Effect of starvation and refeeding on polyamine concentrations and ornithine decarboxylase antizyme in mammary gland of lactating rats

Margaret E. BROSAN,* Rosemary FARRELL,† Hannah WILANSKY* and Dermot H. WILLIAMSON†

*Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9, and †Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

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1. Starvation caused a marked increase in putrescine content in mammary gland of lactating rats, together with a marked decrease in activity of ornithine decarboxylase and appearance of measurable ornithine decarboxylase antizyme. 2. Refeeding for 5 h caused disappearance of free antizyme and ornithine decarboxylase activity returned to the value in fed animals. Putrescine concentration remained elevated. 3. There was no significant change in nucleic acid content of mammary gland from starved rats, but spermidine and spermine contents increased significantly. 4. Refeeding for 5 h returned the spermidine content of mammary glands to 'fed' values, and significantly decreased the content of spermine, although it did not reach control values. Thus changes in polyamine content of mammary gland in starved rats are clearly dissociated from changes in either RNA content or activities of polyamine-synthetic decarboxylases. 5. Starvation caused a fall in the content of spermidine in liver, with no change in spermine content. Refeeding for 5 h returned the spermidine content to 'fed' values.

Spermidine content in the mammary gland of lactating rats was reported to be much higher than in the non-lactating mammary tissue (Neish & Key, 1968; Russell & McVicker, 1972). It was proposed that increases in spermidine were concomitant with, and possibly required for, increases in RNA content (Russell & McVicker, 1972). Premature weaning of the pups caused a rapid fall in both spermidine and RNA contents of mammary gland (Russell & McVicker, 1972).

The metabolism of rat mammary gland is very sensitive to alterations in the dietary state of the animals (Williamson, 1980). During short-term starvation, for example, the synthesis of lactose (Carrick & Kuhn, 1978) and lipid (Robinson *et al.*, 1978), and the release of milk to the pups (Brosnan *et al.*, 1982), are depressed. We have previously shown that the activity of ornithine decarboxylase (EC 4.1.1.17) in the mammary gland of starved rats is barely detectable, and the activity of *S*-adenosyl-methionine decarboxylase (EC 4.1.1.50) is depressed (Brosnan *et al.*, 1982). These changes in enzyme activity would markedly depress the synthesis of spermidine from ornithine in mammary gland during starvation. If depressed synthesis were reflected in decreased spermidine concentration, the ability of the mammary gland to synthesize the milk proteins

casein and α -lactalbumin could be compromised (Oka & Perry, 1974).

It is known that the activity of ornithine decarboxylase can be rapidly decreased by degradation of the enzyme in the absence of continued enzyme synthesis, with a half-life in the order of minutes (Jänne *et al.*, 1976), or by binding and neutralization by ornithine decarboxylase antizyme, an inhibitory protein which is increased in activity by putrescine (Heller & Canellakis, 1981). Putrescine concentration in liver of starved mice has been reported to increase as a result of spermidine degradation (Seiler *et al.*, 1981b).

The aim of the present study was to determine whether the concentration of polyamines alters in mammary gland in situations in which milk production is known to be affected, and to determine whether the marked fall in ornithine decarboxylase activity observed under these conditions is due to the presence of ornithine decarboxylase antizyme.

Materials and methods

Animals

Rats of the Wistar strain with between 8 and 11 pups were used after a lactation period of 9–13 days. Rats were given food and water *ad libitum*, except

where described, and were maintained on a constant lighting schedule (lights on 07:30–19:30h). Starved rats were deprived of food at 09:00h. Pups were allowed to continue to suckle until the mother was killed so as to maintain the normal stimulus for milk production.

Chemicals

L-[1-¹⁴C]Ornithine was obtained from Amersham International, Amersham, Bucks., U.K. Chemicals were of the highest grade available and were obtained from BDH Chemicals, Poole, Dorset, U.K., or from Sigma Chemical Co., Poole, Dorset, U.K.

Analysis of polyamines and nucleic acids

Rats were dissected under pentobarbital anaesthesia (60mg/kg body wt.). One inguinal mammary gland was freed from surrounding tissue and rapidly cut free and clamped between tongs cooled in liquid N₂ (Wollenberger *et al.*, 1960). A sample of liver was also freeze-clamped. Polyamines were extracted from the powdered freeze-clamped tissues by addition of sulphosalicylic acid (50mg/ml, 4ml/g of tissue), followed by homogenization in a Silverson-model laboratory mixer/emulsifier for 30s. After standing for 30min at 4°C, the acid extract was centrifuged at 27000g at 4°C for 20min. Polyamines were assayed as described by Hall *et al.* (1978).

The other inguinal mammary gland was minced and rapidly cooled in homogenizing medium {0.25 M-sucrose/2 mM-EDTA/2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4}, 10ml/g of tissue. Mammary gland was homogenized in a Silverson-model laboratory mixer/emulsifier for 20–25 s. The homogenate was used for the determination of total DNA and RNA. DNA was extracted into hot HClO₄, as described by Schneider (1945), and the concentration was determined with diphenylamine reagent (Burton, 1956)

with calf thymus DNA as standard. RNA was precipitated with 0.5 M-HClO₄ and hydrolysed in 0.3 M-KOH, as described by Seiler & Schmidt-Glenewinkel (1975). The orcinol method (Ashwell, 1957) was used to determine RNA, with *Torula* yeast RNA as standard.

Assay of ornithine decarboxylase and ornithine decarboxylase antizyme

A portion of homogenate was made 5 mM with respect to dithiothreitol and was centrifuged at 27000g at 4°C for 35 min. Ornithine decarboxylase activity in the supernatant was measured by following release of ¹⁴CO₂ from L-[1-¹⁴C] ornithine, as described previously (Murphy & Brosnan, 1976). Reaction mixtures contained 0.1 μmol of pyridoxal phosphate, approx. 2–3 mg of mammary-gland protein, 50 μmol of Tris (pH 7.3), 0.2 μmol of EDTA and 0.8 μmol of L-ornithine (0.2 Ci/mol), in a final volume of 2.0 ml. Antizyme in cytosol was determined by its ability to inhibit a constant amount of ornithine decarboxylase, as described by Heller & Canellakis (1981). To ensure that the inhibition detected was due to a macromolecule, cytosol from two starved rats was dialysed against 50 mM-KCl/50 mM-Tris/HCl, pH 7.3, for 24 h at 4°C. Assays of ornithine decarboxylase and of antizyme were linear with respect to protein concentration up to 5 mg of cytosol protein. Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Starvation of lactating rats for 6–24 h did not cause any significant change in content of DNA or RNA in the mammary gland. These results are given in Table 1. Refeeding of starved rats for 2.5 or 5 h was also without significant effect on these parameters.

Table 1 also gives the content of free polyamines

Table 1. *Effect of dietary manipulations on nucleic acid and polyamine contents of mammary gland from lactating rats*
Experimental details are described in the text. Nucleic acids are expressed as mg/g and polyamines are given as μmol/g fresh wt. of tissue. The results presented are means ± s.d. for (n) determinations. Abbreviation: BLD, less than 5 nmol/g of tissue. Values that are significantly different from those for rats starved (24 h) are shown: **P* < 0.05, ***P* < 0.01.

Condition of dam	DNA	RNA	Putrescine	Spermidine	Spermine
Fed	2.5 ± 0.3 (10)	10.2 ± 3.9 (12)	BLD (8)	1.953 ± 0.232 (8)**	0.156 ± 0.037 (8)**
Starved (6 h)	2.5 ± 0.3 (4)	10.1 ± 4.6 (4)	0.034 ± 0.028 (4)*	2.141 ± 0.427 (4)	0.162 ± 0.038 (4)**
Starved (24 h)	2.8 ± 0.7 (5)	8.3 ± 2.8 (9)	0.161 ± 0.111 (8)	2.332 ± 0.191 (8)	0.250 ± 0.038 (8)
Starved (24 h) + refeed (2.5 h)	2.7 ± 0.2 (4)	8.4 ± 1.8 (6)	0.315 ± 0.221 (6)	2.253 ± 0.391 (6)	0.223 ± 0.011 (6)
Starved (24 h) + refeed (5.0 h)	2.9 ± 0.4 (8)	8.3 ± 2.5 (9)	0.136 ± 0.112 (9)	2.043 ± 0.226 (9)**	0.211 ± 0.030 (9)*

Table 2. *Effect of dietary manipulations on ornithine decarboxylase activity and ornithine decarboxylase antizyme in mammary gland from lactating rats*

Experimental details are described in the text. Ornithine decarboxylase activity is expressed as pmol/h per mg of cytosol protein. Antizyme activity is given as percentage inhibition of a standard preparation of ornithine decarboxylase from fed rats. Approx. 2 mg of cytosol protein containing antizyme was added to the ornithine decarboxylase preparation. The results presented are means $\pm$ s.d. for n experiments. Abbreviation: BLD, below the limits of detection. Values that are significantly different from fed rats are shown: ** $P < 0.01$.

Condition of dam	n	Ornithine decarboxylase	Antizyme
Fed	6	387 $\pm$ 135	2 $\pm$ 6
Starved (24 h)	6	14 $\pm$ 17**	31 $\pm$ 17**
Starved (24 h), cytosol dialysed	2	BLD	51
Starved (24 h) + refed (2.5 h)	4	28 $\pm$ 16**	35 $\pm$ 14**
Starved (24 h) + refed (5.0 h)	4	351 $\pm$ 183	2 $\pm$ 4

measured in mammary gland of these rats. In the fed rats, putrescine was not detectable. Spermidine content was approx. 2 μ mol/g fresh wt. of tissue, and the content of spermine was less than 10% of that of spermidine. When food had been removed from lactating rats for only 6 h, putrescine was readily detectable, and a very marked accumulation of this diamine resulted by 24 h of starvation. Spermidine and spermine also increased significantly in mammary gland of 24 h-starved rats. After refeeding for 5 h, putrescine content was still elevated, but spermidine had returned to 'fed' values and spermine had also significantly decreased, although not yet to control values.

Ornithine decarboxylase activity and the activity of ornithine decarboxylase antizyme are given in Table 2. The activity of ornithine decarboxylase in mammary gland of fed rats was quite high, and declined to barely measurable values after 24 h starvation. At the same time, there was appearance of measurable antizyme activity. Under the conditions of assay, only antizyme in excess of that needed to neutralize endogenous ornithine decarboxylase would be measured (Heller & Canellakis, 1981). When starved rats were refed for 2.5 h, there was no detectable change in ornithine decarboxylase or antizyme activity. However, refeeding for 5 h resulted in complete disappearance of free antizyme, and ornithine decarboxylase activity was returned to the value observed in fed rats.

The polyamine concentration in liver of lactating rats is shown in Table 3. In the fed rats, spermidine

Table 3. *Effect of dietary manipulations on polyamine content of liver from lactating rats*

Experimental details are described in the text. Polyamines are given as μ mol/g fresh wt. of tissue; putrescine was less than 5 nmol/g of tissue. The results presented are means $\pm$ s.d. for n experiments. Values that are significantly different from fed rats are shown: * $P < 0.05$.

Condition of dam	n	Spermidine	Spermine
Fed	5	0.637 $\pm$ 0.043	0.757 $\pm$ 0.223
Starved (24 h)	5	0.532 $\pm$ 0.073*	0.813 $\pm$ 0.288
Starved (24 h) + refed (5.0 h)	6	0.718 $\pm$ 0.110	0.931 $\pm$ 0.196

content of liver was considerably lower, and that of spermine considerably higher, than that in mammary gland. Starvation for 24 h caused a significant fall in the hepatic spermidine content, without effect on the content of spermine. Refeeding for 5 h returned the spermidine content to 'fed' values, but that of spermine remained unchanged.

Discussion

Putrescine content and ornithine decarboxylase antizyme

Despite a marked decrease in ornithine decarboxylase activity by 6 h of food deprivation (Brosnan *et al.*, 1982), the putrescine content of mammary gland increased dramatically over 24 h starvation. In mice starved for 24 h, hepatic putrescine concentrations were increased, as a result of increased activity of spermidine N^1 -acetyltransferase and subsequent oxidation of the N^1 -acetyl-spermidine to putrescine by polyamine oxidase (Seiler *et al.*, 1981b). If this pathway were also present and activated in mammary gland of starved rats, it could account for the increase in putrescine observed in these glands. Alternatively, the increased putrescine in mammary gland could be derived from hepatic spermidine by the acetylase-oxidase pathway in liver and subsequent transport of putrescine to mammary gland. Although we could not detect an increase of putrescine in livers of starved rats, there was a significant decrease in their spermidine content. Mammary-gland explants have been shown to transport putrescine from the surrounding medium (Perry & Oka, 1980).

Elevation of intracellular diamine content by application of putrescine or diaminopropane to cell cultures (Heller *et al.*, 1976) or injection into animals *in vivo* (Grillo *et al.*, 1980) elicits the appearance of a protein inhibitor of ornithine decarboxylase, which has been named ornithine decarboxylase antizyme (Heller *et al.*, 1976). The natural elevation of endogenous putrescine concentration in these mam-

mary glands, which was detectable by 6h, was followed by appearance of readily measurable free antizyme in mammary gland at 24h. This observation, which represents the first report of the appearance of measurable antizyme under normal physiological conditions without the addition of exogenous diamines, lends support to the proposal by Heller & Canellakis (1981) that ornithine decarboxylase antizyme serves to regulate the activity of ornithine decarboxylase *in vivo*.

Heller & Canellakis (1981) have stated that, in cells with high contents of antizyme, there is a delay in appearance of ornithine decarboxylase after a stimulus, since some ornithine decarboxylase will be neutralized by free antizyme and will thus be unmeasurable. Such a mechanism could account for the lag in ornithine decarboxylase induction observed on refeeding. Conversely, free antizyme disappears by 5h refeeding, although putrescine concentrations are still elevated, since it has been completely neutralized by the newly induced ornithine decarboxylase.

Spermidine and spermine content

Fed lactating rats have about 12 times as much spermidine as spermine in their mammary glands, in agreement with previous reports (Neish & Key, 1968; Russell & McVicker, 1972). When lactating rats were starved overnight, the nucleic acid content of the mammary gland did not change significantly, but the contents of spermidine and spermine increased. Thus there is a clear dissociation between the content of polyamines and of RNA in this situation. The activity of ornithine decarboxylase is also decreased (Brosnan *et al.*, 1982, and the present study), and thus it is unlikely that these polyamines are synthesized from ornithine *de novo*. Although the polyamine content of blood of fed lactating rats is low, and the largest part of this is contained in the blood cells (Lundgren & Oka, 1978), the possibility of redistribution of polyamines from liver or other tissues during starvation must be considered. Alternatively it is possible that the increased polyamines observed in the present study could come from degradation of a covalently bound pool which is not detected by our polyamine extraction and analytical procedures. We would detect N^1 - or N^8 -acetyl-spermidine if they were present, but we have not observed significant quantities of these acetylated polyamines in mammary gland of fed rats (results not shown). It was claimed by Ugami *et al.* (1955) that lactating mammary glands contain a peptide, which they called mammamine, which contains a high proportion of spermidine, but this observation could not be confirmed by Neish & Key (1968). Spermidine and/or putrescine have been reported to be covalently attached to other proteins in various cells (Folk *et al.*, 1980; Canellakis *et al.*, 1981;

Haddox & Russell, 1981), although an intracellular store of polyamine which is bound to protein and which could be mobilized as required has not been characterized.

On refeeding, spermidine concentration gradually returned to the 'fed' value, with no significant change in nucleic acid concentrations. Spermine was also significantly decreased by refeeding, but was still higher than in fed rats ($P < 0.05$). There would appear to be three potential routes of disposal of polyamines on refeeding. They could be secreted from the gland, either into the blood or into the milk. In each of these fluids, free putrescine and spermine are below the limits of detection (5 nmol/ml) of our method, and the free spermidine concentration is low in fed rats (blood, 43 ± 13 nmol/ml, $n = 4$; milk 33 ± 13 nmol/ml, $n = 5$), although the concentrations in blood from starved or refed rats are not known. Little milk is being released to the pups at this stage (Brosnan *et al.*, 1982), and thus it is not likely that the secretion of free spermidine into milk is a significant source of its removal. Spermine could be acetylated, followed by oxidation to spermidine, and spermidine to putrescine, as proposed by Seiler *et al.* (1981a). Information is not at present available on the activity of this pathway in mammary gland. Thirdly, if the polyamines were present in a covalently linked form, they could be returned to that form, either in mammary gland itself or in the milk.

The physiological effects of these changes in polyamine content in mammary gland are not evident at present. Spermidine is clearly not limiting for milk protein synthesis in starved rats, nor do changes in its concentration necessarily lead to changes in the concentration of RNA in the mammary gland.

The present report clearly documents for the first time the physiological appearance of the ornithine decarboxylase antizyme in response to increases in endogenous putrescine and demonstrates that it plays a role in the regulation of ornithine decarboxylase.

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