The polyamines are ubiquitous short-chain aliphatic amines essential for the growth and the proper function of normal cells. The multiple roles of polyamines at the molecular level are not completely clarified but due to their cationic nature the polyamines are able to bind to various negatively charged structures and macromolecules such as DNA, RNA, proteins and phospholipids [1]. Rather constant intracellular levels of polyamines are controlled by biosynthetic and catabolic enzymes as well as by transport in response to changes of cel-

Mice with targeted disruption of spermidine/spermine N\(^1\)-acetyltransferase gene maintain nearly normal tissue polyamine homeostasis but show signs of insulin resistance upon aging

Kirsi Niiranen a, Tuomo A. Keinänen a, Eija Pirinen a,b, Sami Heikkinen a,b, Maija Tusa a,b, Szabolcs Fatrai a,b, Suvikki Suppola a, Marko Pietilä a, Anne Uimari a, Markku Laakso b, Leena Alhonen a, Juhani Jänne * a

a A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland
b Department of Medicine, University of Kuopio, Kuopio, Finland

Received: July 20, 2006; Accepted: September 4, 2006

Abstract

The N\(^1\)-acetylation of spermidine or spermine by spermidine/spermine N\(^1\)-acetyltransferase (SSAT) is the ratecontrolling enzymatic step in the polyamine catabolism. We have now generated SSAT knockout (SSAT-KO) mice, which confirmed our earlier results with SSATdeficient embryonic stem (ES) cells showing only slightly affected polyamine homeostasis, mainly manifested as an elevated molar ratio of spermidine to spermine in most tissues indicating the indispensability of SSAT for the spermidine backconversion. Contrary to SSAT deficient ES cells, polyamine pools in SSAT-KO mice remained almost unchanged in response to N\(^1\),N\(^1\)-diethylnorspermine (DENSPM) treatment compared to a significant reduction of the polyamine pools in the wild-type animals and ES cells. Furthermore, SSATKO mice were more sensitive to the toxicity exerted by DENSPM in comparison with wild-type mice. The latter finding indicates that inducible SSAT plays an essential role in vivo in DENSPM treatmentevoked polyamine depletion, but a controversial role in toxicity of DENSPM. Surprisingly, liver polyamine pools were depleted similarly in wild-type and SSAT-KO mice in response to carbon tetrachloride treatment. Further characterization of SSAT knockout mice revealed insulin resistance at old age which supported the role of polyamine catabolism in glucose metabolism detected earlier with our SSAT overexpressing mice displaying enhanced basal metabolic rate, high insulin sensitivity and improved glucose tolerance. Therefore SSAT knockout mice might serve as a novel mouse model for type 2 diabetes.

Keywords: polyamine • knockout • type 2 diabetes

Introduction

The polyamines are ubiquitous short-chain aliphatic amines essential for the growth and the proper function of normal cells. The multiple roles of polyamines at the molecular level are not completely clarified but due to their cationic nature the polyamines are able to bind to various negatively charged structures and macromolecules such as DNA, RNA, proteins and phospholipids [1]. Rather constant intracellular levels of polyamines are controlled by biosynthetic and catabolic enzymes as well as by transport in response to changes of cel-
lular conditions or extracellular environment [2]. An overaccumulation of the polyamines induces apoptosis and cell transformation and their depletion inhibits cell proliferation and migration [3]. The rate-controlling enzymes in polyamine biosynthesis are ornithine decarboxylase (ODC, EC 4.1.1.17) and S-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50) [4], whereas the catabolic enzymes spermidine/spermine N₁-acetyltransferase (SSAT, EC 2.3.1.57) [5] and polyamine oxidase (PAO, EC 1.5.3.11) [6] promote the interconversion of higher polyamines. SSAT catalyses the acetylation of spermine and spermidine in the N₁-position and these acetylated forms can be back-converted to spermidine and putrescine, respectively, by the successive activity of PAO. Recently, an alternative route for spermine degradation has been discovered. The cytosolic spermine oxidase (SMO/PAOh1, EC 1.5.3.3) selectively oxidizes spermine without a prior acetylation producing spermidine, H₂O₂ and 3-aminopropanal [7–10]. Accordingly, SSAT is not required for the backconversion of spermine as was also indicated by our results with SSAT deficient mouse embryonic stem (ES) cells [11]. Abnormally controlled polyamine metabolism is involved in several pathological processes. Initially, cancer chemotherapy was exclusively focused on the inhibition of polyamine biosynthesis and depletion of intracellular polyamines. Later on, the possibilities to modulate the catabolism for a therapeutic use have been explored [5]. A number of polyamine analogues have been identified to downregulate polyamine synthesis and massively induce SSAT, which is normally present in small amounts. The induction of SSAT leads to the depletion of the higher polyamines and accumulation of analogues, which do not support the cell growth. N₁, N₁₁-diethyl-norspermine (DENSPM), one of the most potent inducers of the SSAT, is undergoing clinical trials as an experimental anticancer agent [12]. DENSPM is able to elicit a profound apoptotic response in certain cell types [13, 14] and it effectively inhibits the growth of several tumors in animal models [15]. A role for polyamine catabolism in energy metabolism has been suggested by our studies with transgenic mice overexpressing SSAT [16]. These mice showed metabolic phenotype opposite to that observed in patients with type 2 diabetes (T2D). One of the characteristic features of T2D is insulin resistance *i.e.* impaired glucose transport into muscle and fat cells and impaired insulin secretion [17]. In SSAT transgenic mice, the amount of white adipose tissue (WAT) was greatly reduced, glucose tolerance and insulin sensitivity were improved and basal metabolic rate and mitochondrial biogenesis were enhanced [16]. The results obtained with SSAT-deficient embryonic stem cells are now largely confirmed with SSAT genedisrupted mouse line. The SSAT knockout (SSAT-KO) mice are viable, fertile and phenotypically indistinguishable from the syngenic mice. They were able to maintain their polyamine homeostasis to large extent. Contrary to SSAT-KO ES cells, SSAT-KO mice were more sensitive to the toxicity exerted by DENSPM but resistant towards DENSPM treatment associated polyamine pool depletion. However, both the syngenic and SSAT-KO mice responded to carbon tetrachloride treatment by a depletion of liver polyamine pools. There was no difference in lifespan between SSAT-deficient and wildtype (WT) mice but upon aging the SSAT-KO showed signs of insulin resistance. The latter findings together with the results obtained with SSAT overexpressing mice imply that polyamine catabolism may have a novel role in energy and glucose metabolism.

**Material and methods**

**Generation of SSAT knockout mice**

Mouse spermidine/spermine N₁-acetyltransferase gene was isolated from 129/SvJ mouse genomic library (Stratagene Inc., La Jolla, CA) and targeted to mouse embryonic stem cell line RW4 as described earlier [11]. To generate chimeric mice, C57BL/6J blastocysts injected with SSAT-KO ES cell clones were implanted into pseudopregnant mice. Altogether three chimeric females and one chimeric male were born. The chimeric male passed the mutant allele to his female offspring and after mating (SSAT-/SSAT+) female mice with wildtype males, male mice carrying SSAT null allele (SSAT-/Y) were born. The SSAT genedisrupted offspring were identified by tail biopsies using same PCR oligonucleotides and Southern blot probes as described previously [11]. The male SSATdeficient mouse was backcrossed in C57BL/6J mouse strain to dilute the 129/SvJ genetic background originated from RW-4 embryonic stem cells. Age and sex-
matched C57BL/6J mice, or wildtype littermates obtained from matings, were used as controls. All animals were kept at the National Laboratory Animal Center of the University of Kuopio on a 12h day/night cycle and were fed normal rodent chow (R3, Lactamin AB, Stockholm). All experimental protocols were approved by the by the Animal Care and Use Committee of the University of Kuopio, Finland.

**Determination of polyamines and SSAT activity**

For the determination of polyamine concentrations and SSAT activity, the tissues were homogenized in lysis buffer (25 mM Tris·HCl pH 7.4, 1 mM dithiothreitol and 0.1 mM EDTA). Polyamines and their acetylated derivates were measured from tissue homogenates with the aid of high-performance liquid chromatography [18]. The concentration of DENSPM was also determined by HPLC according to Porter et al. [19]. SSAT activity was measured from centrifuged homogenates [15].

**Histology**

Tissues were fixed in 10% buffered formalin overnight at room temperature, processed, and embedded in paraffin using standard procedures. Fourmicrometer thick sections were prepared and stained with haematoxylin and eosine.

**Metabolic assays, glucose and insulin tolerance tests**

Blood metabolites were determined from 10-week-old and 52-week-old SSAT-KO and WT male mice after 16 h fasting. Plasma glucose was measured microfluorometrically as described [20]. Plasma triglyceride and cholesterol levels were assayed using Microlab 200 analyzer (Merck, Germany) and triglycerides kit or cholesterol kit (Diasys diagnostic Systems GmbH, Germany). For the glucose tolerance test (GTT), mice were fasted for 16 h and blood samples were collected from a tail vein (fasting/ 0 min sample in GTT). Then 2 mg/g D(+)- glucose (BDH, England) was intraperitoneally injected, followed by blood sampling at 15, 30, 60, 90, and 120 min after the injection. For insulin tolerance test (ITT), 0.25 mU/g insulin (Actrapid, Novo Nordic, Denmark) was administered after 12-h fasting and blood samples were collected at time points 0, 20, 40, and 80 min. Plasma glucose levels were measured as described above and plasma insulin levels were determined using a rat insulin ELISA kit (Crystal Chem Inc, USA) with mouse insulin as standards. The tolerance tests were performed without anesthesia and the animals were 16-month-old male mice.

**Chemicals**

The N¹, N¹¹-diethylornorspermine was essentially synthesized as described [21]. The drug was dissolved in saline and administered intraperitoneally.

**Statistical analysis**

For statistical analyses the two-tailed Student's t test was used when applicable. Insulin levels were logarithmically transformed for statistical analyses. Two-way ANOVA was used for multiple comparisons.

**Results**

**Targeting of embryonic stem cells and generation of SSATdeficient mice**

A targeting vector, in which the neo gene cassette was inserted into the exon 1 of the SSAT gene, was used to inactivate the spermidine/spermine N¹-acetyltransferase gene in mice. The targeting vector was electroporated into RW-4 embryonic stem cells and three of the seven clones appeared to be correctly targeted and contained no additional integration sites based on PCR and Southern blot analyses [11]. ES cells from clone 5 were used in blastocyst injections and a total of four chimeric mice were born, three females and one male. The chimeric male gave germ line transmission to his female offspring and after mating (SSAT/-SSAT+) female mice with wild-type males, the male mice carrying SSAT null allele (SSAT/-Y) were born alive at an expected frequency of 25%. The offspring were screened for the presence of the disrupted SSAT gene by PCR and Southern blot analyses (data not shown). The male SSATdeficient mouse was backcrossed in C57BL/6J mouse strain. Null mice appeared to be viable, healthy, fertile and phenotypically indistinguishable from the wild-type and heterozygous (female) littermates.
Effect of CCl₄ treatment to liver polyamine pools

Inducible SSAT was initially characterized from CCl₄ treated rats, in which liver spermidine and spermine pools depleted while putrescine accumulated and N¹-acetyl spermidine was detected in response to the treatment [22, 23]. Therefore, polyamine homeostasis was studied in mice treated with CCl₄ (0.1 ml/kg i.p. 1 % w/v in corn oil) 24 h prior to polyamine pool analysis. SSAT activity was increased in livers of WT mice whereas in SSAT-KO mice the activity remained at low basal level. As a result of SSAT induction, WT mice showed markedly elevated putrescine and acetylated spermidine pools. Surprisingly, the hepatic spermidine and spermine pools decreased similarly in both SSAT-deficient and wildtype mice (Table 1).

Effect of multiple doses of DENSPM to the tissue polyamine pools and survival of wild-type and SSAT-KO mice

DENSPM is one of the most efficient inducers of SSAT. It is known to deplete higher polyamine pools by inducing SSAT and by down regulating the both key enzymes of polyamine biosynthesis, ODC and AdoMetDC [12, 24]. Therefore, SSAT-KO and WT mice were subjected to a 3-day DENSPM treatment (125 mg/kg i.p. daily) to verify the absence of inducible SSAT enzyme activity in knockout animals as well as to study the effects of the analogue on the tissue polyamine pools. Significantly increased SSAT activity was detected in all tissues of drug treated wild-type mice while the enzyme activity remained at basal level in SSAT-deficient animals. The treatment significantly reduced tissue spermidine and spermine pools and enhanced putrescine accumulation in wildtype mice while no such changes were seen in knockout animals (Table 2). The drug accumulated similarly in both the WT and SSAT-KO mice. However, amongst the studied tissues pancreas was most resistant to DENSPM treatment, due to low accumulation of the drug into that organ. Activated polyamine catabolism has been shown to enhance the sensitivity of SSAT transgenic mice to the toxicity exerted by DENSPM as the transgenic animals died about three days earlier than their syngenic littersmates [25]. Thus, the hypothesis was that animals lacking the inducible SSAT enzyme would be more resistant to DENSPM-induced toxicity. Based on the previous studies with the SSAT overexpressing mice, a DENSPM-survival experiment was designed to last ten days with daily injections of 125 mg/kg i.p. of the polyamine analogue [25]. Surprisingly, on the fourth day of the experiment (after three injections of DENSPM) two of the thirteen SSAT-KO animals had died while all ten wild-type mice tolerated the drug without overt signs of toxicity. All the knockout mice had died by day 8 (median = 5). In WT group, the first animal died on the fifth day and on the last day of the experiment.

### Table 1

| Animal          | SSAT activity | Polyamine pools |
|-----------------|---------------|-----------------|
| PUT             |               | SPD             |
| N¹-Ac-SPD       |               | SPM             |
| Untreated WT    | 3.7 ± 1.2     | 13 ± 7          | 0 ± 0          | 897 ± 70 | 855 ± 24 |
| SSAT-KO         | 3.6 ± 0.5     | 16 ± 8          | 8 ± 8          | 955 ± 14 | 914 ± 20 |
| CCl₄-treated WT | 7.7 ± 1.2     | 164 ± 93        | 84 ± 12 **     | 588 ± 28* | 521 ± 17 *** |
| SSAT-KO         | 2.6 ± 1.0     | 34 ± 9          | 14 ± 7          | 601 ± 24 *** | 561 ± 28 *** |

Data are means ± S.E.M. where n = 3. Animals were 16 week old male mice. SSAT activity is expressed as pmol/10 min per mg of tissue and polyamine pools as pmol/mg of tissue. *p<0.05, **p<0.01 and ***p<0.001 refer to the statistical significance of difference between nontreated and treated animals.
Table 2 The effect of DENSPM on polyamine pools in SSAT knockout (SSAT-KO) and wildtype (WT) mice. Mice received 125 mg/kg of DENSPM on three consecutive days and were sacrificed on the fourth day.

| Tissue | SSAT activity | Polyamine pools |
|--------|---------------|-----------------|
|        | PUT N1-Ac-SPD N8-Ac-SPD SPD SPM TOTAL DENSPM | |
| Liver  |               |                 |
| WT     | 0.5 ± 0.4     | 41 ± 50 N.D.    | 22 ± 32 806 ± 153 681 ± 148 1550 |
| WT + DENSPM | 6.2 ± 5.2* | 144 ± 108 10 ± 16 43 ± 27 472 ± 151** 573 ± 100 1250 485 ± 106 |
| SSAT-KO| 0.5 ± 0.3     | 38 ± 32 12 ± 20 17 ± 26 918 ± 125 753 ± 132 1737 |
| SSAT-KO + DENSPM | 0.5 ± 0.4 | 25 ± 29 N.D. 10 ± 24 932 ± 216 645 ± 66 1611 306 ± 88* |
| Kidney |               |                 |
| WT     | 0.3 ± 0.1     | 73 ± 34 N.D.    | 14 ± 18 301 ± 28 491 ± 78 880 |
| WT + DENSPM | 17.1 ± 15.1* | 152 ± 49** 3 ± 7 13 ± 14 220 ± 28*** 319 ± 100** 708 903 ± 123 |
| SSAT-KO| 0.4 ± 0.6     | 79 ± 16 8 ± 18 12 ± 11 418 ± 50 610 ± 180 1126 |
| SSAT-KO + DENSPM | N.D. | 54 ± 34 7 ± 18 14 ± 13 409 ± 47 413 ± 127 898 719 ± 157* |
| Pancreas |               |                 |
| WT     | 1.7 ± 0.7     | 17 ± 24 N.D.    | N.D. 4007 ± 499 819 ± 35 4843 |
| WT + DENSPM | 4.6 ± 3.1* | 20 ± 41 N.D. 26 ± 30 4195 ± 442 662 ± 56** 4904 81 ± 8 |
| SSAT-KO| 1.4 ± 0.6     | 28 ± 61 4 ± 11 N.D. 4621 ± 917 928 ± 133 5581 |
| SSAT-KO + DENSPM | 1.4 ± 1.6 | N.D. N.D. 7 ± 16 4575 ± 757 718 ± 85** 5299 66 ± 13* |
| Spleen |               |                 |
| WT     | 8.2 ± 3.7     | 50 ± 59 N.D.    | N.D. 1145 ± 66 769 ± 85 1964 |
| WT + DENSPM | 71.4 ± 30.9*** | 336 ± 100*** 336 ± 100*** 22 ± 18 759 ± 77*** 519 ± 32*** 1636 256 ± 65 |
| SSAT-KO| 5.8 ± 5.4     | 4 ± 9 N.D. 1388 ± 43 650 ± 31 2042 |
| SSAT-KO + DENSPM | 5.2 ± 4.3 | 9 ± 14 N.D. 1360 ± 268 595 ± 79 1964 183 ± 59 |

Data are means ± S.D. where n = 6 in both genotype. Animals were four months old male mice. PUT, Putrescine; N1-Ac-SPD, N1-acetylspermidine; N8-Ac-SPD, N8-acetylspermidine; SPD, spermidine; SPM, spermine. *p<0.05, **p<0.01, ***p<0.001, in comparison with DENSPM treated and untreated animals. SSAT activity is expressed as pmol/10 min per mg of tissue, polyamine and DENSPM pools as pmol/mg of tissue. N.D., not detectable.
four animals were still alive and were then sacrificed (median = 8) (Fig. 1). Statistical analysis showed highly significant difference in the survival between the genotypes (p<0.001). The cause of higher mortality of SSAT-KO animals in response to DENSPM treatment remained obscure.

**Polyamine pools, SSAT activity and longterm aging in wild-type and SSAT knockout mice**

SSAT knockout and wild-type mice were subjected to a long-term follow-up study in order to study the lifespan and polyamine metabolism upon aging. Tissue spermine levels were found to be practically unaltered in 4- and 12-month-old SSAT-KO mice except in livers of younger mice where spermine was reduced by appr. 12 % as compared with syngenic littermates (Table 3A). Decreased spermine pool was found in liver, kidney and brain of the 24-month-old SSAT-KO females. Significantly enhanced accumulation of spermidine was seen in the young and the oldest SSAT-KO mice liver, kidney and spleen (Table 3A, C). A similar trend, constantly elevated (about 30%) spermidine level was also seen earlier in the SSAT-KO ES cells [11]. The elevated spermidine levels in the knockout mice led to a higher spermidine/spermine ratio in every analyzed tissue except in pancreas in all three age groups (Table 3A, C). Putrescine levels in spleens of SSAT-KO mice were significantly decreased at different ages. In kidney, putrescine concentration was also significantly reduced in older SSAT-KO mice whereas in pancreas, liver and brain only minor alterations in putrescine levels were observed (Table 3B, C). The longterm aging study was terminated after 24 months and the remaining animals were sacrificed. Histological analysis of tissues revealed changes in kidneys of aged knockout animals: dilations of Bowman's space with atrophy of glomeruli and associated cells, dilations of tubules of inner medulla, inflammation and necrosis of renal papillae and inflammation of surrounding fat. The above mentioned changes were scored minimal or absent in the wildtype mice (data not shown, n = 4 in both groups).

**Characterization of metabolic parameters of SSAT-KO mice**

As our earlier studies with SSAT overexpressing mice have shown reduced fat mass and high insulin sensitivity (unpublished), the biochemical parameters like glucose, triglycerides and cholesterol were screened regularly from SSAT-KO mice. There were no changes in body weights and epididymal fat pads (data not shown) between genotypes during aging. In addition, the food intake (WT 5-month-old 4.0 ± 0.3

---

Fig. 1 The effect of DENSPM on the survival of SSAT knock-out (SSAT-KO) and wild-type (WT) mice. Mice received intraperitoneally 125 mg/kg/day of DENSPM. Animals were four months old male mice: ten animals in WT group and 13 animals in SSAT-KO group. SSAT-KO mice are significantly (p<0.001) more sensitive to the toxicity exerted by DENSPM treatment.
vs. SSAT-KO 5-month-old 3.7 ± 0.3 g/day/mouse, WT 14-month-old 4.3 ± 0.2 vs. SSAT-KO 14-month-old 4.4 ± 0.5 g/day/mouse) and water intake (WT 5-month-old 5.4 ± 0.6 vs. SSAT-KO 5-month-old 5.4 ± 1.6 g/day/mouse, WT 14-month-old 4.6 ± 0.9 vs. SSAT-KO 14-month-old 4.7 ± 0.5 g/day/mouse) were also similar in both groups. The triglycerides and cholesterol levels revealed no differences between SSAT-KO and WT mice but elevated plasma glucose levels in SSAT-KO males were detected onwards 12 months (Table 4). In order to further study the glucose metabolism and to determine whether older SSAT-KO males were more insulin resistant or diabetic than the controls, glucose and insulin tolerance tests were carried out. In glucose tolerance test (GTT), mice were intraperitoneally injected with 2 mg/g of glucose. Plasma glucose levels were measured at 0, 15, 30, 60, 90 and 120 min and insulin levels at 0, 15 and 120 min after glucose administration. After 16 h fasting (0 min), SSAT-KO mice showed significantly increased (p<0.05) plasma glucose levels as compared with syngenic littermates (Fig. 2A). The other time points showed no differences in glucose levels between the groups. The insulin levels in glucose tolerance test were, however, significantly elevated in null mice at every time point indicating compensatorily increased insulin secretion and insulin resistance (p<0.05 – <0.01). The insulin tolerance test (ITT) was performed after 12h fasting using 0.25 mU/g insulin. Glucose samples were collected at time points 0, 20, 40, and 80 min. The insulin tolerance test confirmed that knock-

| Tissue | SSAT activity | Polyamine pools |
|--------|---------------|-----------------|
|        | PUT           | SPD            | SPM            | SPD/SPM ratio |
| Liver  |               |                |                |               |
| WT     | 0.5 ± 0.1     | 8 ± 7          | 781 ± 73       | 737 ± 31      | 1.0 ± 0.1 |
| SSAT-KO| 0.4 ± 0.0     | N.D.           | 980 ± 32**     | 650 ± 35**    | 1.5 ± 0.1*** |
| Kidney |               |                |                |               |
| WT     | 0.6 ± 0.1     | 56 ± 19        | 357 ± 68       | 681 ± 125     | 0.5 ± 0.1 |
| SSAT-KO| 0.2 ± 0.1**   | 53 ± 10        | 493 ± 79*      | 688 ± 51      | 0.7 ± 0.1* |
| Pancreas |             |                |                |               |
| WT     | 1.5 ± 0.2     | 1 ± 2          | 3700 ± 725     | 524 ± 33      | 7.0 ± 0.9 |
| SSAT-KO| 1.1 ± 0.3     | 3 ± 7          | 3368 ± 450     | 557 ± 80      | 6.1 ± 0.4 |
| Spleen |               |                |                |               |
| WT     | 5.0 ± 0.5     | 101 ± 12       | 1066 ± 122     | 721 ± 39      | 1.5 ± 0.1 |
| SSAT-KO| 1.2 ± 0.3**   | 24 ± 3***      | 1339 ± 77**    | 636 ± 58      | 2.1 ± 0.1*** |
| Brain  |               |                |                |               |
| WT     | 1.2 ± 0.1     | 11 ± 13        | 377 ± 22       | 279 ± 19      | 1.4 ± 0.1 |
| SSAT-KO| 1.1 ± 0.0     | 7 ± 8          | 370 ± 17       | 261 ± 23      | 1.4 ± 0.2 |

Table 3A Tissue polyamines in SSAT knockout (SSAT-KO) and wild-type (WT) female mice at the age of 4 months.

Data are means ± S.D. where n = 4. PUT, Putrescine; N1-Ac-SPD, N1-acetylspermidine; N8-Ac-SPD, N8-acetylspermidine; SPD, spermidine; SPM, spermine. *p < 0.05, **p < 0.01, ***p < 0.001, in comparison with wildtype and SSAT knockout mice. SSAT activity is expressed as pmol/10 min per mg of tissue and polyamine pools as pmol/mg of tissue. N.D., not detectable.
out mice were more insulin resistant than wild-type mice as their plasma glucose levels were elevated at all time points (Fig. 2B).

**Discussion**

The function of a certain gene can be studied by overexpressing or by knocking out the gene and studying the following metabolic consequences. For studying the consequences of SSAT-silencing in polyamine metabolism and especially in DENSPM evoked cellular events in vitro, antisense oligomers, RNA interference and forced mutation pressure have been used [11, 26–28]. We used homologous recombination technique in the generation of SSATdeficient embryonic stem cells [11], which were subsequently used to generate SSAT knockout mice. The actual role of SSAT in polyamine catabolism has not been studied earlier in vivo due to the lack of specific inhibitors. Contrary to the disruption of other polyamine metabolismrelated genes [29, 30], SSAT-KO mice were viable, fertile and did not display overt phenotypical abnormalities. The knockout gene was inherited in a Mendelian fashion. The absence of the functional SSAT gene in knockout mice was confirmed both by PCR and by Southern blot analysis. Furthermore, DENSPM- and CCl4-treatments were used to study the role of inducible SSAT for polyamine metabolism. SSAT activity remained at basal level in tissues of DENSPM-treated knockout animals whereas significantly enhanced enzyme activity and thus decreased higher polyamine levels and accumulation of putrescine were detected in wild-type mice.

| Tissue | SSAT activity | PUT     | SPD     | SPM     | SPD/SPM ratio |
|--------|--------------|---------|---------|---------|---------------|
| Liver  |              |         |         |         |               |
| WT     | 0.4 ± 0.1    | N.D.    | 1062 ± 106 | 732 ± 60 | 1.5 ± 0.1     |
| SSAT-KO| 0.3 ± 0.0    | N.D.    | 1229 ± 88  | 641 ± 56  | 1.9 ± 0.2**   |
| Kidney |              |         |         |         |               |
| WT     | 0.4 ± 0.1    | 123 ± 24 | 278 ± 15 | 650 ± 104 | 0.4 ± 0.1     |
| SSAT-KO| 0.4 ± 0.0    | 32 ± 32* | 398 ± 86 | 581 ± 38  | 0.7 ± 0.2     |
| Pancreas|             |         |         |         |               |
| WT     | 1.5 ± 0.1    | 34 ± 29 | 3402 ± 312 | 566 ± 66  | 6.0 ± 0.6     |
| SSAT-KO| 1.2 ± 0.6    | 22 ± 19 | 3157 ± 752 | 629 ± 80  | 5.0 ± 0.9     |
| Spleen |              |         |         |         |               |
| WT     | 4.7 ± 0.2    | 96 ± 10 | 1141 ± 231 | 695 ± 169 | 1.7 ± 0.1     |
| SSAT-KO| 1.1 ± 0.2*** | 8 ± 9***| 1426 ± 105 | 633 ± 53  | 2.3 ± 0.0***  |
| Brain  |              |         |         |         |               |
| WT     | 1.7 ± 0.1    | 21 ± 4  | 492 ± 26 | 318 ± 41  | 1.6 ± 0.3     |
| SSAT-KO| 1.4 ± 0.1**  | 9 ± 8   | 506 ± 62 | 295 ± 14  | 1.7 ± 0.1     |

Data are mean value ± S.D. where n = 3.
Similarly, SSAT activity was increased only in CCl₄-treated WT mice leading to increased levels of N¹-acetylated spermidine and putrescine in the liver. The small amount of N¹-acetyl spermidine also in SSAT-KO mice may indicate the presence of acetylases other than SSAT. It has been shown earlier that inducible SSAT represents only some portion of the measured N¹AcSpd activity under basal condition [31]. SSAT was initially characterized from CCl₄-treated rat livers and caused appearance of N¹AcSpd and depletion of higher polyamines in conjunction with putrescine accumulation [22, 23]. Unexpectedly, liver spermine and spermidine pools decreased similarly in both mice lines in response to CCl₄. Thus, it seems that inducible SSAT have no distinct role in depletion of spermidine and spermine in mice liver in response to CCl₄-treatment. Evidently, the role of SSAT-induction in liver damage is an interesting subject of further study. Previous studies with cells have clearly proven essential role of the inducible SSAT in DENSPM sensitivity in vitro and our data with SSAT transgenic mice has further strengthen that view in vivo [11, 25, 26, 28, 32]. Unexpected sensitivity of SSAT-KO mice to the toxicity exerted by DENSPM excludes the possibility that inducible SSAT together with polyamine pool depletion plays an essential role in vivo in sensitizing the animals to the drug. The reason for increased mortality remains obscured as the histological examination did not reveal any organ (liver, spleen, kidney and pancreas) toxicities (data not shown). The DENSPM survival experiment

| Tissue | SSAT activity | PUT | N¹-Ac-SPD | N⁸-Ac-SPD | SPD | SPM | SPD/SPM ratio |
|--------|---------------|-----|-----------|-----------|-----|-----|--------------|
| Liver  |               |     |           |           |     |     |              |
| WT     | 0.4 ± 0.1     | 9 ± 16 | 13 ± 12 | N.D.     | 737 ± 59 | 860 ± 44 | 0.9 ± 0.1    |
| SSAT-KO| 0.4 ± 0.1     | N.D. | N.D.     | N.D.     | 1171 ± 210*** | 688 ± 69*** | 1.7 ± 0.4**  |
| Kidney |               |     |           |           |     |     |              |
| WT     | 0.4 ± 0.1     | 51 ± 6 | N.D.     | 10 ± 9  | 368 ± 24 | 656 ± 35 | 0.6 ± 0.1    |
| SSAT-KO| 0.3 ± 0.2     | 34 ± 6*** | N.D.  | 3 ± 9  | 569 ± 93*** | 557 ± 31*** | 1.0 ± 0.2*** |
| Pancreas|               |     |           |           |     |     |              |
| WT     | 4.3 ± 0.8     | 11 ± 29 | N.D.     | N.D.    | 5133 ± 114 | 1021 ± 55 | 5.0 ± 0.2    |
| SSAT-KO| 2.6 ± 0.3**   | N.D. | N.D.     | N.D.    | 5195 ± 224 | 1040 ± 140 | 5.1 ± 0.6    |
| Spleen |               |     |           |           |     |     |              |
| WT     | 3.8 ± 1.1     | 130 ± 20 | N.D.    | N.D.    | 943 ± 73 | 669 ± 45 | 1.4 ± 0.1    |
| SSAT-KO| 2.2 ± 2.2     | 30 ± 18*** | N.D.  | N.D.    | 1480 ± 314*** | 652 ± 47 | 2.3 ± 0.5*** |
| Brain  |               |     |           |           |     |     |              |
| WT     | 1.8 ± 0.2     | 17 ± 14 | N.D.    | 13 ± 4  | 522 ± 18 | 303 ± 10 | 1.7 ± 0.1    |
| SSAT-KO| 1.6 ± 0.1*    | 3 ± 8* | N.D.    | 12 ± 6  | 558 ± 44 | 283 ± 13** | 2.0 ± 0.2**  |

Data are mean value ± S.D. where n = 7.
was carried out with a daily injections of 125mg/kg i.p. dosage that has not exerted mortality in some studies but proved to be toxic for SSAT transgenic mice UKU165b and for their syngenic littermates [15, 25, 33–35]. Thus, there are some differences between separate mouse strains in sensitivity to DENSPM. In our case, the experimental setup was identical between distinct experiments although the SSAT-KO and SSAT transgenic mice lines were not of the same genetic background. However, the survival curves of both experiments showed that first deaths appeared on day 4 and on day 5 most of the animals died. Moreover, according to the original hypothesis and the published data from SSAT-deficient ES-cells, SSAT-KO mice should have been more resistant to DENSPM than their WT littermates. It seems that inducible SSAT may play a controversial role in the toxicity exerted by DENSPM in vivo. At the moment most likely explanation is related to intracellular accumulation of DENSPM that was greatly enhanced in SSAT overexpressing mice when compared to their syngenic littermates. Simultaneously natural polyamine pools almost disappeared in SSAT overexpressing mice. On the contrary, in SSAT-KO mice intracellular DENSPM concentrations were similar with their WT littermates but natural polyamine pools remained almost unchanged in response to analogue which may cause the observed toxicity. This view is further supported from the data obtained with SSAT-KO ES cells in which DENSPM accumulated and polyamine pools depleted similarly as in control cells but targeted cells proved to be more resistant against this analogue. The tissue polyamine pools remained almost similar during aging in wildtype and knockout mice except of moderately elevated spermidine levels in several tissues of SSAT-KO mice. Increased Spd/Spm ratio reflected therefore the importance of SSAT in spermidine back-conversion. Our previous studies with SSAT [25] and double transgenic (MTDC/MTSSAT) [36] mice lines indicated that accelerated polyamine catabolism reduced lifespan at least by 50 %. The lack of SSAT did not affect the life expectancy of the animals.

Fig. 2 Insulin and glucose tolerance tests of SSAT knockout (SSAT-KO) and wild-type (WT) mice. A: glucose tolerance test. 16-h fasted nonanaesthetized 16-month old male mice were intraperitoneally injected with 2 mg/g glucose. B, insulin tolerance test. 12-h fasted nonanaesthetized 16 months old male mice were intraperitoneally injected with 0.25 mU/g insulin. Blood samples were collected from a tail vein at indicated time points. Data are presented as means ± SEM, where n(WT) = 10, n(SSAT-KO) = 12. Statistically significant differences are indicated as *, p < 0.05; **, p < 0.01.
Type 2 diabetes and related conditions, such as obesity and insulin resistance, are serious public health risks. T2D results from impaired insulin secretion from pancreatic β-cells or insulin resistance of peripheral tissues or both. Although tremendous research efforts have been focused on the pathogenesis of this disease, the underlying mechanisms remain only partly understood. There are several transgenic and knockout mouse models in diabetes research, which provide more sophisticated strategies in resolving the etiology of T2D at molecular level [37]. The SSAT overexpressing mice are metabolically opposite to the type 2 diabetic patients showing reduced amount of WAT, high basal metabolic rate, improved glucose tolerance, high insulin sensitivity, enhanced mitochondrial biogenesis and low accumulation of triglycerides in liver and skeletal muscle [16]. The phenotypical characterization of SSAT-deficient mice showed insulin resistance at older age without obesity or lipid abnormalities. In addition, the histology of old SSAT-KO mice revealed microscopic alterations in kidneys resembling the histopathological changes observed in diabetic nephropathy. Therefore, our SSAT knockout mouse line can provide new insights also in T2D research. However, further studies are required for the determination of mechanisms leading to the development of insulin resistance and diabetic nephropathy in SSAT-KO mice.

In conclusion, the characterization of SSAT genedisrupted mice not only revealed the somewhat confusing role of SSAT in maintaining polyamine homeostasis and as a mediator of DENSPM toxicity but also supported the notion that polyamine catabolism plays a role in glucose metabolism.

Acknowledgements

We thank Karolinska Institutet for blastocyst injections. We are also grateful to Sisko Juutinen, Anne Karppinen, Arja Korhonen, Tuula Reponen and Eila Ruotsalainen for their skilful technical assistance and Dr. Tekele Fashe (DVM) for histological analyses. The research was supported by grants from the Academy of Finland.

References

1. Tabor CW, Tabor H. 1,4-Diaminobutane (putrescine), spermidine, and spermine. Annu Rev Biochem. 1976; 45: 285–306.
2. Seiler N, Delcros JG, Moulinoux JP. Polyamine transport in mammalian cells. An update. Int J Biochem Cell Biol. 1996; 28: 843–61.
3. Chen C, Young BA, Coleman CS, Pegg AE, Sheppard D. Spermidine/spermine N1-acetyltransferase specifically binds to the integrin alpha9 subunit cytoplasmic domain and enhances cell migration. J Cell Biol. 2004; 167: 161–70.
4. Hillary RA, Pegg AE. Decarboxylases involved in polyamine biosynthesis and their inactivation by nitric oxide. Biochim Biophys Acta. 2003; 1647: 161–6.
5. Casero RA, Jr., Pegg AE. Spermidine/spermine N1-acetyltransferasethe-turning point in polyamine metabolism. FASEB J. 1993; 7: 653–61.

| Animal    | Weight (g) | Glucose (mM) | Cholesterol (mM) | Triglycerides (mM) |
|-----------|------------|--------------|------------------|--------------------|
| **10-week-old** |            |              |                  |                    |
| WT        | 23.8 ± 1.8 | 7.0 ± 0.7    | 2.9 ± 0.3        | 1.0 ± 0.2          |
| SSAT-KO   | 22.4 ± 1.9 | 7.7 ± 1.2    | 2.8 ± 0.2        | 0.9 ± 0.2          |
| **52-week-old** |        |              |                  |                    |
| WT        | 32.5 ± 2.3 | 9.0 ± 0.8    | 2.7 ± 0.4        | 0.9 ± 0.1          |
| SSAT-KO   | 31.9 ± 3.8 | 10.0 ± 1.0*  | 3.0 ± 0.4        | 0.8 ± 0.1          |

Data are means ± S.D. where n(10-week-old) = 10-15 in both genotype and n(52-week-old) = 6-10 in both genotype. *p < 0.05 refers to the statistical significance of difference between SSAT knockout and wild-type mice.
6. Vujcic S, Liang P, Diegelman P, Kramer DL, Porter CW. Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine backconversion. *Biochem J.* 2003; 370: 19–28.

7. Bellelli A, Cavallo S, Nicolini L, Cervelli M, Bianchi M, Mariotti P, Zelli M, Federico R. Mouse spermine oxidase: a model of the catalytic cycle and its inhibition by N,N1-bis(2,3-butenadienyl)-1,4-butanediamic. *Biochem Biophys Res Commun.* 2004; 322: 1–8.

8. Wang Y, Devereux W, Woster PM, Stewart TM, Hacker A, Casero RA, Jr. Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res.* 2001; 61: 5370–3.

9. Wang Y, Murray Stewart T, Devereux W, Hacker A, Frydman B, Woster PM, Casero RA, Jr. Properties of purified recombinant human polyamine oxidase, PAOh1/SMO. *Biochem Biophys Res Commun.* 2003; 304: 605–11.

10. Vujcic S, Diegelman P, Bacchi CJ, Kramer DL, Porter CW. Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. *Biochem J.* 2002; 367: 665–75.

11. Niiranen K, Pietilä M, Pirttilä TJ, Järvinen A, Halmekytö M, Korhonen VP, Keinänen TA, Alhonen L, Jänne J. Targeted disruption of spermidine/spermine N1-acetyltransferase gene in mouse embryonic stem cells. Effects on polyamine homeostasis and sensitivity to polyamine analogues. *J Biol Chem.* 2002; 277: 25323–8.

12. Bergeron RJ, Feng Y, Weimar WR, McManis JS, Dimova H, Porter C, Raisler B, Phanstiel O. Properties of the *N*-methyl-N1-acetyltransferase gene expression. *J Med Chem.* 1999; 42: 693–8.

13. Bernacki RJ, Bergeron RJ, Porter CW. Antitumor activity of *N*,*N*′-bis-(ethyl)spermine homologues against human MALME-3 melanoma xenografts. *Cancer Res.* 1992; 52: 2424–30.

14. Porter CW, Ganis B, Libby PR, Bergeron RJ. Correlations between polyamine analogueduced increases in spermidine/spermine N1-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. *Cancer Res.* 1991; 51: 3715–20.

15. Bernacki RJ, Oberman EL, Seweryniak KE, Atwood A, Bergeron RJ, Porter CW. Preclinical antitumor efficacy of the polyamine analogue N1, N11-diethylnorspermine administered by multiple injection or continuous infusion. *Clin Cancer Res.* 1995; 1: 847–57.

16. Pirinen E, Heikkinen S, Virkamäki A, Hohtola E, Pietilä M, Jänne J, Laakso M. Severely reduced white fat deposits without a defect in insulin sensitivity in transgenic mice overexpressing spermidine/spermine N1-acetyltransferase. *Diabetologia* 2002; 45: A83.

17. Laakso M. Insulin resistance and its impact on the approach to therapy of type 2 diabetes. *Int J Clin Pract Suppl.* 2001; 8–12.

18. Hyvönen T, Keinänen TA, Khomutov AR, Khomutov RM, Eloranta TO. Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by highperformance liquid chromatography. *J Chromatogr.* 1992; 574: 17–21.

19. Porter CW, Cavanaugh PF, Jr., Stolovich N, Ganis B, Kelly E, Bergeron RJ. Biological properties of *N*4- and N1,N8-spermidine derivatives in cultured L1210 leukemia cells. *Cancer Res.* 1985; 45: 2050–7.

20. Passonneau JV, Lowry OH. Enzymatic analysis: A practical guide. Ottawa ON Canada: Humana Press, 1993.

21. Rehse K, Puchert E, Leisring S. Antiaggregatory and anticoagulant effects of oligaamines. 12. Alkyl- and arylalkyl-derivatives of putrescine, spermidine and spermine. *Arch Pharm (Weinheim).* 1990; 323: 287–94.

22. Hölttä E, Sinervirta R, Jänne J. Synthesis and accumulation of polyamines in rat liver regenerating after treatment with carbon tetrachloride. *Biochem Biophys Res Commun.* 1973; 54: 350–7.

23. Matsui I, Wiegand L, Pegg AE. Properties of spermidine N-acetyltransferase from livers of rats treated with carbon tetrachloride and its role in the conversion of spermidine into putrescine. *J Biol Chem.* 1981; 256: 2454–9.

24. FogelPetrovic M, Kramer DL, Vujcic S, Miller J, McManis JS, Bergeron RJ, Porter CW. Structural basis for differential induction of spermidine/spermine N1-acetyltransferase activity by novel polyamine analogs. *Mol Pharmacol.* 1997; 52: 69–74.

25. Alhonen L, Pietilä M, Halmekytö M, Kramer DL, Jänne J, Porter CW. Transgenic mice with activated spermidine/spermine N1-acetyltransferase show enhanced sensitivity to the polyamine analog, N1, N11-diethylnorspermine. *Mol Pharmacol.* 1999; 55: 693–8.

26. Chen Y, Kramer DL, Jell J, Vujcic S, Porter CW. Small interfering RNA suppression of polyamine analoginduced spermidine/spermine N1-acetyltransferase. *Mol Pharmacol.* 2003; 64: 1153–9.

27. Fogel-Petrovic, M, Vujcic S, Haner R, Regenass U, Mett H, Porter CW. Sequence specific antisense oligonucleotide analog interfere with spermidine/spermine N1-acetyltransferase gene expression. *Anticancer Res.* 1996; 16: 2517–23.

28. McCloskey DE, Pegg AE. Altered spermidine/spermine N1-acetyltransferase activity as a mechanism of cellular resistance to bis(ethyl)polyamine analogues. *J Biol Chem.* 2000; 275: 2808–14.

29. Nishimura K, Nakatsu F, Kashiwagi K, Ohno H, Saito N,ログザ, M, 河野 G. Essential role of S-adenosylmethionine decarboxylase in mouse embryonic development. *Genes Cells.* 2002; 7: 41–7.

30. Pendeville H, Carpino N, Marine JC, Takahashi Y, Muller M, Martial JA, Cleveland JL. The ornithine decarboxylase gene is essential for cell survival during early murine development. *Mol Cell Biol.* 2001; 21: 6549–58.

31. Persson L, Pegg AE. Studies of the induction of spermidine/spermine N1-acetyltransferase using a specific antisemum. *J Biol Chem.* 1984; 259: 12364–7.

32. McCloskey DE, Pegg AE. Properties of the spermidine/spermine N1-acetyltransferase mutant L156F that decreases cellular sensitivity to the polyamine ana-
logue N1, N11-bis(ethyl)norspermine. *J Biol Chem*. 2003; 278: 13881–7.

33. **Bergeron RJ, Muller R, Bussenius J, McManis JS, Merriman RL, Smith RE, Yao H, Weimar WR.** Synthesis and evaluation of hydroxylated polyamine analogues as antiproliferatives. *J Med Chem*. 2000; 43: 224–35.

34. **Kanter PM, Bullard GA, King JM.** Preclinical toxicologic evaluation of DENSPM (N1,N11-diethylnorspermine) in rats and dogs. Anticancer Drugs. 1994; 5: 448–56.

35. **Porter CW, Bernacki RJ, Miller J, Bergeron RJ.** Antitumor activity of N1,N11-bis(ethyl)norspermine against human melanoma xenografts and possible biochemical correlates of drug action. *Cancer Res*. 1993; 53: 581–6.

36. **Suppola S, Heikkinen S, Parkkinen JJ, UusiOukari M, Korhonen VP, Keinänen T, Alhonen L, Jänne J.** Concurrent overexpression of ornithine decarboxylase and spermidine/spermine N1-acetyltransferase further accelerates the catabolism of hepatic polyamines in transgenic mice. *Biochem J*. 2001; 358: 343–8.

37. **Plum L, Wunderlich FT, Baudler S, Krone W, Bruning JC.** Transgenic and knockout mice in diabetes research: novel insights into pathophysiology, limitations, and perspectives. *Physiology (Bethesda)*. 2005; 20: 152–61.