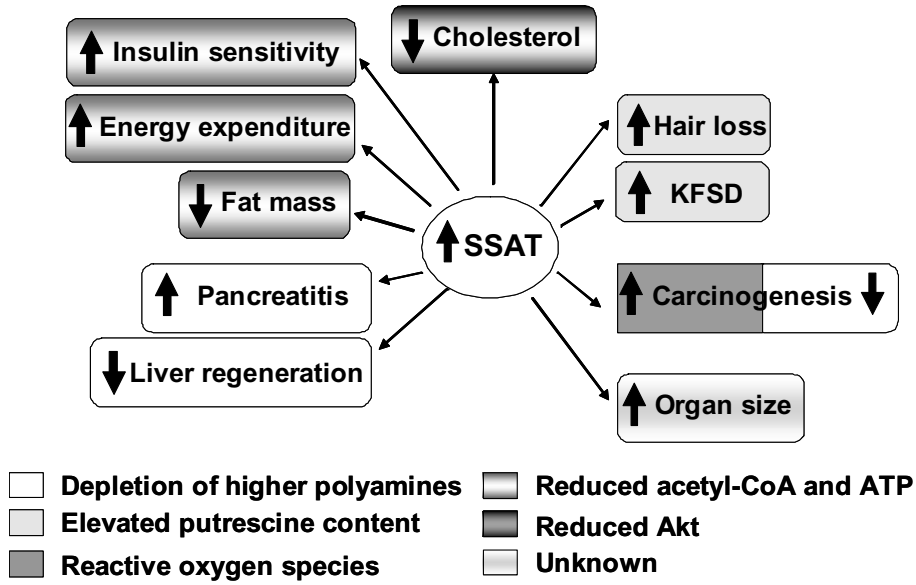

Figure 16. Physiological consequences of SSAT overexpression. KFSD, keratosis follicularis spinulosa decalvans. The shading of the boxes presents the most likely cause for the physiological change.

Our studies revealed for the very first time that the polyamine cycle can be considered as a futile cycle and the acceleration of the rate of polyamine cycle due to SSAT overexpression causes depletion of the cellular ATP pool. The high AMP/ATP-ratio results then in the activation of AMPK with the concomitant induction of PGC-1α which improves glucose and energy metabolism. Moreover, the activation of AMPK suppresses ACC, the enzyme converting acetyl-CoA to malonyl-CoA explaining why the formation of malonyl-CoA from acetyl-CoA is impaired in WAT of SSAT, the previously suggested reason for the lean phenotype of SSAT mice (Jell et al., 2007). Therefore, our findings deepened the understanding of the mechanisms underlying the altered body WAT mass in SSAT mice.

Our results provide evidence that the ideal concept for drug development for obesity and type 2 diabetes is the enhancement of ATP consumption and basal metabolic rate since these are associated with weight loss and beneficial changes in glucose homeostasis. Currently, promising anti-hyperglycemic agents are SIRT1 activators which enhance energy expenditure, increase insulin sensitivity and protect against high-fat diet-induced obesity (Lagouge et al., 2006; Milne et al., 2007).

Another novel finding in our studies was that polyamine catabolism is a potential new target to augment CYP7A1 expression and bile acid synthesis in the liver. In other words, activated polyamine catabolism increased the stability and activity of PGC-1α, the critical activator of CYP7A1, by reducing activity of Akt. Thus, our studies provide support for previous observations that the activation of CYP7A1 and bile acid synthesis is an effective way of lowering circulating total cholesterol levels at least in mice.

Our findings open several possibilities for future studies. For example, it would be important to elucidate the cause for organomegaly and especially for cardiac enlargement in SSAT mice. Second, the molecular mechanisms by which activated and deficiency of polyamine catabolism affect insulin sensitivity and gluconeogenesis on chow and high-fat diet need to be clarified. Furthermore, the detailed mechanisms to explain why activated polyamine catabolism causes low cholesterol levels and the phosphorylation of Akt should also be investigated.

In future studies, to overcome the undesired effect of SSAT overexpression in nontarget tissues, mice having WAT- and liver-specific overexpression of SSAT would be feasible study models. Although the regulation of energy and glucose metabolism is similar in mice and humans, and CYP7A1 is also induced by PGC-1α in humans, it should be investigated whether these results can be duplicated in humans. If the future studies support the role of polyamine catabolism as a potential drug target, the development of WAT- and liver-specific SSAT activators would be of interest.

8 SUMMARY

The main aim of this study was to investigate the effects of activated and inactivated polyamine catabolism on glucose, energy and lipid metabolism in mice. The following results were obtained:

I SSAT mice had reduced fat mass, low tissue TG content, increased mitochondria number, enhanced basal metabolic rate, high insulin sensitivity and OXPHOS accompanied by increased levels of AMPK and PGC-1α. Activated polyamine catabolism caused depletion of the ATP pool in WAT of SSAT mice resulting in the induction of AMPK which in turn activated PGC-1α.

II Activated polyamine catabolism reduced significantly plasma total cholesterol levels in mice by enhancing CYP7A1-mediated conversion of cholesterol to bile acids. The activation of CYP7A1 was attributable to an increased stability and activity of PGC-1α which was most likely elicited by activated polyamine catabolism–induced reduction in the activity of Akt.

III SSAT-KO mice maintained normal polyamine homeostasis but showed abnormal glucose homeostasis during aging. Increased fasting glucose levels and insulin resistance were observed in aged SSAT-KO mice.

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