HORMONAL REGULATION OF SPERMIDINE SYNTHASE DURING THE DEVELOPMENT OF MOUSE MAMMARY EPITHELIUM IN VITRO

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Received October 31, 1977

SUMMARY: The activity of spermidine synthase increases rapidly during the induction of mammary epithelial development by insulin, cortisol and prolactin in vitro. The increase is detectable 2-3 hours after the initiation of culture, and thereafter continues almost linearly up to 72 hours. The enzyme activity remains unchanged in the absence of the hormones during culture. The increase in enzyme activity is dependent only on the actions of insulin and cortisol which appear to act at both transcriptional and translational levels. Studies with cycloheximide suggest that spermidine synthase in mammary cells has a half-life of more than 12 hours.

INTRODUCTION

Spermidine synthase catalyzes the last step of spermidine biosynthesis by transferring the propylamine group of decarboxylated S-adenosylmethionine to putrescine. The formation of the two substrates of the enzyme, putrescine and decarboxylated S-adenosylmethionine, is catalyzed by ornithine decarboxylase and by S-adenosylmethionine decarboxylase, respectively. In contrast to numerous studies on the two decarboxylases, spermidine synthase in a eucaryotic system has not been extensively studied. Only recently (1-3) has mammalian spermidine synthase, which was once considered to be an inseparable component of S-adenosylmethionine decarboxylase, been shown to be a separate protein, as in the case of the bacterial enzyme (4).

Biosynthesis of spermidine is markedly enhanced during the development of mammary gland that occurs in pregnancy and lactation (5). A similar increase in spermidine occurs in mouse mammary explants that are stimulated

ISSN 0006-291X

to undergo developmental changes by the synergistic action of insulin, cortisol and prolactin (6,7). Previous studies with the organ culture system have presented several lines of evidence indicating that spermidine biosynthesis is an important regulatory step in the development of mammary gland (6-9). To elucidate the mechanism whereby the interplay of the hormones stimulates the biosynthesis of spermidine, we have begun to examine the changes in the activities of spermidine-biosynthetic enzymes (3,7,8,10). In the present communication, we present data on the hormonal regulation of spermidine synthase during the development of mouse mammary gland *in vitro*.

MATERIALS AND METHODS

S-adenosyl-L-[carboxyl-^{14}C]methionine (55 mCi/mmole) and [1,4-^{14}C]-putrescine dihydrochloride (52 mCi/mmole) were purchased from New England Nuclear. S-adenosyl-L-methionine, actinomycin D, cycloheximide, crystalline bovine serum albumin and dithiothreitol were purchased from Calbiochem. Spermidine, spermine, putrescine and cadaverine were products of Sigma Chemical Company. Medium 199 was a product of Grand Island Biological Company. Collagenase, type I, was a product of Worthington Biochemical Corporation. Pork crystalline zinc insulin was a gift from Eli Lilly and Company. Ovine prolactin was a gift from the Hormone Distribution Center, National Institute of Arthritis, Metabolism and Digestive Diseases. Cortisol was obtained from ICN Pharmaceuticals, Inc. (Life Sciences Group). Decarboxylated S-adenosyl-methionine was synthesized enzymatically by the method of Tabor (11) with *E. coli* S-adenosylmethionine decarboxylase purified through step 4 by the method of Wickner *et al.* (4). Decarboxylated S-adenosylmethionine was isolated by Dowex 50-H$^+$ column chromatography and purified to homogeneity by preparative paper high-voltage electrophoresis (7).

The animals used were C3H/HeN mice at the 10th-13th day of their first pregnancy. Preparation and culture of mouse mammary explants were described previously (12). Isolation of an epithelial cell fraction free of fat cells was accomplished by collagenase treatment as described previously (12). The mammary epithelial cell fraction was homogenized in 0.3 ml of 25 mM sodium phosphate buffer, pH 7.6 containing 0.1 mM EDTA, and 1 mM dithiothreitol. The homogenate was centrifuged for 60 min at 105,000 x g, and the resultant supernatant was used for the enzyme assay. The activity of spermidine synthase was assayed in 25 mM sodium phosphate buffer, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM decarboxylated adenosylmethionine, 1 mM [1,4-^{14}C]putrescine and the enzyme solution in a volume of 0.15 ml as described by Jänne *et al.* (13). A blank contained an equal amount of phosphate buffer instead of the enzyme solution. The reaction was linear during the 60-min assay period and was ascertained to be proportional to the amount of enzyme by taking two different aliquots of enzyme solution. The activity was expressed as pmole of spermidine formed per hr per mg of tissue explants.

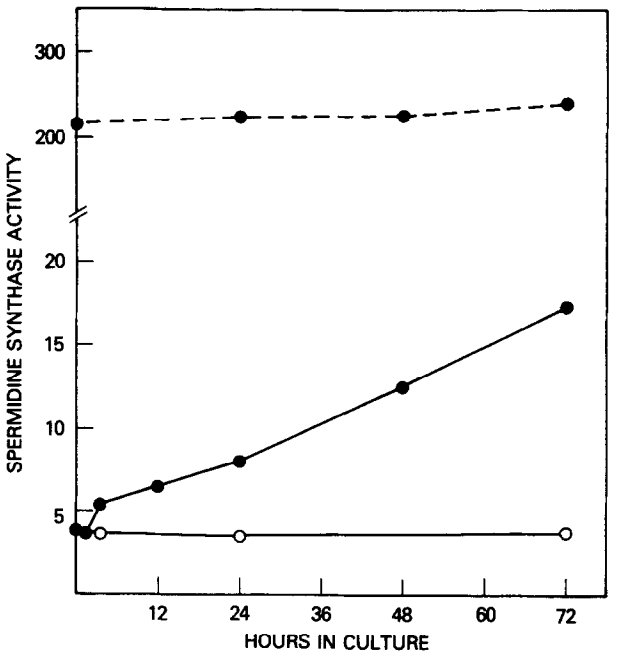

FIGURE LEGEND

Figure 1. Time course of the activity of spermidine synthase in mouse mammary explants in culture. Mammary explants prepared from two to three mid-pregnant mice were cultured in the absence (0) or in the presence of insulin, cortisol and prolactin (●). At the indicated time of culture, mammary explants from each culture system were divided into two portions; one for the determination of enzyme activity in whole explants and the other for treatment with collagenase as described in Materials and Methods. The resultant epithelial cell fraction was assayed for enzyme activity as described in Materials and Methods. (---) represents the activity in whole explants and (——) represents the activity in epithelial cell fraction. Each point represents the mean of two separate experiments. Standard error was in the range of 3-8% for each value.

RESULTS AND DISCUSSION

Mammary gland from mid-pregnant mice contains two major cell types; epithelial cells, which are the prime target of the present study, and fat cells which comprise a large portion of the tissue. This makes it important to ascertain that any biochemical change observed with the tissue reflects the response of epithelial cells. Accordingly, changes in spermidine synthase activity were determined both in whole explants

Table I. Effect of actinomycin D and cycloheximide on activity of spermidine synthase in mammary epithelium of mouse tissue explants in culture

Culture condition	Addition	Enzyme Activity (pmole/mg/hr)
Uncultured control	-	4.4 ± 0.4
Insulin, cortisol and prolactin:		
12 hr	-	6.7 ± 0.5
24 hr	-	11.0 ± 0.5
36 hr	-	14.3 ± 1.2
12 hr	Actinomycin at 0 hr	4.1 ± 0.7
12 hr	Cycloheximide at 0 hr	4.2 ± 0.7
24 hr	Cycloheximide at 12 hr	6.5 ± 0.4
36 hr	Cycloheximide at 24 hr	10.0 ± 0.9

Mammary explants were cultured under the indicated conditions. The final concentration of actinomycin D and cycloheximide was 1 and 4 μg/ml, respectively. The explants from each culture system were weighed and treated with collagenase to prepare the epithelial cell fraction. The epithelial cells were homogenized and assayed for enzyme activity as described in Materials and Methods. Each value represents the mean ± S.E. of two separate culture experiments.

and in the isolated epithelial cells derived from the mammary explants of the corresponding culture. Fig. 1 shows that the activity of spermidine synthase in whole explants remains essentially constant during 72 hours of culture with insulin, cortisol and prolactin. In contrast, the same

hormone treatment stimulated the enzyme activity in epithelial cell fraction, which comprises only a small percentage of the activity in the whole tissue. The increase in enzyme activity occurred within 3 hours of culture and continued up to 72 hours, amounting to about a 3-5 fold increase over the initial level. The epithelial enzyme activity remained unchanged in the absence of the hormones during culture. These results indicate that spermidine synthase activity of epithelial cells and fat cells of mammary explants respond differently to the actions of hormones. The stimulatory effect of hormones on the epithelial enzyme would not have been detected if whole explants alone were assayed, because of the high basal enzyme activity in fat cells and their unresponsiveness to the hormones. Accordingly, in all subsequent studies, the enzyme was assayed using epithelial cell fractions free of fat cells.

As shown in Table I, the addition of actinomycin D or cycloheximide at the onset of culture inhibited completely the hormonal stimulation of spermidine synthase activity, suggesting that the increase in enzyme activity may require both new RNA and protein synthesis. The delayed addition of cycloheximide at any time during culture prevented a further increase in enzyme activity, but caused no significant decline of enzyme activity for 12 hours. This suggests a half-life for the synthase of mammary cells of at least 12 hours, as observed earlier with rat liver enzyme (14). Further attempts to estimate the half-life of the enzyme with cycloheximide were unsuccessful since longer exposure of the explants to the drug produced marked cytotoxic effects and eventual cell death.

To determine the role of insulin, cortisol and prolactin in stimulation of spermidine synthase activity, mammary explants were cultured for 48 hours with various combinations of the hormones. As shown in Table II, enzyme activity remained virtually unaltered in the absence of hormones or in the presence of cortisol and prolactin or each hormone alone (not shown). Addition of insulin caused about a 40% increase. A combination

Table II. Effect of insulin, cortisol, and prolactin on activity of spermidine synthase in mammary epithelium of mouse tissue explants in culture

Culture condition	Enzyme activity
Uncultured control	100
No hormone addition	98 ± 5
Cortisol + prolactin	98 ± 8
Insulin	138 ± 8
Insulin + cortisol	300 ± 25
Insulin + prolactin	180 ± 22
Insulin + cortisol + prolactin	305 ± 35

Mammary explants were cultured for 48 hr in medium containing the indicated combinations of hormones. Other details are described in the legend to Table I. Results are expressed as relative activity by adjusting the activity of the uncultured control to 100. Each value is the mean ± S.E. obtained from three separate culture experiments.

of insulin and cortisol effected the greatest increase of about 200%, whereas a combination of insulin and prolactin produced an increase of approximately 80%. The addition of prolactin with insulin and cortisol did not produce any greater increase in activity than that effected by the combination of insulin and cortisol. In other experiments, not reported here, it was found that the lowest effective concentration of hormones was 50 ng/ml of insulin and 5∿10 ng/ml of cortisol. Maximal response was obtained at a concentration of 5 μg/ml for insulin and 0.5∿ 5 μg/ml for cortisol. These results demonstrate that the increase in spermidine synthase activity in mammary epithelium depends on the action

of insulin and cortisol. The same combination of hormones has been shown to stimulate the activity of S-adenosylmethionine decarboxylase in mammary cells (7).

The observation that the activities of spermidine synthase and S-adenosylmethionine decarboxylase in mammary epithelium are both under the control of insulin and cortisol is noteworthy in several respects. It has been suggested (15,16) that the two enzymes may form a functional complex which would facilitate efficient synthesis of spermidine in cells. This suggestion was based on the observations (3,15,17) that the two enzymes are quite difficult to separate from each other with certain purification procedures. During the development of mammary epithelium, glucocorticoid has been shown to be a key factor for the increase in the concentration of spermidine (7), which, in turn, mimicks the action of the steroid hormone on the lactogenic processes. The observed stimulatory effects of cortisol on spermidine synthase and S-adenosylmethionine decarboxylase lend further support to the view (7,15) that the mode of action of glucocorticoid in this system involves stimulation of the activities of spermidine biosynthetic enzymes and a resultant increase in the intracellular concentration of spermidine, which then acts as a mediator of the steroid hormone action on the formation of milk-proteins.

The present studies complete the first phase of our attempts to elucidate the regulatory mechanisms of spermidine biosynthesis during hormonal induction of mammary development *in vitro*. The data presented here, together with earlier observations (7,8,10) demonstrate that the interplay of insulin, glucocorticoid and prolactin causes the stimulation of a group of spermidine biosynthetic enzymes such as arginase, ornithine decarboxylase, S-adenosylmethionine decarboxylase and spermidine synthase. The increase of the various enzyme activities is not synchronous and differs in extent and stimulatory factors involved. These observations may shed important light on our understanding of the basic control

mechanism of spermidine biosynthesis in this system.

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