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Complex N-Acetylation of Triethylenetetramine[S]

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ABSTRACT:

Triethylenetetramine (TETA) is an efficient copper chelator that has versatile clinical potential. We have recently shown that spermidine/spermine-*N*^{1}-acetyltransferase (SSAT1), the key polyamine catabolic enzyme, acetylates TETA in vitro. Here, we studied the metabolism of TETA in three different mouse lines: syngenic, SSAT1-overexpressing, and SSAT1-deficient (SSAT1-KO) mice. The mice were sacrificed at 1, 2, or 4 h after TETA injection (300 mg/kg i.p.). We found only *N*^{1}-acetyltriethylenetetramine (*N*^{1}AcTETA) and/or TETA in the liver, kidney, and plasma samples. As expected, SSAT1-overexpressing mice acetylated TETA at an accelerated rate compared with syngenic and SSAT1-KO mice. It is noteworthy that SSAT1-KO mice metabolized TETA as syngenic mice did, probably by thialysine acetyltransferase, which had a K_m value of 2.5 ± 0.3 mM and a k_{cat} value of 1.3 s^{-1} for TETA when tested in vitro with the human recombinant enzyme. Thus, the present results suggest that there are at least two *N*-acetylases potentially metabolizing TETA. However, their physiological significance for TETA acetylation requires further studies. Furthermore, we detected chemical intramolecular *N*-acetyl migration from the *N*^{1} to *N*^{3} position of *N*^{1}AcTETA and *N*^{1},*N*^{8}-diacetyltriethylenetetramine in an acidified high-performance liquid chromatography sample matrix. The complex metabolism of TETA together with the intramolecular *N*-acetyl migration may explain the huge individual variations in the acetylation rate of TETA reported earlier.

Introduction

Triethylenetetramine (TETA) is a charge-isosteric analog of spermidine with efficient copper-chelating properties (Fig. 1). Before 1969, the only practical treatment through copper chelation for Wilson's disease was *d*-penicillamine, which produces a wide variety of secondary effects, such as hepatotoxicity, fever, and aplastic anemia, leading to intolerance among a number of patients. Currently, TETA is used as a substitute for *d*-penicillamine (Roberts and Schilsky, 2008), and although it presents some adverse effects as well, they are less severe and less common than those caused by *d*-penicillamine (Gouider-Khouja, 2009). TETA reacts in a stoichiometric ratio 1:1 with copper, and the complex is then excreted in the urine. TETA is also able to chelate and mediate excretion of iron and zinc in vivo (Kodama et al., 1997).

TETA has been found to alleviate secondary complications associated with diabetes. In a streptozotocin model of type 1 diabetes, a daily TETA treatment for 8 weeks after the induction of diabetes suppressed kidney and glomerular hypertrophy (Gong et al., 2008) or cardiac damage without decreasing the circulating glucose levels (Lu et al., 2010a). In the case of type 2 diabetes, Zucker diabetic fatty rats treated with TETA demonstrated significantly reduced development of diabetic cardiomyopathy (Baynes and Murray, 2009). Although the exact mechanism of these beneficial effects is not known, Lu et al. (2010a) suggested that copper chelation by TETA induces antioxidant defense mechanism, thus alleviating diabetes-associated complications.

Kodama et al. (1997) were the first to report that TETA is readily acetylated in humans into N^1-monoacetyltriethylenetetramine (N^1AcTETA; Fig. 1). Ten years later, by using LC-MS-based methodology, Lu et al. (2007b) identified that TETA can also be diacetylated in the form of N^1N^8-diacetyltriethylenetetramine (N^1N^8DiAcTETA; Fig. 1). Actually, most of the urine-excreted TETA appears in the form of N^1AcTETA and N^1N^8DiAcTETA in humans, with huge individual variations (Lu et

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ABBREVIATIONS: TETA, triethylenetetramine (1,8-diamino-3,6-diazaoctane); HFBA, heptafluorobutyric acid; *N*^{1}AcTETA, *N*^{1}-monoacetyltriethylenetetramine; *N*^{3}AcTETA, *N*^{3}-monoacetyltriethylenetetramine; *N*^{1}*N*^{8}DiAcTETA, *N*^{1},*N*^{8}-diacetyltriethylenetetramine; PBS, phosphate-buffered saline; Spd, spermidine; Spm, spermine; SSA, sulfosalisylic acid; SSAT1, spermidine/spermine *N*^{1}-acetyltransferase; hSSAT1, human spermidine/spermine *N*^{1}-acetyltransferase; SSAT2, thialysine acetyltransferase; SSAT1-KO, SSAT1 deficient; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SpmTrien, 1,12-diamino-3,6,9-triazadodecane; FID, free induction decay; TOF, time of flight.

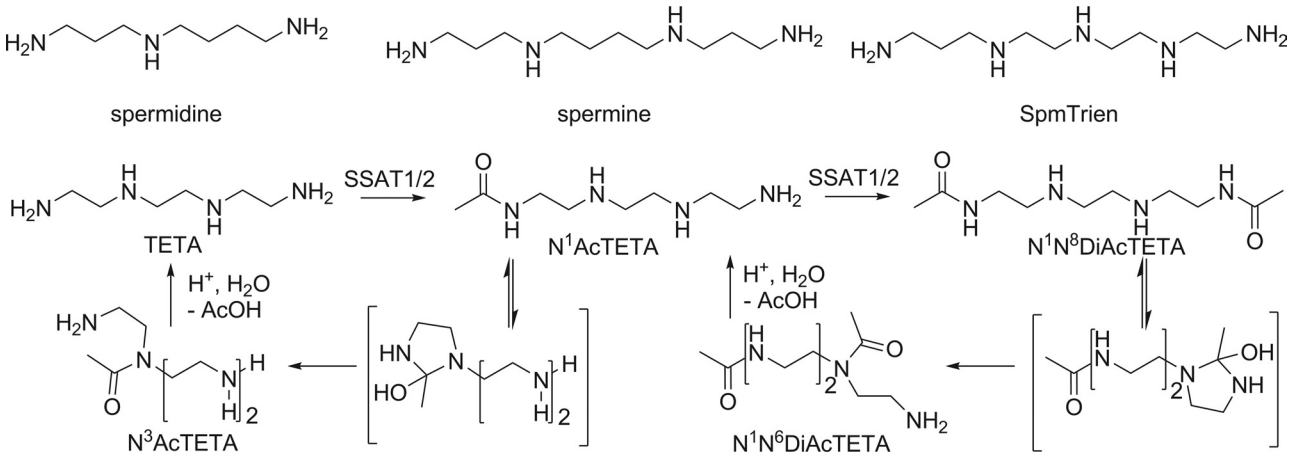


FIG. 1. Chemical structures of polyamines, SpmTrien, TETA and its metabolites, and the postulated pathway for chemical intramolecular *N*-acetyl rearrangement of N^1AcTETA and N^1N^8DiAcTETA.

al., 2007a). Although TETA is acetylated to form N^1AcTETA and N^1N^8DiAcTETA in vivo, to date no enzyme has been definitely identified as responsible for TETA acetylation. Lu et al. (2010b) tested *N*-acetyltransferase 2, which is known to acetylate aromatic amines, as a possible candidate for TETA acetylation. With no conclusive results with *N*-acetyltransferase 2, they suggested that the rate-limiting enzyme in the polyamine catabolism, spermidine/spermine N^1-acetyltransferase (SSAT1), could be responsible for TETA acetylation (Lu et al., 2010b). SSAT1 is indeed a potential candidate because of the close chemical resemblance between its natural substrate spermidine and TETA (Fig. 1). We recently have shown that SSAT1 *N*-acetylates TETA in cell cultures and also in vitro by using a purified mouse recombinant protein (Weisell et al., 2010). To test this hypothesis in vivo, we have now taken advantage of two genetically modified mouse lines, a SSAT1-overexpressing line (Pietilä et al., 1997) and another with disrupted SSAT1 expression (SSAT1-KO) (Niiranen et al., 2006).We used these mouse lines to test whether SSAT1 has a function in the TETA acetylation and whether it is the sole acetylating enzyme of TETA in vivo.

Materials and Methods

Animal Experiments. A transgenic mouse line overexpressing the SSAT1 gene under the control of its own promoter (Pietilä et al., 1997), a mouse line with disrupted SSAT1 gene (Niiranen et al., 2006), and their syngenic littermates were all on the C57BL/6J background. A pilot study using doses of 100, 200, or 300 mg/kg i.p. TETA was performed to study the drug tolerance and tissue accumulation and metabolism of TETA at 2 h. The highest dose used was below the published LD_{50} of 468 mg/kg i.p. TETA for mouse. In additional experiments, the mice were given injections of 300 mg/kg i.p. TETA in phosphate-buffered saline (PBS) (Sigma-Aldrich Finland Oy, Helsinki, Finland), or PBS alone for the control groups. The mice were sacrificed by CO_2 asphyxiation at 1, 2, and 4 h after administration of TETA. Each treatment group contained five age-matched male mice. Blood samples were taken by cardiac puncture and placed on heparinized tubes. Liver and kidney samples were removed, frozen immediately in liquid nitrogen, and stored at −70°C until processing for analyses. The animal experiments were approved by the Animal Care and Use Committee at the Provincial Government of Southern Finland and carried out in accordance with the Declaration of Helsinki.

Analytical Methods. Whole blood samples were processed to obtain plasma. The plasma samples were diluted 9:1 in 50% sulfosalisylic acid (SSA) solution containing 100 μM 1,7-diaminoheptane as an internal standard. Pieces of liver and kidney samples were homogenized in a buffer containing 25 mM Tris, pH 7.4, 0.1 mM EDTA, and 1 mM dithiothreitol using the TissueLyzer II (QIAGEN GmbH, Hilden, Germany). The homogenates were diluted 1:9 in 5% SSA solution containing 10 μM 1,7-diaminoheptane, kept on ice for 20 min, and centrifuged at 14,000*g* for 30 min. Polyamines, TETA, and its acetylated derivates were analyzed by HPLC following the previously published method by Hyvönen et al. (1992). In the biological sample matrix stability test, aliquots of 200 μl of supernatant fractions were neutralized using 14 μl of Na_2CO_3 (2 M) to prevent intramolecular *N*-acetyl migration of N^1AcTETA. Because *ortho*-phtaldialdehyde can be used for primary amines only, the dansyl-Cl method was used to detect N^1N^8DiAcTETA in the 4-h samples (Kabra et al., 1986). SSAT1, SSAT2, and the overall *N*-acetyltransferase activities were assayed as described by Coleman et al. (2004). Kinetic values of TETA acetylation for hSSAT1 were determined as described by Weisell et al. (2010). Recombinant human SSAT1 and SSAT2 were a kind gift from Prof. A. E. Pegg (Pennsylvania State University).

NMR Measurements. NMR samples were prepared in 5% SSA in H_2O (100 mM N^1N^8DiAcTETA or N^1AcTETA) to activate the acid catalyzed *N*-acetyl rearrangement reaction, and spectra were recorded on a DRX spectrometer (Brucker Avance, Milan, Italy) operating at 500.13 MHz using a double-tube system facilitating locking and chemical shift referencing. The external reference tube (o.d., 2 mm; supported by a Teflon adapter) containing the reference substance (40 mM sodium 3-trimethylsilyl[2,2,3,3-d_4]propionate and 0.6 mM $MnSO_4$ in 99.8% D_2O) was placed coaxially into the NMR sample tube (o.d., 5 mm) containing 400 μl of each sample. ^{1}H and ^{13}C NMR spectra were measured using standard protocols to follow-up the reaction. After the reaction was "completed," the NMR samples were dried in vacuo and dissolved into dimethyl sulfoxide-d_6 to detect the NH protons and long-range NH-C couplings in one- and two-dimensional NMR spectra. ^{1}H-^{1}H homonuclear correlation (gradient-enhanced correlation spectroscopy) experiments were carried out in the magnitude mode. For each FID, four transients were accumulated. The ^{1}H-^{13}C gradient-enhanced heteronuclear single quantum correlation experiments were carried out in the phase-sensitive mode using the Echo/Antiecho-TPPI gradient selection. For each FID, 16 transients were accumulated. ^{1}H-^{13}C heteronuclear multiple-bond correlation spectroscopy experiments were carried out with a low-pass J-filter to suppress one-bond correlations. For each FID, 16 transients were accumulated. Window functions and *j* values were according to common practice in each of the experiments.

LC-MS Measurements. Fresh standard samples of N^1AcTETA and N^1N^8DiAcTETA (1 mM) in 0.5% heptafluorobutyric acid (HFBA) were prepared before LC-MS measurements. The acetyl migration samples from the NMR measurements were diluted to 1 mM solution in 0.5% HFBA. Chromatographic separations were performed using the Ultimate/Famos LC system (LC Packings, Amsterdam, The Netherlands) on a Phenomenex Gemini reversed-phase C18 column (3 μm × 50 mm × 2 mm, 110Å) protected with a Phenomenex C18 guard column (4 × 2 mm). A linear gradient was used starting from 98% of 0.1% HFBA in H_2O, 2% of 0.1% HFBA in ACN to 50% of 0.1% HFBA in H_2O, 50% of 0.1% HFBA in ACN in 16 min, at 200 μl/min. The data were recorded on a QSTAR XL hybrid quadrupole TOF instrument (Applied Biosystems, Foster City, CA) in positive ionization mode using information-dependent acquisition experiments for obtaining MS/MS data.

TABLE 1

Hepatic polyamine levels after administration of TETA

Mice were given injections of TETA (300 mg/kg i.p. in PBS) or PBS only and sacrificed at the indicated time points.

Genotype	Time and Treatment	Putrescine	N^1-AcetylSpd	Spd	Spm	N^1AcTETA	TETA
				pmol/μg protein			
Syngenic	Control	26 ± 0	ND	230 ± 36	322 ± 26	ND	ND
	TETA 1 h	34 ± 3	ND	185 ± 4	247 ± 19	352 ± 85	796 ± 63
	TETA 2 h	ND	ND	145 ± 42	287 ± 38	328 ± 37	244 ± 150
	TETA 4 h	ND	ND	145 ± 52	306 ± 19	339 ± 81	135 ± 122
SSAT1	Control	156 ± 50	82 ± 26	345 ± 66*	167 ± 19**	ND	ND
	TETA 1 h	126 ± 19	71 ± 6	311 ± 71**	142 ± 14**	625 ± 77*	934 ± 74*
	TETA 2 h	247 ± 154	485 ± 363	364 ± 172*	136 ± 15**	945 ± 552	423 ± 170
	TETA 4 h	253 ± 335	217 ± 93	233 ± 248	162 ± 29**	244 ± 56	137 ± 133
SSAT1-KO	Control	10 ± 0	14 ± 4	284 ± 50	269 ± 31*	ND	ND
	TETA 1 h	ND	ND	176 ± 9	238 ± 19	400 ± 60	796 ± 29
	TETA 2 h	ND	ND	145 ± 28	302 ± 55	347 ± 44	194 ± 135
	TETA 4 h	ND	ND	154 ± 53	302 ± 29	364 ± 90	146 ± 158

ND, not detectable (<10 pmol/μg protein).
* $P < 0.05$, statistical analysis comparing genotypes (versus syngenic) with the same treatment group.
** $P < 0.01$, statistical analysis comparing genotypes (versus syngenic) with the same treatment group.

During each run, 1 s TOF MS survey scans were recorded for mass range *m/z* 120 to 600 followed by 4 s MS/MS scans of the two most intense singly and doubly charged ions (mass range, *m/z* 30–600). Ion-spray voltage was 5.5 kV. Nitrogen was used as a curtain and nebulizer gas, with flow rates of 16 and 14 l/min, respectively. Declustering and focusing potentials were set at 65 and 200 V, respectively. For the TOF scans, ion release time and width of 6 and 5 μs were used. For MS/MS information-dependent acquisition experiments, nitrogen was used as a collision gas, and the energy was 25 eV. The operation and the spectral processing were performed on Analyst QS version 1.1 software (Applied Biosystems).

Statistical Analysis. Values are expressed as mean ± S.D. ($n = 5$ animals or 3 samples per group). The data were analyzed using the nonparametric Kruskal-Wallis test; when the test was significant, pairwise comparison was performed by the Mann-Whitney *U* test with the aid of the software package SPSS version 14.0 (SPSS Inc., Chicago, IL).

Results

Metabolism of TETA In Vivo. We first studied the tissue accumulation of TETA by giving SSAT1-KO mice injections of 100, 200, or 300 mg/kg i.p. of the drug and determining the tissue and plasma levels of TETA and N^1AcTETA after 2 h. The SSAT1-KO mice were selected for the pilot trials to see whether there were any signs of unexpected toxicity and whether TETA remained unacetylated when SSAT1 activity is not present. There was a significant increase in tissue levels of TETA and N^1AcTETA between the 100 and 200 mg/kg doses, but 300 mg/kg just moderately elevated TETA and N^1AcTETA levels compared with the 200 mg/kg dose (data not shown). However, the highest dose of TETA (300 mg/kg) was used in the subsequent metabolic studies because of the short half-life of TETA in rodents (approximately 2–4 h) (Lu et al., 2010b). Hepatic TETA and N^1AcTETA levels reached the maximum at 1 h after injection, and hepatic N^1AcTETA levels were the highest in SSAT1-overexpressing mice (Table 1), suggesting that SSAT1 acted as an in vivo-acetylating enzyme of TETA. After 1 h, no N^1AcTETA was detected in kidneys, suggesting that the metabolism of TETA is active in liver (Table 2). It is noteworthy that SSAT1-KO mice metabolized TETA similarly to syngenic mice, suggesting that SSAT1 contributes little or no to TETA acetylation in mice not overexpressing the enzyme (Tables 1 and 2). In all genotypes, TETA treatment reduced both Spd and Spm levels in liver and kidney (Tables 1 and 2).

Of the genotypes, SSAT1 mice had the lowest plasma levels of TETA, indicating the fastest clearance of the drug (Table 3). When the 4-h time point samples from liver, kidney, and plasma were subjected to precolumn dansyl-Cl derivatization and further analysis (Kabra et al., 1986), we were not able to detect N^1N^8DiAcTETA in any sample, although the N^1N^8DiAcTETA-dansyl derivative was detectable in the 20-pmol/50-μl level in standard assay mixtures. We also verified the applicability of the used method by mixing control mouse plasma

TABLE 2

Kidney polyamine levels after administration of TETA

Mice were given injections of TETA (300 mg/kg i.p. in PBS) or PBS only and sacrificed at the indicated time points.

Genotype	Time and Treatment	Putrescine	N^1-AcetylSpd	Spd	Spm	N^1AcTETA	TETA
				pmol/μg protein			
Syngenic	Control	59 ± 20	ND	118 ± 24	314 ± 47	ND	ND
	TETA 1 h	49 ± 4	ND	124 ± 12	285 ± 24	ND	1348 ± 147
	TETA 2 h	11 ± 1	ND	90 ± 18	255 ± 36	129 ± 38	422 ± 530
	TETA 4 h	34 ± 29	ND	83 ± 26	263 ± 18	169 ± 26	265 ± 135
SSAT1	Control	138 ± 20	36 ± 11	103 ± 11	260 ± 19*	ND	ND
	TETA 1 h	115 ± 12	47 ± 2	57 ± 18*	267 ± 18	ND	1015 ± 223
	TETA 2 h	88 ± 17	44 ± 8	57 ± 23*	248 ± 12	144 ± 20	240 ± 244
	TETA 4 h	115 ± 39	36 ± 12	57 ± 23	200 ± 24**	124 ± 21	265 ± 98
SSAT1-KO	Control	39 ± 23	ND	154 ± 10*	290 ± 25	ND	ND
	TETA 1 h	ND	ND	128 ± 14	263 ± 25	ND	1137 ± 262
	TETA 2 h	30 ± 23	ND	95 ± 25	264 ± 21	152 ± 56	236 ± 283
	TETA 4 h	37 ± 24	ND	98 ± 30	248 ± 27	173 ± 50	277 ± 141

ND, not detectable (<10 pmol/μg protein).
* $P < 0.05$, statistical analysis comparing genotypes (versus syngenic) with the same treatment group.
** $P < 0.01$, statistical analysis comparing genotypes (versus syngenic) with the same treatment group.

TABLE 3

Plasma levels of TETA and N^1AcTETA after administration of TETA

Mice were given injections of TETA (300 mg/kg i.p. in PBS) or PBS only and sacrificed at the indicated time points. Plasma samples were processed as described under *Analytical Methods*, and TETA and N^1AcTETA concentrations, average micromolars ± S.D. (n = 5 animals), were determined with HPLC.

Genotype	Time and Treatment	N^1AcTETA	TETA
		μM	
Syngenic	TETA 1 h	171 ± 42	1255 ± 272
	TETA 2 h	356 ± 78	1261 ± 730
	TETA 4 h	174 ± 147	205 ± 97
SSAT1	TETA 1 h	224 ± 173	625 ± 211*
	TETA 2 h	279 ± 49	650 ± 159
	TETA 4 h	133 ± 59	103 ± 72
SSAT1-KO	TETA 1 h	195 ± 108	800 ± 393
	TETA 2 h	401 ± 36	1519 ± 523
	TETA 4 h	162 ± 149	228 ± 111

* $P < 0.01$, statistical analysis comparing genotypes (versus syngenic) with the same treatment group.

sample with TETA, N^1AcTETA, and N^1N^8DiAcTETA and derivatizing with dansyl-Cl to detect the added drugs in a biological sample matrix. All the added drugs were detectable in the biological sample matrix at similar levels as in the standards (data not shown).

TETA as a Substrate for Thialysine Acetyltransferase In Vitro. Once we found that SSAT1-KO mice were able to efficiently *N*-acetylate TETA, we performed literature (PubMed) and enzyme database (BRENDA; http://www.brenda-enzymes.info/) searches to find potential *N*-acetylases capable of metabolizing TETA. Thialysine acetyltransferase (SSAT2) was selected as one potential candidate because of its ability to metabolize ethylenediamine and its structural similarity to SSAT1 (Abo-Dalo et al., 2004). SSAT2 has been cloned and is available as a recombinant human protein (Coleman et al., 2004). We performed enzyme kinetic studies with TETA and N^1AcTETA as substrates for SSAT2. SSAT2 had lower affinity for TETA (K_m = 2.5 ± 0.3 mM) than for thialysine (K_m = 0.29 mM), and the V_{max} was 3.96 ± 0.15 μmol · min^{-1} · mg^{-1} SSAT2 as determined by using the P81 disc method (Della Ragione and Pegg, 1982). This method could not be used for thialysine, but by using the spectrophotometric method, the acetylation rate of TETA by SSAT2 was the same as determined for thialysine at 10 mM substrate concentration under the same conditions (Coleman et al., 2004). The acetylation rate of N^1AcTETA was approximately 10% at 10 mM compared with TETA at 10 mM using the spectrophotometric method (Coleman et al., 2004) (data not shown). Furthermore, we determined the kinetic values for acetylation of TETA using hSSAT1 to compare the properties of the human and mouse recombinant enzymes that show high structural homology (Hegde et al., 2007; Montemayor and Hoffman, 2008). hSSAT1 had a K_m value of 83 ± 7 μM and a V_{max} value of 0.90 ± 0.02 μmol · min^{-1} · mg^{-1} (Spd reference, V_{max} = 9.09 ± 0.30 μmol · min^{-1} · mg^{-1}) that were similar to those determined earlier for the mouse recombinant protein K_m value of 169 ± 9 μM and V_{max} value of 1.37 ± 0.02 μmol · min^{-1} · mg^{-1} (Spd reference at 2500 μm, V_{max} = 8.85 ± 0.40 μmol · min^{-1} · mg^{-1}) under the same conditions (Weisell et al., 2010).

Effect of TETA Treatment on Liver *N*-Acetyltransferase Activity. To dissect the *N*-acetylating activity in response to TETA treatment, *N*-acetyltransferase activities were determined from liver homogenates by using the P81 disc method (Della Ragione and Pegg, 1982) with 1 and 10 mM spermidine or TETA as a substrate. As shown in Table 4, TETA treatment induced *N*-acetylating activity similarly in syngenic and SSAT1-KO mice as analyzed with both 1 and 10 mM Spd or TETA. SSAT1-overexpressing mice exhibited the highest induction in N-acetylation activity, but the relative induction was similar in all genotypes.

Acid-Catalyzed Chemical Rearrangement of N^1AcTETA. Charge-deficient polyamine analogs, such as 1,12-diamino-3,6,9-triazadodecane (SpmTrien) and TETA, are interesting because they resemble the natural polyamines spermine and spermidine (Fig. 1), respectively, and are metabolized by SSAT1 in vitro to the corresponding acetylated derivatives (Weisell et al., 2010). When testing the substrate properties of N^1N^8DiAcTETA for acetyl polyamine oxidase [EC 1.5.3.13], we observed that the treatment of N^1N^8DiAcTETA reaction mixture with 5% (w/v) SSA (used to terminate enzymatic reactions) in a nonenzymatic control sample resulted in the formation of a novel compound (retention time, 18.1 min) containing a primary amine group (data not shown). Furthermore, the treatment of N^1AcTETA HPLC standard for 20 h with 5% SSA gave rise to two additional products, i.e., TETA and N^3-monoacetyltriethylenetetramine (N^3AcTETA), which had different retention times than N^1AcTETA when analyzed with HPLC using a polyamine protocol (Fig. 2). Testing the effect of trichloroacetic acid, perchloric acid, and heptafluorobutyric acid, the commonly used acids for HPLC sample preparation or as ion-pairing reagents, revealed that they all induced the chemical rearrangement of N^1AcTETA (Supple-

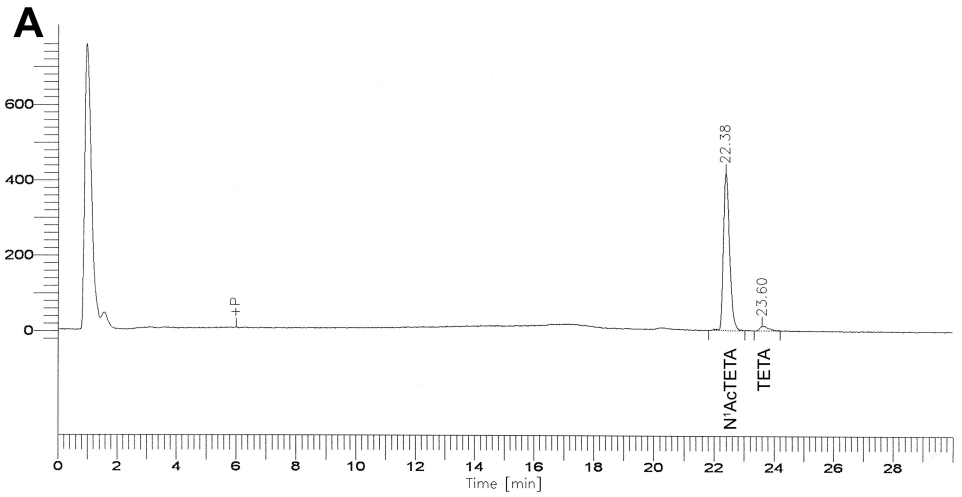
TABLE 4

Effect of TETA on the activity of hepatic N-acetylases

Mice were given injections of TETA (300 mg/kg i.p. in PBS) or PBS only and sacrificed after 1, 2, or 4 h. *N*-acetylase activities were determined by using the P81 disc method using 1 and 10 mM Spd or TETA as a substrate.

Genotype	Time and Treatment	Substrate			
		Spd (1 mM)	Spd (10 mM)	TETA (1 mM)	TETA (10 mM)
		pmol/10 min/mg liver wet weight			
Syngenic	Control	0.4 ± 0.2	1.3 ± 0.9	0.1 ± 0.1	2.6 ± 2.2
	TETA 1 h	2.6 ± 0.8**	5.6 ± 1.8**	1.3 ± 0.4*	3.9 ± 1.6
	TETA 2 h	2.7 ± 1.3**	5.5 ± 2.4**	1.0 ± 1.0	5.9 ± 2.8
	TETA 4 h	1.8 ± 0.6**	4.2 ± 0.9**	1.6 ± 0.5*	4.4 ± 1.2
SSAT1	Control	4.8 ± 1.0	9.0 ± 1.6	1.5 ± 0.5	5.2 ± 1.2
	TETA 1 h	16.6 ± 4.7**	25.7 ± 5.6**	4.0 ± 1.2**	8.2 ± 2.4
	TETA 2 h	28.2 ± 9.0**	45.1 ± 10.1**	4.9 ± 1.6**	11.4 ± 2.3**
	TETA 4 h	15.4 ± 12.5*	23.8 ± 17.3*	3.0 ± 1.7	6.9 ± 3.4
SSAT1-KO	Control	0.3 ± 0.1	1.5 ± 0.3	0.4 ± 0.3	2.9 ± 0.5
	TETA 1 h	1.8 ± 1.2*	4.6 ± 2.5*	1.4 ± 0.9*	3.9 ± 2.2
	TETA 2 h	2.5 ± 1.0**	2.3 ± 1.6	2.5 ± 2.1*	5.1 ± 2.2
	TETA 4 h	1.7 ± 0.4**	4.5 ± 1.3**	1.6 ± 0.2**	4.3 ± 1.2

* $P < 0.05$, statistical analysis comparing the different treatments within each genotype (0 h control versus 1-, 2-, or 4-h TETA).
** $P < 0.01$, statistical analysis comparing the different treatments within each genotype (0 h control versus 1-, 2-, or 4-h TETA).



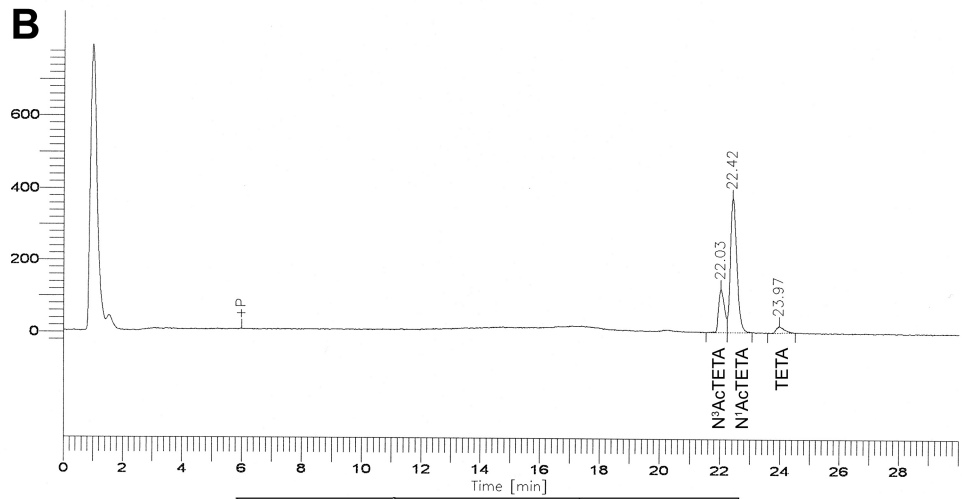


FIG. 2. HPLC graphs of 100 μM N^1AcTETA in 5% SSA at 0 h (A) and 100 μM N^1AcTETA in 5% SSA after 20 h (B) at room temperature. The table containing the respective area analysis (microvolts per second) for individual analytes is shown below B. ND, not detectable.

Analyte	A 0 h	B 20 h
	Area [μV·s]	
N^3AcTETA	ND	1609897
N^1AcTETA	6001708	5530137
TETA	259904	350970

mental Table S1). This led us to hypothesize a possible mechanism of an *N*-acetyl group migration from the terminal position to the neighboring secondary amine group (Fig. 1).

Stability of N^1AcTETA in Biological Sample Matrix. We checked the stability of N^1AcTETA and TETA in a biological sample matrix by supplementing liver homogenate with the drugs (results shown in Supplemental Table S2). TETA was relatively stable for 24 h, and N^1AcTETA was slowly converted into N^3AcTETA as detected by an increase in the peak at the elution position of Spd because N^3AcTETA coelutes with Spd that is present in liver homogenates. N^3AcTETA was quantitated using a standard curve for Spd that clearly seemed to overestimate the formation of N^3AcTETA. Neutralization of the sample matrix strongly retarded the rearrangement but hampered the quantitation of TETA and N^1AcTETA and could not be used for stabilizing HPLC samples containing TETA or its acetylated metabolites. Furthermore, 100 μM N^1N^8DiAcTETA in 5% SSA was estimated to be fully converted into N^1N^6DiAcTETA in 40 h at ambient temperature (data not shown). We quantitated N^1N^6DiAcTETA formation by using the N^1AcTETA standard curve, because we did not have N^1N^6DiAcTETA as a synthesized drug. Rearrangement and relatively low fluorescent yield in dansyl-Cl derivatization may have limited the detection of N^1N^8DiAcTETA in biological samples.

NMR and LC-MS/MS Analysis of Intramolecular *N*-*N*′ Rearrangement. After the initial HPLC studies, a series of NMR and mass spectrometry measurements under acidic conditions were carried out to monitor the acetyl migration for both N^1AcTETA and N^1N^8DiAcTETA. The easiest method was ^{1}H NMR spectroscopy, because a new methyl signal appears at approximately 0.2 ppm downfield from the original acetyl methyl signal (Fig. 3; Supplemental Fig. S1). The novel methyl signal was split into two peaks, which is typical of tertiary amides because of *cis* and *trans* conformers in the structure arising from the partial double-bond character of the C-N bond (Supplemental Fig. S1). Similar doublets have been observed also for N^4-AcetylSpd (Lurdes et al., 1989). The rest of the ^{1}H NMR spectra for the rearranged compounds N^3AcTETA and N^1N^6DiAcTETA were complicated because of the same reason, but all of the novel signals that appeared during the migration process at the NCH_2 region (3.75–3.15 ppm) were confirmed to belong to the rearranged products based on two-dimensional NMR techniques (Supplemental Figs. S3–S5). In addition, the ^{13}C NMR spectra gave

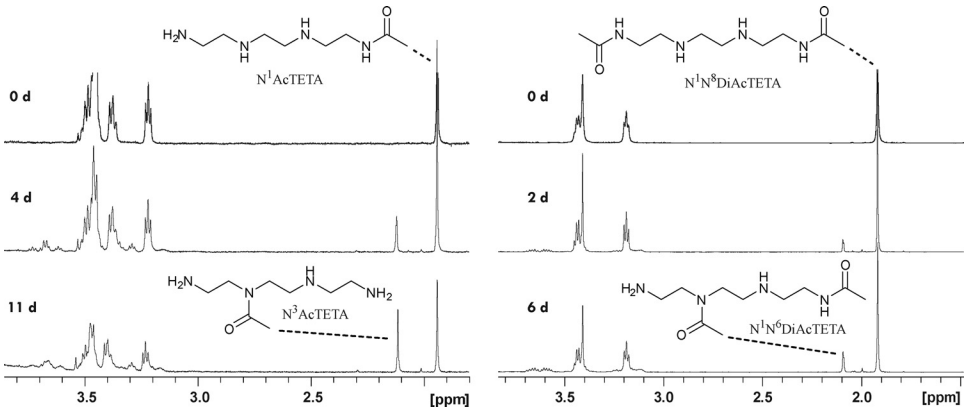


FIG. 3. Accumulation of the rearrangement products during the incubation of the compounds N^1AcTETA (left) and N^1N^8DiAcTETA (right) in aqueous 5% SSA.

the same *cis* and *trans* conformers for the compounds N^3AcTETA and N^1N^6DiAcTETA (Supplemental Fig. S2; data not shown).

After monitoring the acetyl group migration of N^1AcTETA by NMR for 12 days, a LC-MS sample was prepared and immediately analyzed. In this sample, two peaks were separated with the same molecular weight, supporting the hypothesis that the acetyl group has intramolecularly migrated from the terminal position to the nearby secondary amine (Fig. 4; Supplemental Fig. S6). Distinct daughter ions were also observed in the MS/MS spectra, and their expected structures based on the semiexact masses are shown in Fig. 4. Furthermore, only a single peak at *m/z* 189 was observed in the freshly prepared sample of N^1AcTETA. To rule out any possible bias created by LC-MS instrumentation, the first sample was also spiked with N^1AcTETA and measured, because Lu et al. (2007b) reported unresolved shoulder peaks in their LC-MS measurements from human urine sample after the oral administration of TETA.

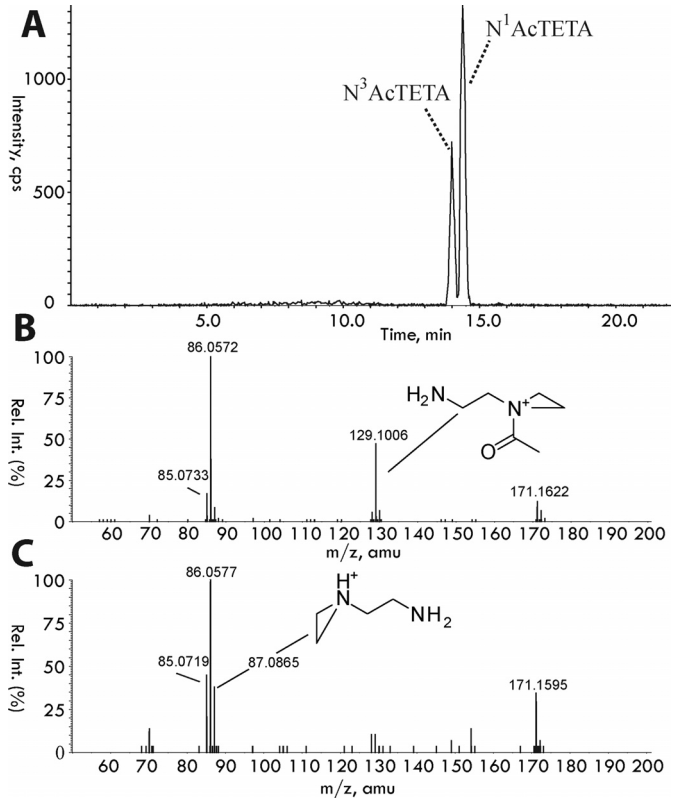


FIG. 4. LC-MS/MS analysis of N^1AcTETA. A, extracted ion chromatogram (*m/z* 189) of N^1AcTETA after 12 days of incubation in aqueous 5% SSA showing separation and detection of N^1AcTETA and N^3AcTETA. B, MS/MS spectrum of N^3AcTETA. C, MS/MS spectrum of N^1AcTETA.

Discussion

TETA has proven to be a very interesting drug molecule having potential in the treatment of several diseases (Lu, 2010). N^1AcTETA and N^1N^8DiAcTETA are characterized as the major metabolites of TETA in humans (Lu et al., 2010b), but the *N*-acetylase(s) responsible for TETA metabolism has remained uncharacterized (Lu, 2010). In this study, we demonstrated that SSAT1 acetylated TETA in vivo, because SSAT1-overexpressing mice metabolized TETA at an accelerated rate compared with syngenic mice. Furthermore, we showed that in addition to SSAT1, TETA was monoacetylated in vivo also by other acetylases, because SSAT1-KO mice metabolized TETA in a similar way than the syngenic mice.

Although N^1AcTETA and N^1N^8DiAcTETA are the major metabolites of TETA in humans (Lu et al., 2010b), we detected only N^1AcTETA from mouse tissue samples. Thus, the metabolism of TETA may be different in rodents compared with humans. Therefore, we will expand our future studies to rats and analyze serum/urine for any N^1N^8DiAcTETA in order validate the animal models in relation to metabolism of TETA in humans. Furthermore, our HPLC method is able to detect primary amines, and our efforts to detect secondary amine-containing compounds using the dansyl-Cl method may have failed because of the low level of N^1N^8DiAcTETA in biological samples treated with 5% SSA. To reliably measure TETA, N^1AcTETA, N^1N^8DiAcTETA and their acetylated rearrangement products, we are developing a feasible LC-MS/MS methodology using deuterium-labeled internal standards (Häkkinen et al., 2008). However, the current data clearly support the view that SSAT1 is not the only enzyme potentially acetylating TETA. Although SSAT1 possesses similar substrate properties for TETA as for natural substrate spermidine, the physiological relevance of SSAT1-mediated acetylation of TETA requires some additional studies.

We found that human recombinant SSAT2 efficiently acetylates of TETA in vitro. SSAT2 enzyme has just recently been characterized, and its exact physiological role is still unknown (Cavallini et al., 1991; Coleman et al., 2004; Cooper, 2004; Han et al., 2006). On the basis of our present data, TETA is clearly a substrate for human SSAT2 and may thus interfere with the enzyme protein and with the connected metabolic pathway in vivo by competing with the natural substrate thialysine. It is noteworthy that both SSAT2 and SSAT1 have been associated with the regulation of hypoxia-inducible factor 1α, which functions as a master regulator of oxygen homeostasis (Vogel et al., 2006; Baek et al., 2007a,b). In a few comparative trials, TETA has exhibited enhanced activity compared with other common metal ch-

elators (Yoshii et al., 2001; Yu et al., 2006; Lu et al., 2010a), which may be attributed to its higher copper chelation selectivity and potency compared with other tested chelators. However, the novel cellular targets of TETA are expected to explain the physiological background of its therapeutic potential in many diseases. Thus, detailed structure-activity studies using structural analogs closely resembling TETA are definitely warranted in distinct disease models to understand its drug action (Feng et al., 2009).

We have recently shown that accelerated polyamine flux by the induction of SSAT1 and simultaneous activation of ornithine decarboxylase increases energy consumption and insulin sensitivity (Pirinen et al., 2007), whereas SSAT1-KO mice develop insulin resistance upon aging (Niiranen et al., 2006). Furthermore, recent studies on TETA metabolism show that its acetylation is accelerated in type 2 diabetic patients (Lu et al., 2007a). Thus, it is tempting to speculate that type 2 diabetic patients display increased SSAT1 activity and, consequently, activated ornithine decarboxylase, i.e., accelerated polyamine flux, as a physiological compensatory mechanism to enhance insulin sensitivity (Kramer et al., 2008). Furthermore, many stressful conditions have been shown to activate SSAT1/polyamine flux (Pegg, 2008), which leads to depletion of cellular ATP and generation of reactive oxygen species. Acetylation of TETA by SSAT1 may reduce reactive oxygen species generation by polyamine-metabolizing enzymes (Babbar et al., 2007; Weisell et al., 2010), thus alleviating peripheral tissue damage among type 2 diabetic patients (Cerrada-Gimenez et al., 2011). In addition, copper-dependent amino oxidases are inhibited by TETA. Copper and/or iron chelation affects the regulation and the functions of hypoxia-inducible factor 1α, and its potential interaction with SSAT1/2 renders TETA action very complicated (Baek et al., 2007a,b; Feng et al., 2009). In light of the previous and present findings, it is clear that additional studies with TETA and SpmTrien in animal models of type 2 diabetes and cancer models are warranted (Yu et al., 2006; Gupte and Mumper, 2009; Lu et al., 2010, 2010a).

In addition to the biochemical findings, we report here for the first time an intramolecular acetyl group migration from the less hindered nitrogen to the more hindered one in the terminally *N*-monoacetylated N^1AcTETA and *N*,*N*'-diacetylated N^1N^8DiAcTETA under acidic conditions. The observed intramolecular acetyl group migration was confirmed to proceed faster in 0.6 M HCl than in 5% SSA, supporting the theory that the rearrangement is acid catalyzed. Furthermore, the rearrangement reaction was extremely slow when trihydrochlorides or dihydrochlorides of N^1AcTETA or N^1N^8DiAcTETA, respectively, were incubated in water compared with the samples incubated in 5% SSA. Moreover, the prolonged incubation of N^1N^8DiAcTETA in 5% SSA resulted in the formation of N^1AcTETA, N^3AcTETA, and, finally, TETA after deacetylations because of the lower stability of the tertiary amide in acidic water solution. Likewise, N^1AcTETA was decomposed to TETA via the same reaction pathway as N^1N^8DiAcTETA. The studies clearly implied that neutralization after acid treatment is required to retard or prevent N^1AcTETA and N^1N^8DiAcTETA rearrangement in HPLC samples. Thus, the observed rearrangement interferes with the quantitative measurements of TETA and its metabolites N^1AcTETA and N^1N^8DiAcTETA in biological samples. The detected chemical rearrangement during HPLC sample analysis may partly explain the huge individual differences reported earlier in TETA metabolism. Our data suggest that the acetyl migration can be retarded or prevented in samples when they are neutralized immediately after protein precipitation, but neutralization with Na_2CO_3 hampers TETA quantification by using HPLC.

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Authorship Contributions

Participated in research design: Cerrada-Gimenez, Weisell, Vepsäläinen, and Keinänen.

Conducted experiments: Cerrada-Gimenez, Weisell, Hyvönen, Park, and Keinänen.

Contributed new reagents or analytic tools: Weisell, Alhonen, and Vepsäläinen.

Performed data analysis: Cerrada-Gimenez, Weisell, Hyvönen, Vepsäläinen, and Keinänen.

Wrote or contributed to the writing of the manuscript: Cerrada-Gimenez, Weisell, Hyvönen, Park, Alhonen, Vepsäläinen, and Keinänen.

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