Polyamine Acetylation Modulates Polyamine Metabolic Flux—
A Prelude to Broader Metabolic Consequences

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Running Title: Polyamine Acetylation Modulates Metabolic Flux

Key words: metabolic flux, polyamine catabolism, polyamine biosynthesis, prostate, spermidine/spermine N1-acetyltransferase, spermidine, spermine.

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Recent studies suggest that over-expression of the polyamine acetylating enzyme spermidine/spermine N1-acetyltransferase (SSAT) significantly increases metabolic flux through the polyamine pathway. The concept derives from the observation that SSAT-induced acetylation of polyamines gives rise to a compensatory increase in biosynthesis and presumably, to increased flow through the pathway. Despite the strength of this deduction, the existence of heightened polyamine flux has not yet been experimentally demonstrated. Here, we use the artificial polyamine precursor 4-fluoro-ornithine to measure polyamine flux by tracking fluorine unit permeation of polyamine pools in human prostate carcinoma LNCaP cells. Conditional over-expression of SSAT was accompanied by a massive increase in intracellular and extracellular acetylated spermidine, and by a 6- to 20-fold increase in biosynthetic enzyme activities. In the presence of 300 μM 4-fluoro-ornithine, SSAT over-expression led to the sequential appearance of fluorinated putrescine, spermidine, acetylated spermidine and spermine. As fluorinated polyamines increased, endogenous polyamines decreased, so that the total polyamine pool size remained relatively constant. At 24 h, 56% of the spermine pool in the induced SSAT cells was fluorine-labeled compared to only 12% in uninduced cells. Thus, SSAT induction increased metabolic flux by ~5-fold. Flux could be interrupted by inhibition of polyamine biosynthesis but not by inhibition of polyamine oxidation. Overall, the findings are consistent with a paradigm whereby flux is initiated by SSAT acetylation of spermine and particularly, spermidine followed by a marked increase in key biosynthetic enzymes. The latter sustains the flux cycle by providing a constant supply of polyamines for subsequent acetylation by SSAT. The broader metabolic implications of this futile metabolic cycling are discussed in detail.

Intracellular levels of the polyamines, putrescine, spermidine, and spermine are thought to be homeostatically maintained within a relatively constant range by three main effector systems: biosynthesis, acetylation/export and transport (1). In mammalian cells, biosynthesis is regulated by ornithine and S-adenosylmethionine decarboxylases (ODC and SAMDC); acetylation and export, by spermidine/spermine N1-acetyltransferase (SSAT); and transport, by a group of unidentified membrane protein(s). Each of these effectors is sensitively responsive to
intracellular polyamine pools with uptake and biosynthesis being negatively regulated by polyamines and acetylation being positively regulated (2,3). In antiproliferative strategies utilizing biosynthetic enzyme inhibitors (4–8), these various homeostatic effectors counter polyamine pool depletion and thus, compromise growth inhibition (9–11). In an alternative approach, we exploited homeostatic responses by using polyamine analogs to create a circumstance of apparent polyamine excess (1,12). The resulting down-regulation of polyamine biosynthesis export was accompanied by rapid depletion of endogenous polyamine pools and inhibition of cell growth (13). Subsequent mechanistic studies showed that rapid polyamine pool depletion was at least partially due to potent induction of the acetylating enzyme SSAT (14,15) which facilitated polyamine oxidation and their export out of the cell (16,17). Thus, induction of SSAT became recognized as a major contributor to analog-mediated growth inhibition and/or apoptosis (18,19).

The cytosolic enzyme SSAT acetylates Spm and Spd to form N1'-acetylspermine (AcSpm), N1',N12'-diacetylspermine (DAS) and N1'-acetylspermidine (AcSpd) which are then rendered susceptible to either export out of the cell or oxidation to lower polyamines by polyamine oxidase (PAO) (20,21). Attempts to define the cellular consequences of selective SSAT induction on cell growth have relied on transfection strategies (22,23). Conditional over-expression of SSAT in LNCaP human prostate carcinoma cells led to growth inhibition but in contrast to expectations, polyamine pools were not depleted (23). Despite intracellular accumulation of huge amounts of acetylated polyamines, intracellular polyamine pools were only marginally reduced, due apparently to a compensatory increase in ODC and SAMDC (23). We now know from other studies that growth inhibition in the absence of polyamine pool depletion, as seen in LNCaP cells, is a typical cellular response to SSAT over-expression (24,25).

Similar biochemical findings to those seen in LNCaP cells were made in transgenic over-expression of SSAT which led to a hairless phenotype (26) and altered tumor growth (27,28). We now believe that the various effects seen in cells and mice may be due to altered metabolic flux through the polyamine pathway (23). That is, SSAT acetylation of polyamines leads to increased biosynthesis and heightened metabolic flux (Fig. 1). The adverse down-stream consequences of sustained metabolic flux that account for growth inhibition and other effects are potentially two-fold: pathway precursor depletion or accumulation of toxic pathway by-products (23,27–30).

Examples of the former could include depletion of the SAMDC substrate and methylation precursor, S-adenosylmethionine (SAM) or depletion of the SSAT substrate and fatty acid precursor, acetyl-CoA (23,27,30). Possible by-product examples could include accumulation of the SAMDC by-product 5'-methylthioadenosine or accumulation of the PAO by-product, hydrogen peroxide (20). Detailed studies in LNCaP cells strongly relate growth inhibition to the fatty acid precursor acetyl-CoA; a finding supported by the lean phenotype seen in SSAT transgenic mice (26,27,29,30) as well as the fatty phenotype seen SSAT knock-out mice (30). More particularly, acetyl-CoA was depleted in SSAT over-expressing LNCaP cells and in the adipose tissue of SSAT transgenic mice. Thus far, the existence of SSAT-induced metabolic flux has only been indirectly inferred on the basis of increased SSAT activity, increased ODC and SAMDC activities, accumulation of acetylated polyamines and persistence of Spd and Spm pools. Although the heightened flux paradigm is compelling and seemingly without alternative interpretation, it has not yet been experimentally demonstrated by direct measurement which is the intention of this present study.

Previously, we reported a method for quantifying polyamine flux that makes use of (a) the ability of fluoro-ornithine (Fl-Orn) to substitute for ornithine as a precursor to Put biosynthesis, (b) the metabolic processing of fluorinated Put to higher polyamines in the absence of overt cytotoxicity, and (c) our ability to distinguish fluorinated polyamines from endogenous polyamines by high performance liquid chromatography (HPLC) (31). The method was used to characterize metabolic flux under various growth states and pharmacological conditions but not during selective induction of SSAT. In similarity to flux measurements based on radioactive tracers, this approach allows for unambiguous separation of newly synthesized
polyamines from pre-existing polyamines. However, fluoro-tagging allows these pools to be visualized and quantified on the same chromatogram and without the need for additional processing steps or equipment. Here, this methodology was used to demonstrate that sustained induction of SSAT increases metabolic flux through the polyamine pathway. Given the many factors that induce SSAT (32-34), these findings could have important implications for better understanding the role of SSAT in drug action and for realizing their potential to affect other metabolic processes.

MATERIALS AND METHODS

**Materials.** 4-Fluro-L-ornithine (Fl-Orn) was synthesized according to a published procedure (31) and provided as a gift by Dr. Janos Kollonitsch (Merck & Co., Rahway, NJ). The PAO inhibitor N\(^{\text{d}}\)-methyl-N\(^{\text{2}}\)-(2,3-butanediyl)butane-1,4-diamine (MDL-72527) and the SAMDC inhibitor 5'-[(Z)-4-amino-2-buteny1]methyl-amino-5'-deoxyadenosine (MDL-73811) were generously provided by Aventis Pharmaceuticals, Inc. (Bridgewater, NJ). The ODC inhibitor DFMO was obtained from ILEX, Inc. (San Antonio, TX). The arginase inhibitor N\(^{\omega}\)-hydroxy-nor-L-arginine (nor-NOHA) was purchased from EMD Chemicals, Inc. (La Jolla, CA). Tetracycline (Tet), aminoguanidine, polyamines and the acetylated polyamines, AcSpd and AcSpm were purchased from Sigma-Aldrich (St. Louis, MO). The 2-fluoroputrescine (Fl-Put) and N\(^{\text{d}}\),N\(^{\text{2}}\)-diacetyl spermine (DAS) was obtained from the late Dr. Nikolaus Seiler (University of Rennes, France). Radioactive [H3]-Spd (27.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

**Cell Culture Conditions.** LNCaP prostate carcinoma cells constitutively expressing the Tet-repressible transactivator (35) were used to transfect human SSAT cDNA and select for conditional overexpression of SSAT (23). Designated SSAT/LNCaP, these cells were routinely grown in RPMI 1640 medium supplemented with 2 mM glutamine (Life Technologies, Inc.), 10% Tet-approved fetal bovine serum (FBS) (Clontech Laboratories, Inc., Mountain View, CA), 100 units/ml penicillin, 100 units/ml streptomycin (Mediatech Inc., Herndon, VA), 150 µg/ml hemoglycin B, 150 µg/ml G418, and 1 µg/ml Tet at 37°C in the presence of humidified 5% CO\(_2\). Of significance, RPMI 1640 medium is ornithine-free. For optimal cell attachment enzyme induction and cell growth, experiments were conducted with poly-D-lysine coated dishes (Becton Dickinson Labware, Bedford, MA). This differs from our previous studies in which LNCaP cells were grown on standard uncoated dishes (23). Aminoguanidine (1 mM) was routinely added to inhibit serum amino oxidases and prevent conversion of extracellular polyamines to toxic products. Cells were harvested with 0.25% trypsin and counted electronically (Coulter Model ZM, Coulter Electronics, Hialeah, FL). Just prior to plating, exponentially growing cells were placed in medium containing 10% NuSerum IV (Collaborative Research, Boston, MA), no addition of hygromycin and G418, and 100 ng/ml Tet. Cells were plated onto 100 mm dishes in the absence (induced SSAT) and presence (basal SSAT) of 1 µg/ml Tet. After 24 h, fresh medium was applied immediately prior to addition of 300 μM Fl-Orn. Control cells were treated with 300 μM Orn in place of Fl-Orn. Some experiments included co-treatments at fixed time points with 50 μM of the polyamine oxidase inhibitor MDL-72527, 30 μM of the SAMDC inhibitor MDL-73811, or 100 μM of the arginase inhibitor nor-NOHA. From 0 to 48 h following addition of Fl-Orn, both medium and cells were collected and frozen at -20°C until HPLC analysis was performed.

**HPLC Detection of Polyamines and Fluoro-polyamines.** Cell samples were extracted with 0.6 M perchloric acid, centrifuged, and the supernatant extract assayed for polyamines by reverse phase HPLC. We obtained sharp polyamine peaks with good separation (Fig. 3) by introducing major modifications to a previously described procedure (36) as detailed below. Polyamines in 50-μl of each acid extract were injected onto an autosampler (Model 717 Plus, Waters Assoc., Milford, MA) using a C-18 column [250 x 3 mm; 5 micron] (Alltech Assoc., Deerfield, IL) with an acetonitrile/sodium acetate gradient buffer system and detected with an o-phthalaldehyde post-column derivatization system.
The column was maintained at 35°C in a column heater (Waters Assoc., Milford, MA). The mobile phase consisted of Buffer A (0.1 M sodium acetate and 15 mM octane sulfonic acid, pH 4.5) and Buffer B (0.25 M sodium acetate and 15 mM octane sulfonic acid, pH 4.5 with 30% acetonitrile) at flow rate of 0.75 ml/min. A low pressure gradient generator (Model 6005 Controller, Waters Assoc., Milford, MA) and a programmable pump (Model 616, Waters Assoc., Milford, MA) were used to mix buffers A and B as a linear gradient: at 0 min, 100% A; at 25 min, 100 % B; and at 30 min, re-equilibration with 100% A. The column eluate was derivatized according to Pickering Laboratories Reagent Bulletin (Mountain View, CA). Briefly, 30% w/v o-phthalaldehyde reagent (Pickering Laboratories, Mountain View, CA) containing 20% w/v Thiofluor (Pickering Laboratories) and 0.3% v/v 30% Brij 35 (Pierce Chemical Co., Rockford, IL), was mixed with column eluates in a flow cell at 0.5 ml/min and passed through a fluorescent detector (Model 2475, Waters Assoc., Milford, MA) with fixed excitation and emission wavelengths of 330 nm and 465 nm, respectively. The data were collected and analyzed using Millennium 32 chromatography software version 3.05 (Waters Assoc., Milford, MA). Authentic standards of the natural polyamines, AcSpd, and AcSpm were analyzed separately to identify and quantitate each peak. Fluorinated putrescine (Fl-Put) was verified by an authentic standard, and the other fluorinated compounds (Fl-Spd, Fl-Spm, Fl-AcSpd, Fl-AcSpm) were presumed from their faster retention time (~1 min ahead) relative the parent natural polyamine. Elution times were as follows: Fl-Put, 14.2 min; putrescine (Put), 14.9 min; Fl-AcSpd, 17.6 min; AcSpd, 18.4 min; 6-Fl-3Spd, 20.4 min; spermidine (Spd), 20.9 min; Fl-AcSpm, 22.2 min; AcSpm, 22.8 min; 6-Fl-3Spm, 23.2 min; and spermine (Spm), 23.6 min. The ratio of Fl-Put/Fl-Put was used for calculating the relative values of all other fluorinated compounds. With sensitivity in the range of 20 pmoles, polyamine pools were expressed as nmol/10^6 cells. The OPA reagent used for post-column detection will only bind to primary amines, thus, monoacetylated polyamines bind the dye, but diacetylated polyamines like DAS (22) do not. DAS was analyzed by rerunning the samples using a pre-column dansylation methodology (37) with additional modifications (38).

**Extracellular Polyamines.** Due to high levels of Put found in fetal bovine serum, duplicate experiments were performed using 10% NuSerum containing 5-fold less Put. Experimental medium was also prepared without the addition of antibiotics hygromycin and G418 due to their interference with peak detection by HPLC analysis. At indicated times, 10 ml of medium collected from each dish was passed through a Millipore Centriprep filter chamber (Millipore Corp., Bedford, MA) per manufacturer’s instructions to remove serum proteins >50kDa. To each filtered sample, 12 ml of 0.01 M ammonium phosphate buffer was added and brought to pH 8.0. Samples were then loaded onto preconditioned Bond Elut LRC CBA columns (Varian Associates, Harbor City, CA) and washed with 2 ml of 0.01 M ammonium phosphate buffer. To elute each sample, 1.5 ml of 0.1 N HCl in methanol was applied. Each eluate was dried on a Savant Speed Vac (Thermo-Savant, Holbrook, NY) for 1.5 h, reconstituted with 400 μl ddH2O, and injected (50 μl) onto the HPLC for analysis as described above. The data expressed as pmol per 50 μl was converted to nmol per ml equivalents for 10^6 cells.

**Enzyme Activities.** SSAT, ODC, and SAMDC enzyme activities were measured with soluble protein extracts as previously described (39,40). SSAT was expressed as pmol of N’-[^14C]acetylsperrmidine generated/min/mg protein. ODC and SAMDC were expressed as nmol[^14C]O2 released/h/mg protein.

**Spd Transport.** This assay was performed as previously described (41) with the following modifications. Cells were grown in six-well coated dishes (Becton Dickinson Labware, Bedford, MA) in the absence and presence of Tet 48 h prior to assay, washed with serum-free RPMI 1640 medium and placed on ice. Triplicate wells were exposed to 2 ml of [3H]-Spd (220 cpm / pmol) mixed with cold Spd to final concentrations of 2 μM and 10 μM in RMPI 1640 medium, and incubated in a 37°C water bath for 30 min (a time within the linear range of uptake for these concentrations). After 3 washes with PBS
containing excess cold Spd, cells were dissolved in 0.1 N NaOH, neutralized with an equal volume of 0.1 N HCl and counted using a scintillation detector (Beckman Coulter, Fullerton, CA). Data was expressed as pmol/min/mg protein.

Measurement of Acetyl-CoA and Malonyl-CoA. Coenzymes A were monitored using a high performance capillary electrophoresis as previously described (30), and data obtained from triplicate samples was expressed as nmol per 10^8 cells.

Statistics. Analysis of data obtained from three or more separate experiments included averages ± standard deviations. Significant differences determined by student t-test are indicated as p values < 0.05 to compare basal SSAT with induced SSAT conditions.

RESULTS

Effects of SSAT Over-expression on Polyamine Metabolic Parameters and Cell Growth. In the presence of Tet, the SSAT/LNCaP cells grew logarithmically with a doubling time of ~25-30 h. Following Tet removal, SSAT activity was maximally induced by ~50-fold at 24 h after which, growth inhibition became apparent (Fig. 1). Although the ~50-fold induction in activity achieved with the Tet-regulatable SSAT plasmid-borne gene (Table 1) is probably higher than most physiological modulation of the endogenous gene, it is less than the increases seen with various pharmacological perturbations (32-34) and particularly those involving polyamine analogs where inductions of >500-fold are not uncommon (14,15).

In the first set of experiments, 300 μM Fl-Orn or Orn was added in order to monitor flux in cells over a 48 h period under conditions of basal and induced SSAT activity. As previously reported (31), 300 μM Fl-Orn had no adverse effects on cell growth over this 48 h period (data not shown). In order to interpret flux findings, polyamine pools and biosynthetic enzyme activities were also measured at the end of this same 48 h period (Table 1, Fig. 2A). There was a 50-fold increase in SSAT activity which was accompanied by compensatory increases in ODC and SAMDC activities of ~7-fold and 20-fold, respectively, relative to basal SSAT cells. The 50-fold induction of SSAT activity is much higher than we previously reported [i.e. 20-fold; (23)] due to the fact that in the present experiments, the SSAT/LNCaP cells were grown on poly-D-lysine coated dishes which increased both enzyme induction and cell tolerance to it. Because regulation of the polyamine transporter typically parallels ODC in response to changes in polyamine pools, we also examined [^3H]-Spd uptake (Fig. 2B). In fact, intracellular Spd uptake increased by 3-fold with SSAT induction.

Taken together, these data suggest that acetylation of Spd and Spm by SSAT initiates changes in polyamine homeostasis that affect biosynthesis and uptake (Fig. 2). Analysis of polyamine pools (Table 1) show that induction of SSAT leads to a modest decrease Spm pools and a huge increase in Put pools. More significantly, intracellular and extracellular AcSpd increase remarkably. Since ODC and SAMDC are known to be repressed by intracellular polyamine pools (1,12,42), it appears that acetylation of Spm and particularly Spd, relieve feedback repression of the enzymes.

Identification of Fl-Labeled Polyamines by HPLC. Previous HPLC studies indicate that Fl-Orn is decarboxylated by ODC to produce Fl-Put which is further processed to the deduced products Fl-Spd and Fl-Spm (31,43). As shown in Figure 3, these various fluorinated and non-fluorinated polyamines were clearly separable and quantifiable in the same HPLC chromatograph. Typically, each fluorinated polyamine analog runs just ahead of its respective endogenous polyamine. The identity and quantitation of the fluorinated polyamines was based on the relative association of the authentic Fl-Put standard to Put, and previous reports by our laboratory and others showing that 2-Fl-Put is metabolically converted to 6-Fl-Spd and 6-Fl-Spm (31,43). As shown in Figure 3, these various fluorinated and non-fluorinated polyamines were clearly separable and quantifiable in the same HPLC chromatograph. Typically, each fluorinated polyamine analog runs just ahead of its respective endogenous polyamine. The identity and quantitation of the fluorinated polyamines was based on the relative association of the authentic Fl-Put standard to Put, and previous reports by our laboratory and others showing that 2-Fl-Put is metabolically converted to 6-Fl-Spd and 6-Fl-Spm (31,43). Here, those findings were confirmed and extended to show that Fl-Spd and Fl-Spm are apparently acetylated by SSAT and form products that similarly elute ahead of the endogenous acetylated polyamines. Interestingly, the intracellular Fl-tagged polyamines were perceived as being similar to natural polyamine pools since the individual polyamine pools remained relatively constant even...
though the proportion of fluorinated polyamines were freely exchanging and increased steadily with time. Because DAS lacks primary amines for binding OPA reagent, it was assayed using a HPLC method based on pre-column derivatization (22). Unfortunately, Fl-DAS and native DAS co-eluted with this method and therefore, could not be separately quantified.

**Effect of Endogenous Orn on Fl-Orn Permeation.** Intracellular Orn, a product of the citric acid cycle, is synthesized by arginase from the essential amino acid arginine (20). To assess the extent to which endogenous Orn might compete with Fl-Orn as a substrate for ODC and hence mask flux, we made use of the inhibitor of arginase, nor-NOHA, to reduce endogenous Orn. In the presence of 100 μM nor-NOHA, each of the Fl-tagged polyamine species examined under basal and induced SSAT conditions was uniformly 2-fold greater than in the absence of the inhibitor, while total pools sized remained relatively constant (data not shown). Thus, nor-NOHA effectively reduced Orn production and increased the ratio of endogenous Fl-Orn/Orn to favor decarboxylation of Fl-Orn, thereby enhancing the rate of Fl permeation. Since Fl permeation of the SSAT/LNCaP cell polyamine pools was rapid and substantial, the inhibitor was not routinely used in the present experiments.

**Time-Dependent Fl-Permeation of Polyamine Pools.** SSAT/LNCaP cells grown in the presence (basal SSAT) or absence (induced SSAT) of Tet for 24 h and then exposed to 300 μM Fl-Orn for 48 h showed a time-dependent increase in the newly synthesized fluorinated polyamines that replaced endogenous polyamines while total pool sizes remained relatively constant. Thus, HPLC analysis of Fl-Orn permeation of pools allows us to distinguish newly synthesized from existing polyamine pools thereby providing indication of metabolic flux rate. Under basal SSAT conditions, Fl-Spd was apparent by 4 h and Fl-Spm, by 12 h after which, both increased steadily. Under conditions of induced SSAT, far greater amounts of Fl-Spd were present at 4 h and Fl-Spm appeared at 4 h instead of 12 h (Fig. 4). In fact, Fl-analogs were significantly greater at every time point in SSAT induced cells relative to cells with basal SSAT. By 48 h, fluorinated polyamines comprised ≤25% of the total Spd and Spm pools under basal SSAT while under induced SSAT, Fl-Spd and Fl-Spm represented >50% of the Spd and Spm pools, respectively. Using fluorine tagging of Spm at 48 h as an indicator, the rate of metabolic flux for SSAT-induced cells was ~4.5 times faster than the uninduced cells. We presume this is due to increased acetylation of polyamines in conjunction with increased biosynthesis of polyamines.

**Acetylated Polyamine Accumulation.** A fixed time point comparison of acetylated and non-acetylated polyamines was conducted in cells under basal and induced conditions. Cells were grown in the presence and absence of Tet for 24 h and then exposed to 300 μM Fl-Orn or Orn for an additional 24 h. As shown in Figure 5, individual polyamine levels were similar whether cells were exposed to either Orn or Fl-Orn indicating that the latter was metabolically processed as efficiently as the natural substrate Orn. While acetylated products were rarely seen under basal conditions, they comprised the majority of polyamines under SSAT-induced conditions with AcSpd being the main species. In fact, AcSpd accumulated to levels that were ~10 times higher than those of Spd. This accumulation indicates that SSAT-induced cells are acetylating polyamines faster than they can be oxidized by PAO and/or exported out of the cell. The fact that ~50% of the AcSpd is Fl-labeled suggests that Fl-Spd is acetylated as efficiently by SSAT as the endogenous Spd. Given the massive amounts of AcSpd that were generated in the induced cells, it would seem that ODC and SAMDC feedback regulation is insensitive to acetylated polyamines since both enzymes remain at high levels (Table 1).

**Time-Dependent Polyamine Acetylation.** Having shown that induced SSAT cells generate acetylated polyamines while basal SSAT cells do not, we next examined the time-dependent Fl-permeation of acetylated polyamine pools. SSAT/LNCaP cells were grown in the absence of Tet for 24 h and then exposed to 300 μM Fl-Orn for 0 to 48 h. Due to SSAT induction during the Tet pre-incubation, cells contained high levels of unlabeled AcSpd pools at the initiation of the Fl-Orn incubation. Thereafter, there is a steady Fl-permeation of both the intracellular Spd and
AcSpd pools that continued with time (Fig. 6A). Although not present in comparable amounts, Spm, AcSpm and DAS were also Fl-labeled in a time-dependent manner but the acetylated Spm products failed to accumulate to levels as high as those of AcSpd (Fig 6A). At every time point, the ratio of AcSpd/Spd is ~5, whereas the ratio of AcSpm/Spm is <0.25. Since intracellular Spm pools are 2-3 times higher than Spd and since SSAT has a lower K_m for Spm than Spd (32,39), these ratios are opposite to expectations. One explanation is that the endogenous Spm pool is bound (32) or sequestered in the nucleus (or elsewhere) and hence, less available than Spd for SSAT acetylation in the cytosol.

Medium collected from the aforementioned experiments was analyzed by HPLC (Fig. 6B). By far, the most abundant extracellular polyamine was AcSpd. Accumulation of extracellular AcSpd began immediately following the medium change between the pre- and post-incubations and rose to ~50 nmol/10^6 cell equivalents over the 48 h post-incubation. By comparison, only minor amounts of acetylated Spm products appeared in the medium. Non-acetylated Fl-Spd and Fl-Spm also appeared in the medium, whereas Spd and Spm did not. This suggests that newly synthesized Fl-Spd and Fl-Spm is more susceptible to export than preexisting Spd and Spm.

Role of PAO Inhibition in Flux. The above data are consistent with the idea that acetylation of Spd and its excretion out of the cell may be responsible for initiating the heightened metabolic flux that was experimentally shown to take place. It is also possible that a significant portion of AcSpd and AcSpm may be oxidized to Put, and this could help to drive flux. In order to examine the possible contribution of polyamine oxidation in perpetuating flux, SSAT-induced cells were exposed to 50 μM of the PAO inhibitor MDL-72527 during the 24 h post-incubation period with the expectation that if acetylated polyamines were being oxidized to a significant degree, the level of intracellular and extracellular acetylated polyamines would increase (44). As shown in Figure 7, intracellular AcSpd levels were similar in the presence or absence of MDL-72527 and extracellular AcSpd actually decreased with MDL-72527. Minor increases were observed for AcSpm and DAS, suggesting some oxidation and back-conversion of these products to Spd and AcSpd, respectively. However, since AcSpm and DAS represent only a small portion of the total SSAT-acetylated products, they would seem at best, to be minor contributors to the heightened flux. Instead, the data indicate that acetylation functions independently of oxidation to initiate the flux cycle. Nearly identical results were obtained if cells were exposed to Fl-Orn during MDL-72527 treatment (data not shown).

Effects of Biosynthetic Enzyme Inhibition on Flux. As shown above, SSAT induction induces flux as indicated by the increased rate of Fl permeation of polyamine pools. For flux to be sustained, new polyamines must be synthesized, taken up or both. To examine the contribution of biosynthesis, SSAT-induced cells were exposed for 48 h to 300 μM Fl-Orn in the absence and presence of the SAMDC inhibitor MDL-73811. The more commonly used ODC inhibitor, DFMO, was not suitable for these experiments since the high levels of Fl-Orn would be expected to compete with it for uptake and enzyme binding. In cells not treated with MDL-73811 (Table 2), the levels of Fl-Spd, Fl-Spm, and Fl-AcSpd are about equal (~1:1 ratio) to native polyamine levels. MDL-73811 treatment blocked synthesis of these three Fl-tagged polyamines while the pools of native Spd and Spm decreased by half. This confirms (a) that new synthesis is required for flux to be sustained and (b) that the Fl-tagged pools derive from newly synthesized Spd and Spm. In addition, we noted that significantly less AcSpd was produced and exported in the treated cells indicating that these processes are also dependent on increased biosynthetic flux.

Treatment of cells with SAMDC inhibitors typically increases Put significantly due to a compensatory increase in ODC (45,46) and the inability of the enzyme product Put to be processed forward to Spd due to SAMDC inhibition. Thus, MDL-78311 treatment gave rise to intra- and extracellular accumulation of both Put and Fl-Put. While the native Put seemed to be partitioned evenly between the cells and medium, most of the Fl-Put was rapidly exported into the medium, reaching levels 2.5-fold those of extracellular Put.
Effects of Flux on Coenzymes A. We previously reported (23) that SSAT induction in the SSAT/LNCaP cells depletes the SSAT cofactor, acetyl-CoA pools. Since the downstream metabolite of acetyl CoA, malonyl-CoA, is known to potently regulate fatty acid oxidation (47), we measured both acetyl- and malonyl-CoA pools at 48 h and 72 h following Tet removal. These time points were chosen to coordinate with flux measurements indicated in Figure 1. Relative to basal SSAT cells, we found significant time-dependent decreases in both CoA molecules such that 72 h after SSAT induction, acetyl-CoA pools were reduced from 17 ± 2 to 8.7 ± 1.2 nmol/10^6 cells (50%) and malonyl-CoA pools were reduced from 35.3 ± 2.8 to 12.0 ± 1.6 nmol/10^6 cells (65%).

DISCUSSION

Except for a 1995 publication from this laboratory (31), little attention has been given in the literature to the issue of polyamine metabolic flux. The hypothesis being tested here is that induction of SSAT gives rise to heightened and sustained metabolic flux through both the biosynthetic and acetylation arms of the polyamine pathway. Prior to this study, the notion that induced SSAT might lead to heightened metabolic flux was deduced on the basis of what we now designate as four hallmark observations: (a) increased SSAT activity, (b) a rise in biosynthetic enzyme activities and (c) persistence of polyamine pools despite (d) accumulation of intracellular and extracellular acetylated polyamines. In the face of these events, the most compelling conclusion is increased metabolic flux through the pathway. Despite the logic of this deduction, this concept has not yet been experimentally confirmed. Thus, we measured fluorine permeation of polyamine pools in basal SSAT versus induced SSAT cells incubated for various times in the presence of Fl-Orn. As shown in Figure 4, the rate of Fl-Spm accumulation in cells growing under basal conditions was increased ~5-fold by induction of SSAT. In actuality, this value markedly underestimates the rate of flux. For example, it does not account for the huge amounts of intracellular and extracellular AcSpd (~45 nmol) generated during the first 24 h was 22 times that of the intracellular Spd levels (~2 nmol). Since Spd pools remained relatively constant over this period and since the amount of AcSpd generated by uninduced cells was insignificant, by this analysis flux could have increased by >20-fold in SSAT-induced cells.

The basis for this increase in flux in induced SSAT cells is initiated by increased acetylation of polyamines. This is then perpetuated by an increase in the biosynthetic enzymes ODC and SAMDC. These enzymes are known to be negatively repressed at the level of protein translation and degradation by basal intracellular levels of polyamines (12,48-50). We believe that acetylation of Spm and particularly Spd following SSAT induction relieves enzyme repression in some manner, giving rise to an overall increase in biosynthetic capabilities. Thus, polyamines lost to acetylation (and eventually, export) are rapidly replaced by biosynthesis and made available for continued recycling via acetylation. Under conditions of heightened metabolic flux, sustained acetylation of these polyamines gives rise to new steady-state in which ODC and SAMDC activities are homeostatically set at higher levels so that intracellular polyamine pools are maintained despite massive acetylation and export. The critical role of biosynthesis in sustaining flux was clearly demonstrated by the ability of SAMDC inhibition to interrupt flux (see Table 2). Thus, while flux is initiated by induction of SSAT, it is critically sustained by increases in biosynthetic enzyme activity for as long as SSAT remains over-expressed. This sequence of events is diagrammed in Figure 8.

The basis for de-repression of ODC and SAMDC following SSAT induction is unclear. While there is a modest decrease in Spm, we have previously reported that Spd and Spm pools actually increase during the same time when ODC and SAMDC are increasing (23). It seems likely that, repression is controlled by a relatively small unbound portion of the total intracellular polyamine pool and acetylation could affect polyamine binding by reducing the net molecular charge (32). More certainly, AcSpd is incapable of substituting for Spd in repressing the biosynthetic enzymes since huge amounts accumulate within the cell (Table 1, Figs. 5 and 6).
Our findings also show that in addition to de-repressing ODC and SAMDC, acetylation of intracellular polyamines increases polyamine transport. Although the latter is known to increase in response to polyamine pool depletion by biosynthetic enzyme inhibitors (41,51,52), it has not been shown to occur in response to SSAT induction. In both cases, the response is consistent with an attempt by the system to homeostatically normalize polyamine imbalances by importing extracellular polyamines. Up-regulation of transport by SSAT could have significant implications in vivo where in the case of enzyme inhibitors, salvage of exogenous polyamines is known to compromise antitumor effects (10,11,53).

We also addressed the possible relationship of polyamine oxidation to SSAT-induced flux. The SSAT products, AcSpd and AcSpm are preferred substrates of PAO which converts them to Put and Spd, respectively, via the back-conversion pathway (20,32). By making these products readily available, SSAT could contribute to flux by increasing their oxidation. Using the PAO inhibitor, MDL-72527, we expected intracellular acetylated polyamines to increase if they were being oxidized (44) but they did not. This confirms that flux is initiated by polyamine acetylation and not by polyamine oxidation.

It is informative to consider the potential of SSAT as a modulator of metabolic flux relative to other polyamine enzymes. Intuitively, it would seem that as key regulators of polyamine biosynthesis, ODC and SAMDC might be better suited as regulators of flux. However, they are not because, as noted earlier, both are repressed at the levels of protein translation and degradation by intracellular Spd and Spm levels (12,48). Thus, if their expression were up-regulated, enzyme activity (and hence, flux) would be rapidly repressed by rising levels of Spd and Spm. This does not preclude the possibility that forced expression of ODC, particularly if it is rendered stable to degradation (54), can induce flux.

Polyamines have been traditionally thought to represent functional entities and the processes of biosynthetic, catabolism, and transport, as means to homeostatically control and maintain their intracellular concentrations. While the findings here do not dispute this view, several recent studies have provided evidence to indicate that SSAT-induced changes in metabolic flux appear to have much broader metabolic consequences, particularly in vivo. For example, the futile metabolic cycling initiated by SSAT and sustained by increases in polyamine biosynthesis can deplete pathway precursors such as ornithine, methionine, S-adenosylmethionine and acetyl-CoA. Alternatively, it can lead to overproduction of toxic pathway by-products such as 5'-methylthioadenosine, hydrogen peroxide and aldehydes. These various possibilities have been recently identified as “enhanced flux” by Kee et al., (23,27), as a “metabolic ratchet” by Tucker et al., (28) and as a “paddle-wheel” by Jänne et al. (55).

Several independent pieces of evidence support the idea that heightened polyamine flux leads to deprivation of the SSAT co-enzyme and fatty acid precursor acetyl-CoA in both cultured cells and mice. Summarized, the evidence is as follows: (a) conditional over-expression of SSAT in LNCaP cells leads to a significant decrease in acetyl- and malonyl-CoA pools (23); (b) transgenic over-expression of SSAT in TRAMP mice is accompanied by a significant decrease in acetyl-CoA in prostatic tumors (27), (c) transgenic over-expression of SSAT in C57Bl/6 mice depletes acetyl- and malonyl-CoA pools in adipose tissue, increases fatty acid oxidation, and results in a distinctly lean phenotype (30) and lastly, (d) genetic deletion of SSAT in C57Bl/6 mice leads to an accumulation of acetyl- and malonyl-CoA, decreased fatty acid oxidation and a fat-prone phenotype, particularly on a high fat diet (30). The importance of the malonyl-CoA finding in both SSAT transgenic and SSAT knock-out mice is that it is synthesized from acetyl-CoA by acetyl-CoA carboxylase and it is known to regulate β-oxidation of fatty acids at the level carnitine palmitoyltransferase 1 (47). Thus, a decrease in malonyl-CoA in adipose tissue is consistent with increased fatty oxidation and a lean phenotype in SSAT transgenic mice. Coincidentally, the SSAT transgenic phenotype is nearly identical to that of mice lacking the enzyme responsible for malonyl-CoA synthesis, acetyl-CoA carboxylase-2, and they too have reduced levels of malonyl-CoA in the adipose tissue (56) and increased fatty acid oxidation in isolated adipocytes (57). Finally, the probable relationship
between malonyl-CoA depletion and growth inhibition has been recently reinforced by studies showing that small molecule inhibition of acetyl-CoA carboxylase increases fatty acid oxidation and inhibits growth of LNCaP cells (58).

While the findings with SSAT overexpression in cells and mice represent an exaggeration of normalcy that may or may not be physiologically relevant, the finding that SSAT knock-out mice are fat prone has clearer physiological implications. In particular, they imply that in normal cells and tissues of wild type mice and at physiological enzyme levels, SSAT prevents fat accumulation. Taken together, our past and present findings strongly relate polyamine acetylation to fatty acid metabolism via acetyl- and malonyl-CoA. We emphasize that the metabolic consequences of increased polyamine flux may vary according to cells and tissues and that in some contexts, other metabolic and cellular consequences may obtain. As an example, we have reported that global over-expression of SSAT suppresses growth of prostate tumors (23) and enhances growth of intestinal tumors (28) even though both effects appear to be due to increased flux. Thus, while acetyl-CoA depletion appears to be involved in the former, alternative metabolic disturbances are likely to be responsible for the latter. Similarly, a recent study found that selective SSAT expression in mouse skin increased sensitivity to chemical carcinogens due to elevations in ODC activity and Put levels, both indicative of increased flux with increased liberation of toxic metabolic by-products (59).

In summary, this study provides direct evidence for the premise that conditional over-expression of SSAT leads to heightened metabolic flux through the polyamine pathway. It also confirms the validity of (a) increased SSAT activity, (b) a rise in biosynthetic enzyme activities and (c) persistence of polyamine pools despite (d) accumulation of intracellular and extracellular acetylated polyamines as hallmark indicators of metabolic flux. For now, the broader metabolic consequences of heightened polyamine flux are best characterized by effects on the SSAT-coenzyme and fatty acid precursor, acetyl-CoA (30) in association with profound phenotypic effects involving fat accumulation in mice. We point out that these consequences are probably context-dependent and alternative metabolic perturbations are likely to be revealed in other cells and tissues. In either case, it is significant that SSAT-induced changes in polyamine flux can impact other metabolic pathways such as fat metabolism.

ACKNOWLEDGEMENTS

The authors dedicate this manuscript to the memory of Professor Nikolaus Seiler, an outstanding scientist who before others, understood the biological importance of polyamine acetylation and catabolism and whose pioneering metabolic studies in this area greatly facilitated the studies and understandings presented here. This work was supported by NIH Grants CA-22153 and CA-76428 and NIH Predoctoral Training Grant CA-09072.

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FOOTNOTES

1The abbreviations used are: DAS, $N^1,N^{12}$-diacetylspermine; DFMO, α-difluoromethylornithine; Fl-Orn, 4-fluoro-L-ornithine; Fl-Put, 2-fluoroputrescine; Fl-Spd, 6-fluorospermidine; Fl-Spm, 6-fluorospermine; Fl-AcSpd, 6-fluoro-$N^1$-acetylspermidine; Fl-AcSpm, 6-fluoro-$N^1$-acetylspermine; HPLC, high pressure liquid chromatography; ODC, ornithine decarboxylase; Orn, ornithine; Put, putrescine; SAM, $S$-adenosylmethionine; SAMDC, $S$-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine; AcSpd, $N^1$-acetylspermidine; AcSpm, $N^1$-acetylspermine; SSAT, spermidine/spermine $N^1$-acetyltransferase; PAO, polyamine oxidase; Tet, tetracycline.
Table 1. Effects of SSAT Induction on Polyamine Metabolism

<table>
<thead>
<tr>
<th>SSAT / LNCaP</th>
<th>Enzyme Activities a</th>
<th>Polyamine Pools b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SSAT</td>
<td>ODC</td>
</tr>
<tr>
<td>Basal Cells</td>
<td>23 ± 5</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Medium</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Induced Cells</td>
<td>1165 ± 270***</td>
<td>20 ± 4***</td>
</tr>
<tr>
<td>Medium</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

aSSAT units, pmol/min/mg protein; ODC and SAMDC units, nmol/h/mg protein. Data represent mean ± SD where n = 6. ***, p < 0.0001 designate student t-test differences between basal and induced enzyme levels.

bIntracellular pools, nmol/10⁶ cells; extracellular (medium) pools, nmol/10⁶ cell equivalents. Data represent mean ± SD where n ≥ 4. *, p < 0.01; **, p < 0.001; ***, p < 0.0001 designate student t-test differences between basal and induced pools.
<table>
<thead>
<tr>
<th>Induced SSAT / LNCaP Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Polyamine Pools&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Put</td>
</tr>
<tr>
<td>Control Cells</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Medium</td>
<td>2.2</td>
</tr>
<tr>
<td>MDL-Rx Cells</td>
<td>18.6</td>
</tr>
<tr>
<td>Medium</td>
<td>26.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Following 24 h Tet removal, cells were treated for 48 h with 300 µM Fl-Orn +/- 30 µM MDL-73811 (MDL-Rx). Data is the average of two experiments performed in duplicate with SD <15%.

<sup>b</sup> Intracellular pools, nmol/10<sup>6</sup> cells; extracellular (medium) pools, nmol/10<sup>6</sup> cell equivalents.
Figure 1. Effects of SSAT induction on cell growth. SSAT/LNCaP cells were grown in the presence and absence of Tet for a total of 72 h. Cell growth (left y axis) and SSAT activity (right y axis) were determined at 24, 48 and 72 h. Note that the 70-fold SSAT induction is accompanied by growth inhibition. The shaded bar drawn from 24-72 h indicates the period of time in subsequent experiments when cells were treated with 300 μM Fl-Orn to monitor flux (shaded bar). Data represent average values ± SD (n = 6).

Figure 2. Compensatory increases in polyamine biosynthetic enzymes and uptake following SSAT induction. SSAT/LNCaP cells were grown in the presence (+; basal SSAT) and absence (−; induced SSAT) of Tet for 48 h. (A) ODC (left y axis), SAMDC (left y axis) and SSAT (right y axis) activities were determined and fold increase above each set of bars represent the activity ratio of induced versus basal SSAT. (B) Spd uptake was determined by exposing +/− Tet SSAT/LNCaP cells to 2 μM and 10 μM [H3]-Spd and quantifying the amount of intracellular radioactivity after a 30 min incubation at 37°C. Data represents the average ± SD of three separate assays performed in triplicate. Fold increases are the average ratios of induced to basal activities.

Figure 3. Representative Chromatogram of FL-tagged polyamines. (A) Polyamine pool analysis of SSAT/LNCaP cells incubated in the absence of Tet for 24 h and then exposed to 300 μM Fl-Orn for an additional 24 h. Post-column fluorescent derivatization of primary amines in acid soluble cell extracts were detected by HPLC methodology outlined in Methods. (B) Polyamine standard mix containing authentic FL-Put for verification. Identity of fluorinated compounds (FL-Spd, FL-Spm, FL-AcSpd, FL-AcSpm) were presumed from peaks with retention times ~1 minute ahead of the parent natural polyamine. Production of DAS, not detectable by this method, was determined using pre-column dansylation methodology outlined in Methods.

Figure 4. Effects of SSAT induction on metabolic flux as indicated by fluorine permeation of polyamine pools. SSAT/LNCaP were grown in the presence and absence of Tet for 24 h, then exposed to 300 μM Fl-Orn for 0, 4, 8, 12, 24 and 48 h after which the relative levels of Put, Fl-Put, Spd, Fl-Spd, Spm, and Fl-Spm were determined. Stacked bars represent total polyamine pool depicted as fluorinated polyamine (hatched upper) and native polyamine (solid lower) pool. It is relevant that the basal SSAT cells continue to divide while the induced SSAT cells do not (see Fig. 1). Data represents the average values (n = 3) with SD < 15%.

Figure 5. Fixed time point comparison of polyamine pools in SSAT/LNCaP cells exposed to Orn or Fl-Orn. SSAT/LNCaP cells were grown in the presence and absence of 300 μM Tet for 24 h and then exposed to either 300 μM Orn or 300 μM Fl-Orn for 24 h prior to analyzing polyamine pool content. The data depict the total level of each polyamine with Orn treatment (solid bars) graphed beside Fl-Orn treatment (endogenous polyamine solid and FL-analog hatched, stacked bars) for comparison. Note that the individual polyamine levels are relatively constant for Orn and Fl-Orn-exposed cells. Data represents the average of three separate determinations with SD < 15%.

Figure 6. Time-course analysis of SSAT effects on metabolic flux as indicated by fluorine permeation of intracellular (A) and extracellular (B) polyamines. Following 24 h in the absence of Tet (induced SSAT), SSAT/LNCaP cells were placed in fresh medium and exposed to 300 μM Fl-Orn from 0 to 48 h. Medium and cells were collected for polyamine analysis. (A) Comparison of intracellular Spd and AcSpd (left panel) and intracellular Spm, AcSpm and DAS (right panel) showing time-dependent fluorine-labeling. The stacked bars depict levels of natural (lower) and FL-analog (upper) and their acetylated products. Data expressed as nmol/10⁶ cells represents 4 separate determinations with SD <15%. (B) Comparison of extracellular Spd and AcSpd (left panel) and extracellular Spm, AcSpm and DAS showing...
time-dependent labeling with fluorine. Stacked bars depict the natural and the acetylated Spd (left panel) and Spm (right panel) products and the Fl-analogs (hatched upper) for both. Note that the major product exported into the medium with SSAT activation is AcSpd and Fl-AcSpd. Data expressed as nmol/10^6 cell equivalents represents the average of 4 separate determinations with SD <15%.

**Figure 7.** Effects of PAO inhibition on accumulation and export of acetylated polyamines. SSAT/LNCaP cells were grown in the absence of Tet for 24 h, and then exposed for an additional 24 h to the presence (+72527) or absence (−72527) of the PAO inhibitor MDL-72527 at 50 μM. The cells and medium were processed to determine intracellular (left panel) and extracellular (right panel) levels of AcSpd, AcSpm and DAS. Note that PAO inhibition failed to increase intracellular accumulation or export of acetylated Spd or Spm. Data represent average values (n = 3) with SD < 10%.

**Figure 8.** SSAT-induced increases in polyamine metabolic flux. Polyamine biosynthesis is regulated by ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) which generate, respectively, the polyamine putrescine (Put) and decarboxylated S-adenosylmethionine (dcSAM). The latter is used as an aminopropyl donor by spermidine synthase (Spd Syn) to form spermidine (Spd) and by spermine synthase (Spm Syn) to form spermine (Spm). This forward-synthesis can be reversed by a back-conversion pathway in which Spm and Spd are acetylated by spermidine/spermine N^1-acetyltransferase (SSAT) to form N^1-acetylsperrmine (AcSpm) and N^1-acetylsperrmidine (AcSpm) which are then oxidized by polyamine oxidase (PAO) to form Spd and Put, respectively. A major portion of AcSpd is exported out of the cell. Data presented here suggest that the following sequence of events mediates SSAT-induced metabolic flux: (1) SSAT is induced; (2) Spm and particularly Spd are acetylated; (3) ODC and SAMDC activities increase (4) new polyamines are synthesized and become available for continued acetylation and (5) heightened metabolic flux (large arrow) is established for as long as SSAT remains induced. This heightened metabolic flux is initiated by SSAT acetylation of polyamines and sustained by ODC and SAMDC regulation of new polyamine biosynthesis. The end result of these events is futile metabolic cycling which have untoward consequences to the cell. More specifically, it can cause depletion of pathway precursors such as S-adenosylmethionine (SAM), acetyl-CoA, or accumulation of potentially toxic by-products such as 5’-methylthioadenosine (MTA). [Figure adapted from Jell *et al.*, (30)].
FIGURE 2.

A. Polyamine Enzymes

B. Spd Transport

[Graph showing enzyme activity and uptake comparison]
FIGURE 3.

A

Induced SSAT/LNCaP
300 µM Fl-Orn, 24 h

mV

FL-PUT
PUT
FL-N'AcSPD
N'AcSPD
FL-SPD
SPD
FL-N'AcSPM
N'AcSPM
FL-SPM
SPM

Minutes

16
20
24

B

mV

FL-PUT
PUT
N'AcSPD
FL-SPD
SPD
N'AcSPM
STD

Minutes

16
20
24
FIGURE 4.
FIGURE 6.

A. Intracellular

B. Extracellular
FIGURE 7.

![Graph showing polyamine pools (nmol/10^6 cells) for Intracellular and Extracellular states. The graph compares the levels of AcSpd, AcSpm, and DAS between control (−72527/ Induced SSAT) and treated (+72527/ Induced SSAT) conditions.](image-url)
FIGURE 8.
Polyamine acetylation modulates polyamine metabolic flux - a prelude to broader metabolic consequences
Debora L. Kramer, Paula Diegelman, Jason Jell, Slavoljub Vujcic, Salim Merali and Carl W. Porter

J. Biol. Chem. published online December 18, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M706806200

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