Altered Polyamine Metabolism in the PRO/Re Strain of Inbred Mice

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The PRO/Re strain of inbred mice are characterized by abnormally high concentrations of proline in both blood (hyperprolinaemia) and urine (prolinuria). They excrete increased amounts of polyamines in their urine. Male PRO/Re mice excreted putrescine at 175% and spermidine at 300% the amount of male C57BL/6J controls. Female PRO/Re mice excreted putrescine at 115% and spermidine at 150% of the amount in the urine of female controls. Examination of the enzymes involved in polyamine biosynthesis revealed that ornithine decarboxylase, the initial enzyme in the polyamine-biosynthetic pathway, was increased by 150% in the kidneys and by 100% in the liver of male PRO/Re mice. There was no significant difference between PRO/Re and C57BL/6J male mice for either putrescine- or spermidine-stimulated *S*-adenosylmethionine decarboxylase activity. Female PRO/Re mice showed no significant difference from female C57BL/6J mice for any of the enzymes examined. When the concentrations of the polyamines in the tissues of the PRO/Re mice were determined, spermidine and spermine concentrations in the kidneys of the male PRO/Re mice were twice those of the controls. Spermidine concentration in the livers of both male and female PRO/Re mice was approx. 130% that of the controls. Polyamine concentrations in the brains were similar in controls and mutants. The increased polyamine biosynthesis and excretion in the PRO/Re mutant mice may be a mechanism to decrease the extent of proline accumulation.

The PRO/Re strain of inbred mice is characterized by a markedly higher concentration of proline in both blood (hyperprolinaemia) and urine (prolinuria) (Blake & Russell, 1972). Since proline is intimately involved in the regulation of ornithine synthesis (Scheme 1), it seemed possible that the increased intracellular proline concentration could affect the metabolism of ornithine and thus the biosynthesis and accumulation of the polyamines.

Mammalian polyamine biosynthesis is dependent on the decarboxylation of ornithine by ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) to form putrescine. This is the rate-limiting step in the formation of the polyamines. Putrescine, in turn, acts as an acceptor for a propylamine moiety transferred from decarboxylated *S*-adenosylmethionine by *S*-adenosylmethionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50) to form spermidine, which can accept a second propylamine moiety derived from a second molecule of decarboxylated *S*-adenosylmethionine to form spermine (Scheme 1).

A preliminary screening indicated that the PRO/Re mice, which are phenotypically normal, did indeed excrete significantly greater amounts of putrescine and spermidine in their urine. Investigation of ornithine decarboxylase and *S*-adenosylmethionine

decarboxylase activities, as well as the concentrations of the polyamines in various tissues, indicated that polyamine biosynthesis and accumulation in liver and kidney were increased in the PRO/Re strain of inbred mice as compared with the C57BL/6J mice. The increased biosynthesis, accumulation and excretion of the polyamines seen in the PRO/Re mice may represent a mechanism for lowering the elevated concentration of proline.

Experimental

Animals

C57BL/6J and PRO/Re mice (5–6 weeks old) were obtained from the Jackson Laboratory, Bar Harbor, ME, U.S.A. Strain C57BL/6J is one of the parental lines used to develop strain PRO/Re and has been reported to have normal amounts of proline in the urine and blood (Blake & Russell, 1972). Mice of the C57BL/6J strain were therefore used as the controls in this study.

Materials

DL-[1-¹⁴C]Ornithine (7.7 mCi/mmol) and *S*-adenosyl-L-[carboxyl-¹⁴C]methionine (11.0 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Dithiothreitol and pyridoxal phosphate were purchased from Cal-

Scheme 1. Schematic involvement of proline in the metabolism of ornithine and the biosynthesis of the polyamines

biochem, San Diego, CA, U.S.A. The hydrochloride salts of putrescine, spermidine and spermine were obtained from Sigma Chemical Co., Boston, MA, U.S.A.

Determination of the amount of putrescine, spermidine and spermine excreted in urine

Urine was collected from male and female PRO/Re and C57BL/6J mice selected at random from the Jackson Laboratory mouse colony. Samples were collected for 24 h with 0.5 ml of toluene. The pH was adjusted to 3.0 with 5 M-HCl at the end of the collection period. Collected urine (50 μ l) was spotted on a filter-paper disc and dried. The sample was eluted from the disc with 0.5 ml of water and hydrolysed for 14–16 h at 110°C in 6 M-HCl. The sample was centrifuged, transferred and evaporated. The residue was dissolved in 200 μ l of 0.1 M-HCl, and a 50 μ l portion of this was applied to the column of the Durrum D-500 amino acid analyser. The polyamines were then separated and measured as described by Russell & Russell (1975).

Enzymic assay procedures

Mice were killed by cervical dislocation and the livers and kidneys rapidly excised, chilled and weighed. The tissues were homogenized in 4 vol. of 0.05 M- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 7.2, containing 1.0 mM-dithiothreitol.

Ornithine decarboxylase activity was determined by

measuring the liberation of $^{14}\text{CO}_2$ from DL-[1- ^{14}C]-ornithine as described by Russell & Snyder (1969), except that 1 mM-dithiothreitol was used in the 0.05 M- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 7.2. Although the substrate concentration used was non-saturating (0.2 mM), the same changes were noted when an excess of ornithine (2 mM) was used as the substrate in some experiments.

S-Adenosylmethionine decarboxylase activity was determined by measuring the release of $^{14}\text{CO}_2$ from S-adenosyl-L-[carboxyl- ^{14}C]methionine in the presence of either 2.5 mM-putrescine or 5.0 mM-spermidine as acceptors of the propylamine moiety (Russell, 1972).

Determinations of the concentrations of putrescine, spermidine and spermine in various tissues

Mice were killed by cervical dislocation. The brains, kidneys and livers were rapidly excised, chilled on ice, blotted, weighed and frozen until assayed. The frozen tissues were sonicated for 1 min at 0–4°C with an ultrasonic cell disrupter equipped with a 4.5 in (11.2 cm) probe (Electro-Mechanic Instrument Co., Perkasi, PA, U.S.A.) in 4 vol. of 5% (w/v) trichloroacetic acid at 0–4°C. A sample of the supernatant (1–5 mg of tissue) was applied to the column of the Durrum D-500 amino acid analyser (Palo Alto, CA, U.S.A.). This methodology has been described previously (Marton *et al.*, 1973; Russell & Russell, 1975).

Other determinations

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co., Chicago, IL, U.S.A.) as the standard. Creatinine was measured by a micro-adaptation of the Jaffé technique (Hawk *et al.*, 1954). There was no significant difference between PRO/Re and C57BL/6J mice of the same sex in the excretion of creatinine.

Statistics

The significance of differences between means was assessed by Student's *t* test.

Results

Concentration of putrescine, spermidine and spermine excreted in the urine

Male PRO/Re mice excreted putrescine at 175% and spermidine at 300% of the amounts by C57BL/6J controls (Table 1). PRO/Re females excreted slightly more putrescine and spermidine, at 150% of control values. Spermine was not detectable in any of the urine samples examined.

Ornithine decarboxylase activity in the kidney and liver

Kidney. Ornithine decarboxylase activity in the kidneys of PRO/Re males was 250% of the control value (Table 2). When the cortex and medulla of each kidney were assayed separately, the difference in enzyme activity was found to be in the kidney cortex. From mean values of triplicate determinations in two mice, enzyme activity in the medullae of the PRO/Re and control mice was approximately equal (8.73 μ mol of $^{14}\text{CO}_2$ /30 min per mg of protein), but activity in the PRO/Re-mouse kidney cortex was 150% that of the

control (21.86 versus 13.9 μ mol of $^{14}\text{CO}_2$ /30 min per mg of protein). There was no significant difference between female mutant and control mice.

Liver. PRO/Re males had ornithine decarboxylase activity in the liver 200% that of controls (Table 2).

Putrescine-stimulated *S*-adenosylmethionine decarboxylase activity in the kidney and liver

Kidney. Putrescine-stimulated *S*-adenosylmethionine decarboxylase activity in mutant males was 80% that of the control males. The mutant and control females had similar enzyme activities (Table 2).

Liver. Putrescine-stimulated *S*-adenosylmethionine decarboxylase activity was slightly higher in mutant male mice (113% of control, Table 2).

Table 1. Urinary polyamine concentrations in male and female C57BL/6J and PRO/Re mice

Analyses were as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of duplicate determinations done on samples from five or more mice.

	Polyamines (nmol/ μ mol of creatinine)		
	Putrescine	Spermidine	Spermine
Males			
PRO/Re	*245 $\pm$ 18	*21 $\pm$ 2	<0.02
C57BL/6J	140 $\pm$ 21	7 $\pm$ 1	<0.02
Females			
PRO/Re	36 $\pm$ 4	†17 $\pm$ 2	<0.02
C57BL/6J	31 $\pm$ 4	11 $\pm$ 2	<0.02

* Data differ from control ($P < 0.005$).

† Data differ from control ($P < 0.05$).

Table 2. Activity of the polyamine-biosynthetic enzymes in the kidney and liver of male and female C57BL/6J and PRO/Re mice

Analyses were as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of triplicate determinations on pools of three or more mice.

Enzyme activity (pmol of $^{14}\text{CO}_2$ /30 min per mg of protein)			
	Ornithine decarboxylase	Putrescine-stimulated <i>S</i> -adenosylmethionine decarboxylase	Spermidine-stimulated <i>S</i> -adenosylmethionine decarboxylase
Kidney			
Male			
PRO/Re	*16.47 $\pm$ 0.82	1.35 $\pm$ 0.18	0.69 $\pm$ 0.03
C57BL/6J	6.94 $\pm$ 0.42	1.98 $\pm$ 0.06	0.94 $\pm$ 0.15
Female			
PRO/Re	0.12 $\pm$ 0.02	0.66 $\pm$ 0.05	0.19 $\pm$ 0.01
C57BL/6J	0.12 $\pm$ 0.01	0.70 $\pm$ 0.08	0.29 $\pm$ 0.01
Liver			
Male			
PRO/Re	0.26 $\pm$ 0.05	0.86 $\pm$ 0.05	0.29 $\pm$ 0.06
C57BL/6J	*0.12 $\pm$ 0.03	0.74 $\pm$ 0.09	0.31 $\pm$ 0.03

* Data differ from control ($P < 0.005$).

Table 3. Polyamine concentrations in brain, kidney and liver of male and female C57BL/6J and PRO/Re mice

Analyses were as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of duplicate determinations from five mice.

	Polyamine concentrations (nmol/mg wet weight)		
	Putrescine	Spermidine	Spermine
Brain			
Male C57BL/6J	0.04 $\pm$ 0.01	0.55 $\pm$ 0.01	0.31 $\pm$ 0.02
Male PRO/Re	0.03 $\pm$ 0.01	0.55 $\pm$ 0.05	0.29 $\pm$ 0.04
Female C57BL/6J	0.03 $\pm$ 0.01	0.44 $\pm$ 0.01	0.26 $\pm$ 0.01
Female PRO/Re	0.03 $\pm$ 0.01	0.45 $\pm$ 0.01	0.28 $\pm$ 0.03
Kidney			
Male C57BL/6J	0.04 $\pm$ 0.01	0.32 $\pm$ 0.04	0.35 $\pm$ 0.06
Male PRO/Re	0.05 $\pm$ 0.01	*0.43 $\pm$ 0.04	†0.70 $\pm$ 0.11
Female C57BL/6J	0.03 $\pm$ 0.01	0.14 $\pm$ 0.02	0.36 $\pm$ 0.04
Female PRO/Re	0.03 $\pm$ 0.01	0.13 $\pm$ 0.01	0.27 $\pm$ 0.02
Liver			
Male C57BL/6J	0.03 $\pm$ 0.00	0.63 $\pm$ 0.01	0.46 $\pm$ 0.05
Male PRO/Re	0.03 $\pm$ 0.00	†0.80 $\pm$ 0.10	0.42 $\pm$ 0.01
Female C57BL/6J	0.05 $\pm$ 0.00	0.66 $\pm$ 0.03	0.44 $\pm$ 0.01
Female PRO/Re	0.04 $\pm$ 0.01	†0.89 $\pm$ 0.04	0.42 $\pm$ 0.01

* Data differ from control ($P < 0.01$).

† Data differ from control ($P < 0.025$).

Spermidine-stimulated S-adenosylmethionine decarboxylase activity in the kidney and liver

Kidney. Neither male nor female mutant showed a significant difference from controls (Table 2).

Liver. Spermidine-stimulated S-adenosylmethionine decarboxylase activity was similar in both mutant and control male mice (Table 2).

Concentrations of putrescine, spermidine and spermine in various tissues

Kidney. Male PRO/Re mice had 200% as much spermine and 134% as much spermidine as the control males, but similar amounts of putrescine. Female mutant and control mice showed no significant difference (Table 3).

Liver. Male PRO/Re mice had 127% as much spermidine as control males but similar amounts of putrescine and spermine. The female mutant mice had 135% as much spermidine as the female controls but similar amounts of putrescine and spermine.

Brain. Concentrations of putrescine, spermidine and spermine in the brains of mice of both PRO/Re and C57BL/6J strains were similar.

Discussion

The altered polyamine biosynthesis and accumulation seen in the PRO/Re strain of inbred mice probably directly reflects the hyperprolinaemia which characterizes this strain of mice. The differing concentrations of putrescine and spermidine ex-

creted by male and female mice of the same inbred strain, whether PRO/Re or C57BL/6J, are consistent with previous reports of sexual dimorphism in the excretion of free amino acids in man (Liappis, 1973). For the mice, however, this dimorphism is evidenced in both the accumulation of the polyamines and the activities of the polyamine-biosynthetic enzymes as well as the excretion of the polyamines. The differences in excretion patterns, in this case at least, seem to directly reflect differences in the activities of the polyamine-biosynthetic enzymes. That this dimorphism is more marked in the PRO/Re mice than the C57BL/6J mice is probably the result of a general increase in polyamine biosynthesis in the PRO/Re strain.

Studies of polyamine biosynthesis and metabolism indicate that there is a rapid interconversion of one polyamine into another (Rosenthal & Tabor, 1956; Siimes, 1967; Jänne, 1967; Clark & Fair, 1975). For example, within 1 h of the administration of [^{14} C]-ornithine, all of the acid-soluble radiolabel was accounted for by [^{14} C]putrescine, [^{14} C]spermidine and [^{14} C]spermine (Russell *et al.*, 1970). Not only can spermidine be converted into spermine, but spermine can be reconverted into spermidine (Siimes, 1967) and both spermidine and putrescine are metabolized by being oxidized to CO₂ or excreted in the urine (Rosenthal & Tabor, 1956; Jänne, 1967; Siimes, 1967). Reports on the nephrotoxicity of spermine might explain why this polyamine is not excreted but rather metabolized to spermidine with subsequent increases in spermidine accumulation and excretion (Tabor & Rosenthal, 1956).

An increase in the intracellular concentrations of the polyamines, such as that found in tumour-bearing individuals, is decreased by excretion of the polyamines into the interstitial fluid and eventually appears as increased concentrations in both serum and urine (Russell *et al.*, 1974, 1975). An acceleration in this system of polyamine synthesis and metabolism could be regarded as a possible mechanism for decreasing the overload of proline characteristic of PRO/Re mice. The higher concentrations of proline would result in an increase in the intracellular concentration of ornithine (Fig. 1). This hypothesis is supported by the report that patients with type I hyperprolinaemia and prolinuria, the type postulated for PRO/Re mice (Blake, 1972), also excreted significantly greater amounts of both glutamic acid and ornithine in their urine (Efron, 1965).

PRO/Re mice may have a unique system by which biosynthesis, accumulation and excretion of polyamines may aid in decreasing the extent of hyperprolinaemia.

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