Transgenic mice over-producing putrescine in their tissues do not convert the diamine into higher polyamines

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We recently described a transgenic mouse line over-expressing the human ornithine decarboxylase gene virtually in all tissues. Despite strikingly elevated tissue putrescine concentrations, no or minimal changes were found in the levels of the higher polyamines spermidine and spermine. We have now extended these studies by further increasing tissue putrescine with the aid of 5-fluoromethylornithine, a specific inhibitor of ornithine transaminase and hence the catabolism of L-ornithine. As a result of the treatment with the latter drug, the concentration of putrescine was further increased by a factor of 2–3 without any changes in the concentrations of spermidine and spermine. In the testis of transgenic mice treated with 5-fluoromethylornithine,

the concentration of putrescine was nearly 60 times that in non-transgenic untreated animals, yet the concentration of spermidine was only 1.5-fold higher. A similar small increase in brain spermidine was accompanied by a 40-fold elevation in the concentration of putrescine. The apparent blockade between putrescine and spermidine was in all likelihood not attributable to an inhibition of S-adenosylmethionine decarboxylase, the rate-controlling enzyme in the biosynthesis of spermidine and spermine. Our results are more compatible with the view that in non-dividing adult tissues putrescine is sequestered through some unknown mechanisms in a way that makes it unavailable for the synthesis of the higher polyamines.

INTRODUCTION

The biosynthetic machinery of the polyamines (putrescine, spermidine and spermine) in mammalian cells and tissues is usually responding to mitogenic or anabolic stimuli in a sequential fashion. The activity of ornithine decarboxylase (ODC) is the first to react, leading to a rapid accumulation of cellular putrescine, followed by a much slower build-up of increased concentrations of spermidine, and eventually resulting in a decrease in spermine content (Jänne et al., 1978; Pegg, 1986). Although the biosynthetic enzymes required for the synthesis of spermidine and spermine, namely S-adenosylmethionine decarboxylase, spermidine synthase and spermine synthase, may be induced in response to growth-promoting stimuli (Hannonen et al., 1972), it is possible that the increased accumulation of tissue putrescine is sufficient to initiate the enhanced accumulation of spermidine, as this diamine acts as a low-molecular-mass activator of S-adenosylmethionine decarboxylase (Pegg and Williams-Ashman, 1968), and its ambient concentration, and not the amount of the enzyme, may control the rate of the decarboxylation of S-adenosylmethionine *in vivo* (Hölttä and Jänne, 1972). Experimental findings, some of them dating back to the late 1960s, have indicated that under certain circumstances, such as during the pre-replicative phase of rat liver regeneration (Jänne, 1967) and in cultured cells following hypo-osmotic stress (Käpyaho and Jänne, 1982; Poulin et al., 1991), the conversion of putrescine into spermidine is effectively blocked. This blockade appeared to be, at least partly, attributable to decreased S-adenosylmethionine decarboxylase activity (Käpyaho and Jänne, 1982; Poulin et al., 1991), but also to some unknown mechanisms involving a marked prolongation of the metabolic half-life of putrescine (Käpyaho and Jänne, 1982).

We have recently generated a number of transgenic mice over-expressing the human ODC gene (Halmekytö et al., 1991a,b,c).

In spite of the fact that these animals display a strikingly enhanced ODC activity and grossly elevated tissue putrescine levels, the concentrations of spermidine and spermine remained virtually unaltered in the tissues of the transgenic mice (Halmekytö et al., 1991b). We have not extended these studies by further increasing the concentration of tissue putrescine by preventing the catabolism of L-ornithine through a specific inhibition of ornithine transaminase by 5-fluoromethylornithine, which is known to elevate dramatically tissue ornithine concentrations (Daune et al., 1988). The treatment increased the concentration of putrescine to a level amounting to 50 % of the total polyamines in some tissues (testis) of transgenic animals, yet the concentrations of tissue spermidine did not change to any appreciable extent.

EXPERIMENTAL

Transgenic animals

The transgenic mice used in this study were adult males of the K2 line harbouring 24 copies of the human ODC gene in their genome (Halmekytö et al., 1991c). As regards the metabolism of the polyamines, the animals have been described in details (Halmekytö et al., 1991b). Syngenic littermates served as controls.

Analytical methods

The concentrations of the polyamines were determined by a h.p.l.c. (Hewlett-Packard, Waldbronn, Germany) method as described in detail by Hyvönen et al. (1992). The activities of ODC (Jänne and Williams-Ashman, 1971) and S-adenosylmethionine decarboxylase (Pegg and Williams-Ashman, 1968) were assayed by published methods. The two-tailed *t* test was used for statistical analyses.

Materials

5-Fluoromethylornithine was generously given by Dr. Nikolaus Seiler (Merrell Dow Research Institute, Strasbourg, France). L-[1-¹⁴C]Ornithine (sp. radioactivity 61 Ci/mol) and DL-[1-¹⁴C]-methionine (sp. radioactivity 50 Ci/mol) were purchased from Amersham International (Amersham, Bucks., U.K.).

RESULTS

Table 1 shows the effect of 5-fluoromethylornithine (0.03% in drinking water for 14 days) on the concentration of tissue polyamines in non-transgenic and transgenic mice. As indicated in Table 1, the drug increased the concentration of tissue putrescine in both syngenic (in some tissues the concentration of putrescine was just at or below the detection limit) and

Table 1 Effect of 5-fluoromethylornithine on the concentrations of putrescine, spermidine and spermine in tissues of non-transgenic and transgenic mice

5-Fluoromethylornithine (5-FMO) was administered in drinking water (0.03%) for 14 days. Results are means $\pm$ S.D. from four animals in the group; n.d., not detected. Significance of the differences (produced by the treatment): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Tissue	Mice and treatment	Content (nmol/g wet wt.)		
		Putrescine	Spermidine	Spermine
Testis	Non-transgenic	15 $\pm$ 3	291 $\pm$ 10	488 $\pm$ 28
	Non-transgenic + 5-FMO	30 $\pm$ 7**	270 $\pm$ 24	519 $\pm$ 24
	Transgenic	412 $\pm$ 68	447 $\pm$ 17	417 $\pm$ 11
	Transgenic + 5-FMO	865 $\pm$ 58***	457 $\pm$ 28	441 $\pm$ 29
Brain	Non-transgenic	3 $\pm$ 1	226 $\pm$ 37	221 $\pm$ 20
	Non-transgenic + 5-FMO	2 $\pm$ 0	269 $\pm$ 30	214 $\pm$ 13
	Transgenic	47 $\pm$ 10	295 $\pm$ 4	233 $\pm$ 11
	Transgenic + 5-FMO	122 $\pm$ 37**	322 $\pm$ 40	241 $\pm$ 12
Liver	Non-transgenic	n.d.	1610 $\pm$ 228	1280 $\pm$ 46
	Non-transgenic + 5-FMO	n.d.	1000 $\pm$ 77	1090 $\pm$ 61
	Transgenic	n.d.	1350 $\pm$ 377	1240 $\pm$ 50
	Transgenic + 5-FMO	32 $\pm$ 6***	1440 $\pm$ 337	1190 $\pm$ 123
Spleen	Non-transgenic	72 $\pm$ 32	1500 $\pm$ 235	888 $\pm$ 47
	Non-transgenic + 5-FMO	91 $\pm$ 1	1170 $\pm$ 267	797 $\pm$ 102
	Transgenic	142 $\pm$ 12	1370 $\pm$ 204	775 $\pm$ 121
	Transgenic + 5-FMO	291 $\pm$ 64**	1940 $\pm$ 719	997 $\pm$ 165
Heart	Non-transgenic	n.d.	211 $\pm$ 11	352 $\pm$ 29
	Non-transgenic + 5-FMO	n.d.	159 $\pm$ 26	366 $\pm$ 62
	Transgenic	25 $\pm$ 8	164 $\pm$ 20	371 $\pm$ 25
	Non-transgenic + 5-FMO	45 $\pm$ 11*	162 $\pm$ 27	424 $\pm$ 65

Table 2 Effect of 5-fluoromethylornithine on the activity of ODC in tissues of non-transgenic and transgenic mice

5-Fluoromethylornithine (5-FMO) treatment was as in Table 1. Results are means $\pm$ S.D. from four animals in the group; n.d., not detected. Significance of the differences (produced by the treatment): ** $P < 0.01$; *** $P < 0.001$.

Mice and treatment	ODC activity (pmol/mg wet wt./h)				
	Testis	Brain	Liver	Spleen	Heart
Non-transgenic	17 $\pm$ 9	0.1 $\pm$ 0.1	0.3 $\pm$ 0.2	0.2 $\pm$ 0.3	n.d.
Non-transgenic + 5-FMO	8 $\pm$ 3	0.1 $\pm$ 0.1	0.2 $\pm$ 0.2	n.d.	n.d.
Transgenic	666 $\pm$ 138	18 $\pm$ 5	11 $\pm$ 1.3	19 $\pm$ 3.9	13 $\pm$ 2.1
Transgenic + 5-FMO	163 $\pm$ 39***	10 $\pm$ 3**	4.2 $\pm$ 1.4**	6.7 $\pm$ 1.2**	1.9 $\pm$ 1.0***

Table 3 Effect of 5-fluoromethylornithine on the activity of S-adenosylmethionine decarboxylase in testis and brain of non-transgenic and transgenic mice

5-Fluoromethylornithine (5-FMO) treatment was as in Table 1. Results are means $\pm$ S.D. from four animals in the group. Significance of the differences (produced by the treatment): * $P < 0.05$; ** $P < 0.01$.

Mice and treatment	S-Adenosylmethionine decarboxylase activity (pmol/mg wet wt./h)	
	Testis	Brain
Non-transgenic	16.8 $\pm$ 2.2	19.9 $\pm$ 3.1
Non-transgenic + 5-FMO	10.8 $\pm$ 0.7**	19.5 $\pm$ 2.6
Transgenic	26.6 $\pm$ 0.6	14.3 $\pm$ 1.0
Transgenic + 5-FMO	29.2 $\pm$ 1.8*	15.3 $\pm$ 1.5

transgenic mice. If anything, the effect was more pronounced (2–3-fold) in the tissues of transgenic animals. It is noteworthy that in testis, for instance, the concentration of putrescine was nearly 60 times higher in transgenic animals receiving 5-fluoromethylornithine than in non-treated syngenic animals, yet the concentration of spermidine was only increased by a factor of about 1.5 (Table 1). The changes in putrescine and spermidine contents were very much the same also in the brain (Table 1). In no tissues of non-transgenic or transgenic animals was the concentration of spermidine (or spermine) significantly increased in response to the treatment with 5-fluoromethylornithine (Table 1).

Table 2 indicates that the elevated putrescine content depressed ODC activity in all (where reliably detectable) tissues of both non-transgenic and transgenic mice. The effect of the drug appeared to be more pronounced on the transgene-derived activity (Table 2).

As the decarboxylation of S-adenosylmethionine is in all likelihood the rate-controlling reaction in the biosynthesis of spermidine and spermine, we measured the effect of 5-fluoromethylornithine on the activity of S-adenosylmethionine decarboxylase in testis and brain. The results presented in Table 3 clearly indicate that the blockade between putrescine and spermidine is not attributable to a decreased S-adenosylmethionine decarboxylase activity in testis and brain, the tissues with most massive putrescine accumulation. Although the enzyme activity was marginally, yet significantly, decreased in testis of non-transgenic mice in response to 5-fluoromethylornithine, the opposite, i.e. a significant increase in the enzyme activity in

Table 4 Urinary concentrations of the polyamines in transgenic and non-transgenic mice

The results are means $\pm$ S.D. from four animals in the group.

Mice	Concn. (nmol/ml of urine)				
	Putrescine	Spermidine	Spermine	N ¹ -Acetyl-spermidine	N ⁸ -Acetyl-spermidine
Non-transgenic	79 $\pm$ 48	16 $\pm$ 14	0.7 $\pm$ 0.8	23 $\pm$ 12	3 $\pm$ 2
Transgenic	99 $\pm$ 33	18 $\pm$ 8	1.1 $\pm$ 1.3	26 $\pm$ 13	2 $\pm$ 1

response to the drug, took place in the transgenic animals (Table 3). No changes whatsoever were found in *S*-adenosylmethionine decarboxylase activity in the brain after 5-fluoromethylornithine administration (Table 3).

As one of the mechanisms for clearing the excess of polyamines formed as a result of transgene-derived ODC activity could be an enhanced urinary excretion, we determined the urinary concentrations of putrescine, spermidine and spermine together with monoacetylated (N¹ and N⁸) spermidine in non-transgenic and transgenic mice. The results presented in Table 4 do not support this possibility, for there were no significant differences between syngenic and transgenic animals in urinary excretion of the polyamines.

DISCUSSION

One of the most intriguing phenotypic features of the transgenic mice harbouring the human ODC gene is the finding that, in spite of grossly elevated ODC activity sometimes associated with a massive tissue putrescine accumulation, the concentrations of spermidine and spermine remained practically unaltered (Halmekytö et al., 1991b). An extensive survey of the metabolism of the polyamines, including determination of the activities of all biosynthetic and catabolic enzymes, did not reveal any meaningful explanation for the apparent blockade of putrescine conversion into the higher polyamines (Halmekytö et al., 1991b). The activity of *S*-adenosylmethionine decarboxylase, which probably is critical for the further metabolism of putrescine to spermidine and spermine, was significantly changed in some tissues of the transgenic animals, but these changes were inconsistent (Halmekytö et al., 1991b), as likewise seen in the present study (Table 3). Although tissue putrescine levels were elevated in the transgenic mice by a factor of 15–25 (brain and testis), we were able to expand tissue putrescine pools further by inhibiting the catabolism of L-ornithine with 5-fluoromethylornithine, a specific inhibitor of ornithine transaminase (Daune et al., 1988). Ornithine transaminase is the major 'consumer' of L-ornithine in mammalian tissues, switching this amino acid to the oxidative metabolism of glutamate (Daune et al., 1988). An inhibition of ornithine transaminase is known to result in a dramatic elevation of ornithine concentrations in virtually all tissues (Daune et al., 1988; Seiler et al., 1989). As ornithine concentrations in most tissues under normal conditions are well below the K_m value for ODC (Seiler et al., 1989), elevated tissue ornithine pools lead to an enhanced formation of putrescine (Daune et al., 1988; Seiler et al., 1989). Interestingly, the moderately elevated tissue putrescine contents in response to the administration of 5-fluoromethylornithine were not associated with changes in the concentrations of spermidine and spermine

(Seiler et al., 1989). The latter authors found that the drug enhanced urinary excretion of both putrescine and spermidine. This seems not to be the case in our transgenic mice, as, despite the strikingly elevated tissue putrescine pools, we have not found any signs of enhanced urinary excretion of putrescine or spermidine (Table 4). Similarly, we found that the activity of spermidine/spermine acetyltransferase was unaltered in transgenic mice (Halmekytö et al., 1991b) and that the increase in putrescine content in any of the tissues with or without 5-fluoromethylornithine was not associated with elevations of N¹-acetylspermidine content (results not shown), which is a metabolic marker of enhanced secretion.

Our present results are very much in line with the views expressed by Davis et al. (1992) in their recent review that one of the major functions of the polyamine homeostatic system in non-stressed (or non-dividing) mammalian cells is to prevent an excessive accumulation of the higher polyamines spermidine and spermine. Thus mammalian cells appear to tolerate remarkably high concentrations of putrescine, but may not be able to survive in the presence of extreme pools of spermidine and spermine. An enhanced accumulation of the latter polyamines would be appropriate only after cell division, providing more intracellular chemical binding sites for their sequestration (Davis et al., 1992). The mechanisms by which an excessive accumulation of spermidine and spermine can be prevented under conditions of elevated ODC activity and enhanced putrescine accumulation are only incompletely known. These mechanisms may include an enhanced secretion and urinary excretion of putrescine and spermidine as a result of polyamine acetylation, as demonstrated by Seiler et al. (1989) in connection with their experiments with 5-fluoromethylornithine. These likewise include a marked fall of the activity of *S*-adenosylmethionine decarboxylase, as shown to occur during hypo-osmotic stress (Käpyaho and Jänne, 1982; Poulin et al., 1991), probably also contributing to the prolonged metabolic half-life of putrescine under these conditions (Käpyaho and Jänne, 1982). None of these mechanisms is readily applicable to the conservation of putrescine in the transgenic animals over-expressing ODC. It is, however, possible that the enzyme activities assayed *in vitro* may not necessarily reflect the enzyme activities *in vivo*, for the substrate or coenzyme concentrations could become limiting factors under these conditions. This is exemplified by the fact that testicular ODC could produce 666 pmol of putrescine/h per g of tissue (Table 2), yet the steady-state putrescine concentration was only 412 nmol/g (Table 1). A further mechanism, so far entirely speculative, would be a chemical sequestration of putrescine in non-dividing cells making it unavailable as a precursor for the higher polyamines. This seclusion would only be released upon cell division, possibly by a dilution of the sequestering component. Direct experimental evidence to prove this possibility, however, may be difficult to obtain.

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REFERENCES

- Daune, G., Gerhart, F. and Seiler, N. (1988) *Biochem. J.* **253**, 481–488
- Davis, R. H., Morris, D. R. and Coffino, P. (1992) *Microbiol. Rev.* **56**, 280–290
- Halmekytö, M., Hyttinen, J.-M., Sinervirta, R., Utriainen, M., Myöhänen, S., Voipio, H.-M., Wahlfors, J., Syrjänen, S., Syrjänen, K., Alhonen, L. and Jänne, J. (1991a) *J. Biol. Chem.* **266**, 19746–19751
- Halmekytö, M., Alhonen, L., Wahlfors, J., Sinervirta, R., Eloranta, T. and Jänne, J. (1991b) *Biochem. J.* **278**, 895–898

- Halmekeytö, M., Alhonen, L., Wahlfors, J., Sinervirta, R., Jänne, O. A. and Jänne, J. (1991c) *Biochem. Biophys. Res. Commun.* **180**, 262–267
- Hannonen, P., Raina, A. and Jänne, J. (1972) *Biochim. Biophys. Acta* **273**, 84–90
- Hölttä, E. and Jänne, J. (1972) *FEBS Lett.* **23**, 117–121
- Hyvönen, T., Keinänen, T. A., Khomutov, A. R., Khomutov, R. M. and Eloranta, T. O. (1992) *J. Chromatogr.* **574**, 17–21
- Jänne, J. (1967) *Acta Physiol. Scand. Suppl.* **300**, 1–71
- Jänne, J., Pösö, H. and Raina, A. (1978) *Biochim. Biophys. Acta* **473**, 241–293
- Jänne, J. and Williams-Ashman, H. G. (1971) *J. Biol. Chem.* **246**, 1725–1732
- Käpyaho, K. and Jänne, J. (1982) *Biochim. Biophys. Acta* **714**, 93–100
- Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- Pegg, A. E. and Williams-Ashman, H. G. (1968) *Biochem. Biophys. Res. Commun.* **30**, 76–82
- Poulin, R., Wechter, R. S. and Pegg, A. E. (1991) *J. Biol. Chem.* **266**, 6142–6151
- Seiler, N., Daune, G., Bolkenius, F. N. and Knödgen, B. (1989) *Int. J. Biochem.* **21**, 425–432

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