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ORIGINAL ARTICLE

Polyamine flux analysis by determination of heavy isotope incorporation from ^{13}C, ^{15}N-enriched amino acids into polyamines by LC–MS/MS

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Abstract The study of polyamine flux, i.e. the circulating flow of polyamines through the interconnected biosynthetic and catabolic pathways, is of considerable interest because of the established links between the polyamine metabolism and many diseases, such as cancer and diabetes. To study polyamine flux in detail, a novel method based on following the label incorporation from the ^{13}C, ^{15}N-labeled polyamine precursors, arginine, methionine and ornithine, into polyamines by LC–MS/MS was implemented. This methodology was tested on three distinct cell lines with different spermidine/spermine-N^1-acetyltransferase (SSAT) expression levels, i.e. non-transgenic, transgenic and knockout. These trials allowed the identification of the critical conditions for the successful polyamine flux measurement, such as the functional time frame of label incorporation, until plateau phase with the selected precursor is reached. The novel LC–MS/MS-based method for polyamine flux overcame the limitations of previous existing methodologies, with baseline separation of the different polyamine species and the exact quantification of the incorporated label. Moreover, the obtained results clearly show that the increased SSAT expression is associated with accelerated polyamine flux.

Keywords Polyamines · Polyamine flux · LC/MS-MS · Fetal fibroblasts · SSAT · MDL 72527

Abbreviations

AdoMet	S-adenosyl-L-methionine
AdoMetDC	S-adenosyl-L-methionine decarboxylase
AdoMetSY	S-adenosyl-L-methionine synthetase
APAO	Acetylpolyamine oxidase
ARG	Arginase
dcAdoMet	Decarboxylated S–adenosyl–L–methionine
HPLC	High performance liquid chromatography
LC–MS/MS	Liquid chromatography coupled with tandem mass spectrometry
MDL 72527	N^1,N^4-bis(2,3 butadienyl) 1,4-butanediamine
ODC	Ornithine decarboxylase
Put	Putrescine
SMO	Spermine oxidase
Spd	Spermidine
SPDSY	Spermidine synthase
Spm	Spermine
SPSY	Spermine synthase
SSAT	Spermidine/spermine–N^1–acetyltransferase
SSA	5-sulfosalicylic acid

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Introduction

From the seven enzymes that form the polyamine metabolism, only three are regarded as rate-limiting. Two of the rate-limiting enzymes, ornithine decarboxylase (ODC) and S-adenosyl-L-methionine decarboxylase (AdoMetDC), are found in the biosynthetic pathway, while spermidine/spermine-N^1-acetyltransferase (SSAT) is considered the rate-limiting enzyme from the polyamine catabolism. One of the precursors for the biosynthesis of polyamines is L-ornithine, which is produced from L-arginine during the urea cycle. L-ornithine is decarboxylated into putrescine by ODC. AdoMetDC regulates the decarboxylation of S-adenosyl-L-methionine (AdoMet), the second precursor, producing decarboxylated AdoMet (dcAdoMet). The decarboxylation of AdoMet is sealing its fate as the aminopropyl donor to be used in the polyamine biosynthesis. Spermidine synthase (SPDSY) and spermine synthase (SPSY) combine the aminopropyl moiety provided by dcAdoMet with putrescine or spermidine to produce spermidine and spermine, respectively (for a schematic representation of the polyamine metabolism see Fig. 1).

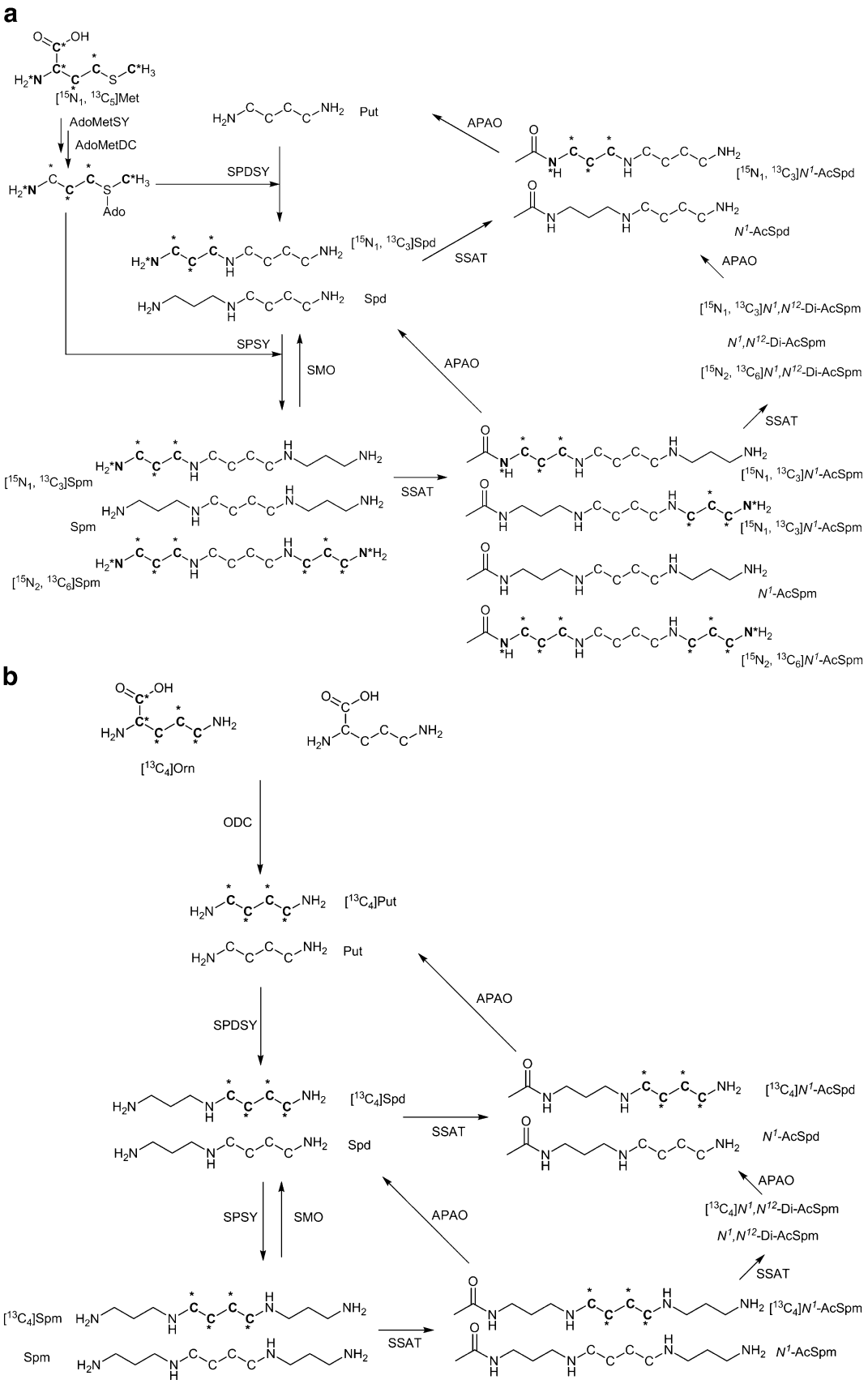
The fact that the amino groups of polyamines are positively charged at physiological pH is fundamental for their diverse physiological functions. This allows for the ubiquitous polyamines to interact with negatively charged molecules, such as DNA, RNA, proteins or phospholipids (Jänne et al. 2004). Among their different functions, polyamines allow for the correct folding of DNA to form nucleosomes (Smirnov et al. 1988; Balasundaram and Tyagi 1991), they are able to change the DNA conformation from the inactive B-form into the transcriptionally active Z-form (Thomas et al. 1985; Liu and Wang 1987), and interact and regulate ion channels, such as potassium inward rectifying channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors, and *N*-methyl-D-aspartate receptors (Johnson 1996; Williams 1997). Additionally, spermidine presents the unique function of being the only known naturally occurring precursor of the unusual amino acid hypusine, which is synthesized during the posttranslational modifications of the eukaryotic translation initiation factor 5A (Shiba et al. 1971; Park et al. 1981, 2010).

Due to the wide range of polyamine functions, alterations on the polyamine metabolism and polyamine levels are found in many different disease states. In fact, the boost on polyamine research during the 1970's was sparkled after the discovery that polyamine levels are altered in hyperproliferative cells, such as cancer (Bachrach et al. 1967; Williams-Ashman et al. 1972; O'Brien et al. 1975). More recently, it has been shown that the loss of function of the adenomatous polyposis coli gene in the familial adenomatous polyposis colon cancer is a direct cause of polyamine accumulation, which precedes the uncontrolled cellular proliferation and development of cancer (Gerner and Meyskens 2004). Since the recognition of the link between polyamines and cancer, numerous approaches aiming to harness the polyamine metabolism into a potential cancer therapy have been developed (Casero and Marton 2007).

Fig. 1 Schematic representation of the polyamine metabolism. Depiction of the different labeled polyamine compounds formed when the cells were grown in the presence of **a** [$^{13}C_5$, ^{15}N]methionine, and **b** [$^{13}C_4$]ornithine. The labeled atoms are marked as *bold letters* with an *asterisk*. A detailed description of the polyamine metabolism is found in the first paragraph of the "Introduction" ▶

More recently, alterations on the polyamine metabolism have been linked with the metabolic syndrome. We and others have shown that transgenic mice overexpressing the SSAT enzyme present enhanced insulin sensitivity, extremely reduced levels of white adipose tissue, and are protected against the negative effects of a high-fat diet (Pirinen et al. 2007; Cerrada-Gimenez et al. 2009; Jell et al. 2007). An opposite phenotype, a mouse line devoid of SSAT expression, became insulin resistant upon aging and presented elevated white adipose tissue accumulation after high-fat diet (Niiranen et al. 2006; Jell et al. 2007). The acute activation of the polyamine catabolic pathway in the SSAT transgenic mice produces a compensatory activation of the biosynthetic pathway, resulting in accelerated polyamine flux, and increased consumption of ATP and acetyl–CoA. The elevated energetic needs of the transgenic cells triggers the activation of, among others, the transcription factor peroxisome proliferator activator receptor cofactor 1-α (PGC-1α) and 5′-AMP-activated protein kinase (AMPK) responsible for some of the beneficial effects seen in these transgenic mice (Pirinen et al. 2007, 2010; Cerrada-Gimenez et al. 2009).

To increase our knowledge on the polyamine flux, we have followed the incorporation of the ^{13}C and/or ^{15}N-labels from natural polyamine precursors, such as methionine, ornithine or arginine, into the polyamine pools by taking advantage of a recently developed method of polyamine detection by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) (Häkkinen et al. 2007, 2008, 2010). The used methodology allowed quantifying the natural and the ^{13}C, ^{15}N-labeled polyamines with a proper baseline separation. The LC–MS/MS methodology was used to analyze the polyamine flux in three different cell lines derived from the same mouse lines used in previous studies, a non-transgenic, SSAT overexpressing (MT-SSAT), and SSAT-deficient (SSATKO) (Suppola et al. 1999; Niiranen et al. 2006).


a
[15N1, 13C5]Met
AdoMetSY
AdoMetDC
Ado
SPDSY
Put
APAO
[15N1, 13C3]N1-AcSpd
[15N1, 13C3]Spd
SSAT
N1-AcSpd
APAO
Spd
[15N1, 13C3]N1,N12-Di-AcSpm
N1,N12-Di-AcSpm
[15N2, 13C6]N1,N12-Di-AcSpm
SPSY
SMO
APAO
SSAT
[15N1, 13C3]Spm
SSAT
[15N1, 13C3]N1-AcSpm
Spm
[15N1, 13C3]N1-AcSpm
N1-AcSpm
[15N2, 13C6]Spm
[15N2, 13C6]N1-AcSpm
b
[13C4]Orn
ODC
[13C4]Put
Put
APAO
SPDSY
[13C4]Spd
SSAT
[13C4]N1-AcSpd
Spd
N1-AcSpd
APAO
SPSY
SMO
APAO
[13C4]N1,N12-Di-AcSpm
N1,N12-Di-AcSpm
SSAT
[13C4]Spm
SSAT
[13C4]N1-AcSpm
Spm
N1-AcSpm

Experimental

Cell culture experiments

Fetal fibroblasts (d13) were isolated from three different mouse lines (MT-SSAT transgenic, SSATKO, and non-transgenic littermates) with the same background (C57Bl/6 J) (Alhonen et al. 1998). The cells were cultured in DMEM (Sigma–Aldrich, Steinheim, Germany) containing 10% fetal calf serum (Sigma) for two passages. After the second passage, the cells were transferred into 100 mm plates and cultured for an additional 24 h until the original medium was replaced with DMEM without L-methionine (GIBCO, Carlsbad, CA, USA) with 10% fetal calf serum (Sigma). The cells were further cultured for 6, 12, 24, 48 or 72 h in the presence of [$^{13}C_6$, $^{15}N_4$] arginine (136 μM Isotec, Steinheim, Germany), [$^{13}C_5$, ^{15}N] methionine (193 μM, Isotec Steinheim, Germany) or [$^{13}C_5$] ornithine (173 μM, Cambridge Isotopes Laboratories Inc., Andover, MA, USA). According to the manufacturer the DMEM used for the experiments contained a concentration of 399 μM of non-labeled arginine. For the ornithine and arginine labeling experiments 0.03 g/l of unlabeled methionine were added to the growth media. N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine MDL 72527 (Hoechst–Roussel Pharmaceuticals Inc., Somerville, NJ, USA) was used at a concentration of 20 μM. Triplicate plates were cultured for each line, time point and treatment.

Sample preparation

Cells were collected by trypsinization and sonicated in 5 % (w/v) 5-sulfosalicylic acid solution (SSA). Cell samples were centrifuged on a refrigerated tabletop centrifuge for 30 min at 13,000 rpm (Biofuge Fresco). Supernatants were used in the LC–MS/MS sample preparation either directly or after diluting them on 5% (w/v) SSA. Aliquots from the growth media were collected at the same time for the analysis of excreted polyamines. Media samples were treated with 50% (w/v) SSA (9 + 1 sample/acid v/v ratio) and centrifuged like cell samples prior to use for the LC–MS/MS sample preparation.

Reagents

Ultra-gradient HPLC-grade acetonitrile (ACN) was from J.T. Baker (Avantor, Deventer, Netherlands), heptafluorobutyric acid (HFBA, >99%) from Fluka (Sigma), ultrapure water was prepared using a Milli-Q Gradient system (Millipore, Milford, MA, USA). Butane-1,4-diamine dihydrochloride (Put, 98%), N^1-(3-aminopropyl)butane-1,4-diamine trihydrochloride (Spd, 98%), *N*,*N*'-bis-(3-aminopropyl)butane-1,4-diamine tetrahydro-chloride (Spm, >95%) were from Sigma. *N*-(3-Amino-1,1,2,2-$^2H_4$-propyl)-*N*'-(3-amino-1,1-$^2H_2$-propyl)butane-1,4-diamine tetrahydrochloride (Spm-6D) was prepared starting from N^1-benzyl-bis-N^1,$N^{3'}$-(2-nitrobenzenesulfonyl)-2,2,3,3-$^2H_4$-propane-1,3-diamine using the previously described methods (Häkkinen et al. 2009) to give Spm-6D (yield 37% for 5 steps) as a colorless solid. ^{1}H NMR (D_2O): δ 3.22–3.00 (8H, m), 2.17–2.00 (2H, m), 1.88–1.70 (4H, m); ^{13}C NMR (D_2O): δ 49.8 (2C), 46.8 (2C, m), 39.4, 39.3, 26.4, 25.6 (3C, s + m). All the other deuterated reference compounds were prepared as previously described (Häkkinen et al. 2009, 2010).

Instrumentation

LC separations, MS/MS detection and analysis of the compounds were achieved with Agilent 6410 Triple Quad LC/MS equipped with Agilent 1200 Series Binary Pump SL pumping system and Agilent 1200 Autosampler. Data acquisition and analysis were performed using the Agilent MassHunter Workstation software (Agilent Corporation, MA, USA). ^{1}H and ^{13}C-NMR spectra were measured on a Bruker Avance 500 DRX 219 spectrometer (Bruker, Rheinstetter, Germany) as described earlier (Häkkinen et al. 2009).

Analytical conditions

LC–MS/MS experiments were based on selected reaction monitoring (SRM) analysis and were performed essentially as described earlier (Häkkinen et al. 2008, 2010). The chromatographic separations were carried out using a Phenomenex Gemini reversed phase C18 column (3 μm, 50 mm × 2 mm, 110 Å) (Phenomenex, Torrance, CA, USA) protected with a Phenomenex Gemini C18 guard column (4 mm × 2 mm) (Phenomenex). The elution was performed using a linear gradient from 2% A (0.1% (v/v) HFBA in water) and 98% B (0.1% (v/v) HFBA in acetonitrile) to 50% B over 12 min at a flow rate of 0.2 ml/min as before (Häkkinen et al. 2010). Four time segments were used for analyses, turning points being 5, 7 and 8.4 min. In first time segment were measured putrescines (dwell times 50 ms, 2–3 ion transitions), in the second time segment were measured N^1-acetylspermidines and diaminoheptane (dwell times 50 ms, 5 ion transitions), in the third time segment were measured spermidines and N^1-acetylspermines (dwell times 40 ms, 10–16 ion transitions) and in the fourth time segment were measured spermines (dwell times 40, 7–10 ion transitions). The precursor and the product spectra of each unlabeled and deuterated polyamine were recorded similarly as described earlier (Häkkinen et al. 2007). Fragmentor voltage value was set to 60 V for unlabeled and labeled putrescines and diaminoheptane, and to

90 V for rest of the analytes. Ion transitions (precursor ions and selected product ions) for quantification and qualification, and collision energy values for all analytes used in the quantitative SRM analysis are given in Supplementary Material (Supplementary Table 1 for [$^{13}C_5$, ^{15}N]methionine, Supplementary Table 2 for [$^{13}C_5$]ornithine, and Supplementary Table 3 for [$^{13}C_6$, $^{15}N_4$]arginine).

Preparation of standards and quality controls

Standard working solutions (concentrations of 0.03, 0.1, 0.3, 1, 3, 10, 30 and 60 μM) and working solutions for quality control (QC) samples (concentrations of 0.05, 0.2, 2 and 20 μM) both in 5 % (w/v) SSA, and internal standard (IS) working solution 1. For methionine and ornithine samples containing 1 μM of Put-8D, Spd-2D, Spm-6D and DAH in water or IS working solution 2. For arginine samples containing 1 μM of Put-8D, Spd-2D, Spm-4D and DAH in water, were prepared essentially as described earlier (Häkkinen et al. 2008). Two types of calibration standards and QC samples were prepared using these working solutions by adding 50 μl of each standard working solution or QC working solution, 50 μl of one of the 1 μM IS working solutions and 25 μl of 10% HFBA in water. Samples were transferred into polypropylene vial inserts (Agilent) for the LC–MS/MS analysis. The calibration curves included also a blank sample and a "zero" sample as described earlier (Häkkinen et al. 2008).

Preparation of cell and cell culture medium samples

LC–MS/MS samples from cells and cell culture media were prepared similarly as calibration standards for calibration curves, and QC samples.

Calibration curves and assay validation

Calibration curves were constructed from accurate concentrations of working solutions of each unlabeled analyte in *x*-axis, and peak-area ratio sample versus deuterated internal standard in *y*-axis using 1/x weighted linear or quadratic least-squares regression model. Assay validation was performed according to the FDA guideline for bioanalytical method validation (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf) essentially as described earlier (Häkkinen et al. 2008). Calibration curves of unlabeled analytes were also used to measure ^{13}C and ^{15}N-labeled analytes. Some labeled analytes, like [$^{13}C_3$, $^{15}N_1$]spermine, generated two different fragments in MS/MS conditions, whereas the unlabeled analyte used in quantification produced only one. In these cases, both of the generated fragments from labeled analyte were counted up and used for quantification. A list of the possible labeled analytes produced from each of the three different polyamine precursors can be found in the Supplementary Material (Fig. 1a and Supplementary Table 1 for [$^{13}C_5$, ^{15}N]methionine, Fig. 1b and Supplementary Table 2 for [$^{13}C_5$]ornithine, and Supplementary Fig. 1 and Supplementary Table 3 for [$^{13}C_6$, $^{15}N_4$]arginine).

Results

The analysis of the polyamine flux by LC–MS/MS was tested using three different cell lines and three different labeled polyamine precursors. Each one of the cell lines had the particular characteristic of having a different SSAT expression level. One cell line transgenically overexpressed the SSAT enzyme (MT-SSAT), the second cell line presented a totally opposite genotype with disrupted SSAT (SSATKO), and the third was a control non-transgenic cell line with unaltered SSAT expression. The particular cell lines were selected to represent different polyamine flux rates. Moreover, the three different polyamine precursors selected for the test were universally ^{13}C and ^{15}N or ^{13}C-labeled, i.e. [$^{13}C_5$, ^{15}N]methionine, [$^{13}C_5$]ornithine or [$^{13}C_6$, $^{15}N_4$]arginine.

Incorporation of [$^{13}C_5$, ^{15}N]methionine into polyamine pools

During the methionine labeling experiment, the three cell lines were grown in the presence of 0.03 g/l (193 μM) [$^{13}C_5$, ^{15}N]methionine, the possible contribution of the fetal calf serum to the methionine levels was considered negligible. The total levels of the individual polyamine pools consisting in unlabeled and labeled polyamines species as pmol/μg DNA together with the percentage of labeled polyamines from the total pool are presented in Table 1. Because the cells were incubated with the [$^{13}C_5$, ^{15}N]methionine as the sole methionine source, the theoretical maximum label incorporation in each polyamine pool was calculated to be approximately 100%. It is clear that already by 6 h, the MT-SSAT transgenic fibroblasts accumulated higher amounts of label in each one of the polyamines pools than the non-transgenic and SSATKO cells did. By 24 h, the spermidine and acetyl-spermidine pools in the transgenic cells consisted of 90% labeled polyamines while 75% of the spermine pool was labeled. Interestingly, the LC–MS/MS methodology allowed us to separate the pools of labeled spermine between the mono-labeled, which is an indicative of how much spermine is de novo produced from spermidine, and the di-labeled pool, which represents the amount of spermine catabolized into spermidine and then re-converted to spermine. In the non-transgenic and SSATKO cells the ratio of labeled

Table 1 Total individual polyamine pools and the percentage of ^{13}C, ^{15}N-label incorporated into the different polyamine pools after incubation with [$^{13}C_5$, ^{15}N]methionine

Cell line/timepoint	Put[a]	Spd (% [$^{13}C_3$, $^{15}N_1$]Spd)[a]	N^1-AcSpd (% [$^{13}C_3$, $^{15}N_1$]N^1-AcSpd)[a]	Spm (% [$^{13}C_3$, $^{15}N_1$]Spm and [$^{13}C_6$, $^{15}N_2$]Spm)[a]
Non–transgenic 6 h	79 ± 3	577 ± 29 (27)	14 ± 1 (30)	129 ± 9 (14; 3)
Non–transgenic 12 h	59 ± 7	584 ± 74 (51)	12 ± 1 (52)	138 ± 19 (25; 12)
Non–transgenic 24 h	71 ± 2	630 ± 53 (76)	16 ± 1 (74)	166 ± 14 (28; 35)
MT–SSAT 6 h	206 ± 18	530 ± 37 (68)	140 ± 17 (66)	104 ± 10 (15; 13)
MT–SSAT 12 h	184 ± 46	456 ± 43 (83)	114 ± 6 (82)	100 ± 9 (18; 31)
MT–SSAT 24 h	184 ± 57	442 ± 16 (90)	158 ± 9 (89)	112 ± 9 (18; 57)
SSATKO 6 h	54 ± 4	647 ± 32 (13)	ND	192 ± 2 (7; 1)
SSATKO 12 h	34 ± 2	581 ± 29 (22)	ND	182 ± 12 (12; 3)
SSATKO 24 h	49 ± 2	668 ± 29 (74)	ND	221 ± 9 (28; 30)

Cell cultures were grown in the presence of 193 μM [$^{13}C_5$, ^{15}N]methionine for 6, 12 or 24 h. The total polyamine amount is presented as pmol/μg DNA, values are presented as mean ± S.D. of three replicates. The value in brackets represents the percentage of labeled polyamine from the total polyamine pool, SD for the percentage values were smaller than 4%. Mono- and di-aminopropyl labeled Spm species are separated with semi-colon

ND not detected

[a] pmol/μg DNA (% of labeled polyamine)

spermidine and spermine was similar in both lines. As expected, due to the lack of SSAT activity, the SSATKO cells completely lacked acetylated spermidine, while the pool from the non-transgenic line was 74% labeled. Additionally, and as indicative of the accelerated polyamine flux produced by the activation of SSAT, the label accumulation in the spermidine pool of MT-SSAT cells reached the plateau phase just after the 6 h timepoint. Nevertheless, the label accumulation in the non–transgenic and SSATKO cells was slower, stabilizing at around 24 h after incubation with labeled methionine.

Incorporation of [$^{13}C_5$]ornithine into polyamine pools

[$^{13}C_5$]ornithine was supplemented at a concentration of 0.03 g/l (173 μM) and the cells were cultured for 24, 48 or 72 h. The total levels of the individual polyamine pools consisting in unlabeled and labeled polyamines species as pmol/μg DNA together with the percentage of labeled polyamines of the total pool are presented in Table 2. The label derived from [$^{13}C_5$]ornithine behaved similarly in all three different cell lines. At the first time point, 24 h, the percentage of labeled putrescine and spermidine were around 60–70% of total polyamines. At later time points the amounts of labeled putrescine and spermidine decreased slightly while the total amounts of putrescine and spermidine remained unchanged, even increasing at the latest time point, 72 h. Although the percentage of putrescine and spermidine label had practically reached the plateau phase by 24 h, the amount of label in the spermine pool kept increasing until 48 h. This was taken as an indicator for the activation of polyamine biosynthetic pathway. Moreover, in agreement with the hypothesis that activation of polyamine catabolism accelerates polyamine flux, the label derived from [$^{13}C_5$]ornithine accumulated into the spermine pool at an accelerated rate in the MT-SSAT transgenic cells when compared to the non-transgenic and SSATKO cells.

When the three cell lines were treated with a combination of [$^{13}C_5$]ornithine and 20 μM MDL 72527, an APAO inhibitor, the "futile cycle" was clearly observed as the combined accumulation of acetylated spermidine and acetylated spermine and decreased spermidine and spermine levels in the non-transgenic and MT-SSAT cells. Due to the total lack of SSAT expression, the SSATKO cells were not able to accumulate acetylated polyamines, instead the spermidine and spermine pools increased over time. Moreover, in the two cell lines with functional SSAT enzyme the accumulation of acetylated polyamines was accompanied with increased excretion of those polyamines.

Incorporation of [$^{13}C_6$, $^{15}N_4$]arginine into polyamine pools

During the arginine labeling experiment, the cells were incubated with 0.03 g/l of [$^{13}C_6$, $^{15}N_4$]arginine (136 μM). Compared to the other two labeled polyamine precursors used, [$^{13}C_6$, $^{15}N_4$]arginine was greatly diluted among the arginine present in the growth media, which had a concentration of 399 μM of arginine. This combined with the wide range of metabolic pathways where arginine has a role, produced that the levels of label incorporation in the polyamine pools were even lower than the calculated theoretical 25%. In fact, from the three tested polyamine

Table 2 Total individual polyamine pools and the percentage of ^{13}C–label incorporated into the different polyamine pools after incubation with [$^{13}C_5$]ornithine

Cell line/timepoint/treatment	Put (% [$^{13}C_4$]Put)[a]	Spd (% [$^{13}C_4$]Spd)[a]	N^1–AcSpd (% [$^{13}C_4$] N^1–AcSpd)[a]	Spm (% [$^{13}C_4$]Spm)[a]	N^1-AcSpm (% [$^{13}C_4$]N^1–AcSpm)[a]
Non–transgenic 24 h	259 ± 11 (72)	1539 ± 51 (66)	168 ± 5 (64)	509 ± 23 (27)	ND
Non–transgenic 48 h	518 ± 26 (71)	2223 ± 133 (73)	277 ± 12 (71)	854 ± 44 (54)	ND
Non–transgenic 72 h	879 ± 34 (67)	3487 ± 103 (70)	395 ± 11 (68)	1254 ± 27 (62)	ND
Non–transgenic 24 h MDL	217 ± 17 (74)	1315 ± 109 (67)	747 ± 25 (61)	290 ± 11 (24)	38 ± 2 (19)
Non–transgenic 48 h MDL	443 ± 51 (70)	1941 ± 166 (73)	960 ± 3 (68)	501 ± 37 (51)	72 ± 2 (44)
Non–transgenic 72 h MDL	448 ± 46 (69)	2108 ± 266 (72)	1055 ± 8 (66)	842 ± 98 (55)	90 ± 2 (55)
MT–SSAT 24 h	1222 ± 22 (72)	1861 ± 69 (71)	1195 ± 21 (67)	574 ± 11 (39)	8 ± 0 (41)
MT–SSAT 48 h	3774 ± 262 (70)	2774 ± 168 (71)	1838 ± 16 (67)	1112 ± 74 (63)	33 ± 1 (65)
MT–SSAT 72 h	10357 ± 227 (66)	3875 ± 121 (66)	4217 ± 65 (63)	1772 ± 28 (66)	45 ± 3 (66)
MT–SSAT 24 h MDL	752 ± 40 (71)	1308 ± 42 (77)	2261 ± 16 (71)	510 ± 22 (46)	165 ± 6 (41)
MT–SSAT 48 h MDL	1389 ± 16 (67)	1782 ± 35 (72)	4604 ± 46 (69)	917 ± 8 (66)	306 ± 7 (62)
MT–SSAT 72 h MDL	2144 ± 58 (64)	2301 ± 54 (67)	7840 ± 177 (65)	1312 ± 24 (67)	427 ± 17 (65)
SSATKO 24 h	367 ± 11 (63)	3037 ± 119 (59)	ND	949 ± 12 (26)	ND
SSATKO 48 h	894.5 ± 68 (60)	6251 ± 414 (67)	ND	2211 ± 95 (52)	ND
SSATKO 72 h	1849 ± 154.4 (56)	10371 ± 1043 (63)	ND	3634 ± 222 (59)	ND
SSATKO 24 h MDL	252 ± 28 (64)	2918 ± 60 (51)	11 ± 0	959 ± 50 (19)	ND
SSATKO 48 h MDL	559 ± 19 (62)	4902 ± 189 (64)	ND	1765 ± 66 (40)	ND
SSATKO 72 h MDL	932 ± 25 (61)	7826 ± 324 (65)	ND	2671 ± 150 (51)	ND

Cell cultures were grown in the presence of 173 μM of [$^{13}C_5$]ornithine for 24, 48 or 72 h with or without the presence of 20 μM MDL 72527. The total polyamine amount is presented as pmol/μg DNA, values are presented as mean ± S.D. of three replicates. The value in brackets represents the percentage of labeled polyamines from the total polyamine pools, SD for the percentage values were smaller than 6%

ND not detected/determinable

[a] pmol/μg DNA (% of labeled polyamine)

precursors, [$^{13}C_6$, $^{15}N_4$]arginine was the one showing the lowest incorporation rate, with maximum incorporation levels ranging just 10%. The total levels of the individual polyamine pools consisting in unlabeled and labeled polyamines as pmol/μg DNA together with the percentage of labeled polyamines of the total polyamine pool for the arginine labeling experiment are presented in Supplementary Table 4.

Excretion of polyamines

When the cells were treated with [$^{13}C_5$]ornithine or [$^{13}C_6$, $^{15}N_4$]arginine in combination with MDL 72527 the level of acetylated spermidine in the cell culture media was vastly increased in the non-transgenic and MT-SSAT cell lines, while the amount of excreted putrescine was slightly reduced (see Table 3 and Supplementary Table 5, respectively). Essentially, the treatment with MDL 72527 doubled the total amount of excreted polyamines per μg of DNA on the cell lines that present a functional SSAT gene, i.e. non-transgenic and MT–SSAT. However, the MDL 72527 treatment did not have the same effects on the SSATKO cells. Although the SSATKO cells excreted putrescine in lower amounts than non-transgenic or MT-SSAT cells, only traces of acetylated spermidine appeared in the culture media. Nonetheless, the total polyamine excretion levels, i.e. labeled and unlabeled, were much higher during the incubation of the different cell lines with [$^{13}C_5$]ornithine than when [$^{13}C_6$, $^{15}N_4$]arginine was used, especially for the non–transgenic and MT–SSAT cell lines.

Discussion

The particular biochemical characteristics of polyamines allow them to participate in many different cellular processes. That is the reason why alterations on the polyamine metabolism and pools have been linked with many disease states, such as cancer (Gerner and Meyskens 2009), metabolic syndrome (Pirinen et al. 2007; Niiranen et al. 2006), inflammation (Babbar et al. 2007), or pancreatitis (Hyvönen et al. 2007; Merentie et al. 2007), among others. Thus, harnessing the polyamine metabolism may prove beneficial for treating some of these affections. In fact, the ODC inhibitor DFMO has been efficiently used for the treatment of the African sleeping sickness (van Nieuwenhove et al.

Table 3 Quantities of excreted polyamines after [$^{13}C_5$]ornithine labeling

Cell line/timepoint/treatment	Put (%[$^{13}C_4$]Put)[a]	Spd (%[$^{13}C_4$]Spd)[a]	N^1–AcSpd (%[$^{13}C_4$]N^1–AcSpd)[a]	Total μM
Non–transgenic 48 h	358 ± 35 (71)	ND	965 ± 89 (57)	0.9 ± 0.0
Non–transgenic 72 h	518 ± 50 (71)	ND	1296 ± 119 (65)	1.9 ± 0.1
Non–transgenic 48 h MDL	271 ± 7 (70)	58 ± 1 (100)	5110 ± 149 (59)	2.8 ± 0.1
Non–transgenic 72 h MDL	400 ± 26 (67)	48 ± 1 (100)	7265 ± 264 (65)	5.0 ± 0.1
MT–SSAT 48 h	357 ± 8 (72)	ND	4218 ± 162 (64)	7.1 ± 0.2
MT–SSAT 72 h	503 ± 38 (69)	ND	5322 ± 417 (65)	14.1 ± 0.4
MT–SSAT 48 h MDL	146 ± 8 (69)	ND	10235 ± 353 (67)	12.1 ± 0.6
MT–SSAT 72 h MDL	169 ± 8 (68)	ND	10591 ± 710 (66)	18.6 ± 0.8
SSATKO 48 h	226 ± 12 (69)	ND	25 ± 1 (ND)	0.6 ± 0.0
SSATKO 72 h	447 ± 31 (64)	12 ± 2 (100)	18 ± 0 (ND)	1.7 ± 0.2
SSATKO 48 h MDL	135 ± 13 (68)	63 ± 2 (59)	45 ± 4 (ND)	0.4 ± 0.0
SSATKO 72 h MDL	242 ± 18 (67)	66 ± 4 (62)	52 ± 6 (30)	0.8 ± 0.0

Cell cultures were grown with 173 μM [$^{13}C_5$]ornithine for 48 or 72 h with or without 20 μM MDL 72527. Individual polyamine levels are expressed as pmol/μg DNA, values are presented as mean ± S.D. of three replicates. The value in brackets represents the percentage of labeled polyamines from the total polyamine pools, SD for the percentage values were smaller than 1.1%. The total levels are expressed as μM

ND not detected

[a] pmol/μg DNA (% of labeled polyamine)

1985), and is currently undergoing clinical trials in combination with sulindac as a cancer chemoprevention agent (Gerner et al. 2007; Meyskens et al. 2008).

Since polyamine precursors and polyamines are found in any normal diet, in the current study we have taken the advantage of using a readily available methionine-free medium formulation that facilitated to a great extent the efficient incorporation of the label derived from [$^{13}C_5$, ^{15}N]methionine into the polyamine pool. By contrast, during the arginine labeling experiment, the [$^{13}C_6$, $^{15}N_4$]arginine was greatly diluted with the arginine available in the growth media, greatly limiting the incorporation of the arginine-derived label into the polyamine pool. The label incorporation can be modeled as a curve with three different interconnected phases: in the first one the cells are still using the non-labeled precursors found in the growth media, thus the label incorporation follows a lag phase; during the second phase, the label rapidly accumulates into the polyamine pools, this phase follows an exponential phase; and the third phase appears when the label accumulation reaches its maximum, depicted with a stationary phase. It is just during the second phase, or exponential, when polyamine flux can be accurately measured. Therefore, the time points for measuring polyamine flux are determined by the nature of the cell lines and the labeled precursor used. When the cells were incubated with [$^{13}C_5$, ^{15}N]methionine the non-transgenic and SSATKO cells reached the maximum label incorporation after 24 h, while the MT-SSAT transgenic cells reached the maximum incorporation already by 6 h. Nonetheless, the necessary time for each cell line to reach the maximum label incorporation is an important piece of information because it clearly correlates with the rate of the polyamine flux. Thus, the cell lines that reach the maximum label incorporation rate in the shortest time, i.e. MT–SSAT, are the ones that possess accelerated polyamine flux.

The original methodology for studying polyamine metabolism was based on following the movement of the label from H^3 or C^{14}-labeled radioisotopes, such as methionine, ornithine, and polyamines, into the polyamine pool (Höltta et al. 1971; Russell and McVicker 1971; Höltta and Jänne 1972; Paulus and Davis 1983). However, this technique has many drawbacks. The necessary radioisotopes with high specific activities are not chemically stable over long-term. Moreover, the used radioisotopes are beta emitters, thus, they require specific handling and waste disposal. In order to accurately quantify the amount of incorporated label the specific activity (total polyamine and DPM counts) of each polyamine pool should be determined. In the specific case of using a methionine radioisotope as a precursor, it is not possible to quantify the single and double labeled spermine. And, the fact that the sample matrix could cause variable effects to the counting efficiency creates the need for internal standards. Altogether, these render the radiometric methodology very laborious and not very practical. This technique was then further refined with the use of fluorinated polyamine analogues combined with HPLC measurements (Kramer et al. 1995, 2008). This improvement is considered the current standard technique for analysis of polyamine flux.

However, this introduced method presents two main disadvantages that undermine the validity of the results obtained with it. First, fluoro-putrescine, which is directly derived from the unnatural amino acid analogue fluoro-ornithine, has a lower K_m value for spermidine synthase than the natural putrescine, 12 μM versus 40 μM, with a 60 % maximal reaction velocity compared to putrescine (Dezeure et al. 1988). This is translated into the fact that cells treated with different doses of fluoro-ornithine do not accumulate fluoro-putrescine in a dose dependent manner while the accumulation of fluoro-spermidine and fluoro-spermine appears to be dose-dependent (Kramer et al. 1995, 2008). In the presented methodology, the use of ^{13}C and ^{15}N or ^{13}C-labeled precursors allowed to overcome this very important drawback, as the minor modifications that these labels impose into the original molecules did not alter their original biochemical characteristics. The second limitation of the HPLC-based method affects the ability to properly detect/quantify the different polyamines, natural and fluorinated. From the HPLC analysis standpoint, the addition of the fluorine label into each polyamine adds only a minor modification to the chemical structure of each polyamine. Thus, the retention times of the fluorine-labeled and the natural polyamines are very close, hampering the accurate separation of each species by HPLC. In fact, the results presented by Kramer and colleagues (Kramer et al. 2008) showed that the peaks of the natural and labeled species of spermidine and spermine clearly overlap, and on a lower extent also the putrescine and acetylated spermidine and acetylated spermine peaks also overlap. Therefore, this HPLC-based methodology cannot be regarded as quantitative. The LC–MS/MS methodology does not suffer from this drawback, allowing for the efficient MS/MS separation of the labeled and unlabeled species of a given polyamine and allowing their precise quantification in the original sample.

During the current experiments we documented that treatment with MDL 72527 greatly increased polyamine excretion in the non-transgenic and MT–SSAT cells. This fact is already known since the mid 80's when it was shown that blocking APAO increases polyamine excretion (Seiler et al. 1985). However, recently, it was found that the amino acid transporter SLC3A2 is involved in the excretion of putrescine and acetylated spermidine. Moreover, SLC3A2 co-localizes with SSAT on the plasma membrane of colon cancer cells (Uemura et al. 2008). All these facts combined with the current results, indicate that activated polyamine excretion is present in the cell lines with an active SSAT enzyme, and suggest that in order for the cells to be able to efficiently excrete polyamines they need to have a functional SSAT enzyme.

The study of polyamine flux combining ^{13}C, ^{15}N–labels and LC–MS/MS presents a major improvement over the current HPLC-based methodology. The use of ^{13}C, ^{15}N-labeled precursor allowed overcoming the problematic of not using natural polyamine precursors, such as fluoro-ornithine. Moreover, the LC–MS/MS methodology was quantitative and avoided the interferences caused by analyzing closely eluting molecules as seen with the HPLC methodology using fluoro-ornithine. Additionally, the developed LC–MS/MS methodology possesses the advantage of measuring polyamines having different amounts of incorporated labels from the same sample. Thus, the incorporation of one or two novel labeled aminopropyl moieties to de novo synthesized Spm or AcSpm could be determined separately, which is practically impossible using earlier described methods exploiting radioactive isotopes.

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