Spermidine as a Possible Mediator of Glucocorticoid Effect on Milk Protein Synthesis in Mouse Mammary Epithelium in Vitro

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SUMMARY

Several lines of evidence are presented for the involvement of spermidine in milk protein synthesis during hormone-dependent differentiation of mouse mammary epithelium in vitro. (a) When mammary explants from mid-pregnant mice are cultured in the presence of insulin, hydrocortisone, and prolactin, synthesis of casein and α-lactalbumin increases markedly during the initial 48 hours. The requirement for hydrocortisone, but not for insulin or prolactin, can be met by spermidine at a concentration as low as 10^{-4} M. Thus the combination of insulin, spermidine, and prolactin results in an increase in synthesis of the milk proteins similar to that produced by the triple hormone combination. The related polyamines, spermine and putrescine, and divalent cations such as Mg^{2+} are ineffective. (b) The concentration of spermidine in mammary epithelium increases by about 3-fold when mammary explants are incubated for 48 hours in the presence of insulin, hydrocortisone, and prolactin, the same combination of hormones which causes marked stimulation of milk protein synthesis. Significant increases in the spermidine concentration occur prior to the accelerated synthesis of milk proteins. The combination of insulin and prolactin, which results in a slight stimulation of milk protein synthesis, correspondingly effects a smaller increase in the intracellular concentration of spermidine. (c) Methylglyoxal bis(guanylhydrazone), a potent inhibitor of spermidine synthesis, at a concentration of 2 μM, prevents increases in both the cellular spermidine concentration and the milk protein synthesis that occur in the presence of the three hormones. The simultaneous presence of spermidine, but not spermine or putrescine, however, overcomes the inhibitory effect of methylglyoxal bis(guanylhydrazone) on milk protein synthesis.

The mechanism whereby glucocorticoid, together with insulin and prolactin, causes the large increase in the concentration of spermidine in cultured mammary cells appears to be mediated through stimulation of the activity of S-adenosyl-L-methionine decarboxylase, which plays a key role in the biosynthesis of spermidine. The increase in the cellular concentration of spermidine may, at least in part, be elicited by the ability of the glucocorticoid to enhance the activity of S-adenosyl-L-methionine decarboxylase, an enzyme which plays a key role in the biosynthesis of spermidine.

EXPERIMENTAL PROCEDURES

Chemicals

Pork crystalline zinc insulin was a gift from Eli Lilly and Co. Hydrocortisone, aldosterone, 17β-estradiol, progesterone, and testosterone were obtained from Nutritional Biochemicals Corp. Ovine prolactin was obtained from the National Institutes of Health. Spermidine, spermine, putrescine, polylysine, and polyarginine were Sigma products. S-Adenosyl-L-[carboxyl-14C]-methionine (7.7 mCi per mmol), [14C]spermidine trihydrochloride (10.7 mCi per mmol), [14C]spermine tetrachloride (9.9 mCi per mmol), and UDP-[14C]galactose (296 mCi per mmol) were obtained from New England Nuclear. Carrier-free [32P]orthophosphate...
phate and bovine casein (Hammersten) were purchased from Schwars-Mann. Methylglyoxal bis(guanylhydrazone) was obtained from Aldrich Chemical Co. Medium 199 was a product of Grand Island Biological Co. Crude collagenase (type I) was obtained from Worthington Biochemical Corp.

METHODS

Organ Culture—The abdominal mammary glands were removed aseptically from C3H/HeN mice in the middle of their first pregnancy. Tissue explants were cultured in Medium 199 containing penicillin G (35 μg per ml) with appropriate addition of hormones and other chemicals as described previously (3). Each hormone was used at a concentration of 5 μg per ml of medium unless stated otherwise. The culture medium was changed every 48 hours.

Casein Synthesis—The extent of casein synthesis was estimated by labeling the explants with carrier-free 32P (10 to 15 μCi per ml of the medium) for 3 hours before the termination of the incubation. Isotopically labeled casein was isolated from the 105,000 X g supernatant of the tissue homogenate by precipitation with rennin and calcium ions in the presence of bovine casein carrier as described previously (3). Casein precipitates were dissolved in 1 ml of 1 NCS solubilizer (Amerasham-Searle), and radioactivity was determined as described earlier (8). The amount of tissue used per determination was normally in the range of 4 to 8 mg. Starch uge gel electrophoresis of the material precipitated by rennin and calcium ions showed that it was indistinguishable from authentic mouse-milk casein (12). Although such precipitate also contained non-casein phosphoproteins, the ratio of synthesis of these proteins was not stimulated by the hormones used herein and therefore remained relatively constant during culture, i.e. 100 to 150 cpm per mg of tissue per 3 hours.

α-Lactalbumin Determination—Activity of α-lactalbumin, the B protein component of the lactose synthetase system (13) was determined by measuring the formation of lactose by the transfer of galactose from uridine diphosphogalactose to glucose as described previously (14). The reaction was run for 30 min at 37°, with a blank containing no glucose to correct for endogenous hexose formation. The increase in α-lactalbumin was similar to that produced by the combination of insulin, hydrocortisone, and prolactin, whereas the increase in casein synthesis was about half of that in the triple hormone system. When casein synthesis was measured using [3H]amino acids instead of [32P]orthophosphate, spermidine was again about half as effective as hydrocortisone.

In experiments not shown here, the combination of insulin, hydrocortisone, prolactin, and spermidine caused no greater increase in α-lactalbumin and casein synthesis than that produced by the combination of insulin, spermidine, and prolactin. Furthermore, spermidine alone, or in combination with any one of the three hormones or with both insulin and hydrocortisone, was ineffective in enhancing milk protein synthesis.

Spermidine in combination with insulin and prolactin was consistently effective at a concentration as low as 4 X 10^{-4} M in producing an increase in α-lactalbumin (Table I). Larger

![FIG. 1](http://www.jbc.org/) Effect of hormones and spermidine on (A) α-lactalbumin and (B) casein synthesis in mammary epithelial cells in culture. Mammary gland explants from C3H/HeN mice in the middle of their first pregnancy were cultured in Medium 199 containing various combinations of insulin (I), hydrocortisone (F), prolactin (P), and spermidine (S). Spermidine was used at a concentration of 4 mg/ml. Activity of α-lactalbumin and the extent of casein synthesis were measured as described under "Experimental Procedures." Each point represents the average of duplicate determinations. Standard error is in the range of 10 to 15% for each point. •, insulin plus hydrocortisone plus prolactin (IFP); ▲, insulin plus prolactin plus spermidine (ISP); ▲, insulin plus prolactin (IP); ○, insulin plus hydrocortisone (IF).
Effect of various concentrations of spermidine on α-lactalbumin activity in cultured mammary gland

Mammary gland explants from midpregnant mice were cultured for 48 hours under the conditions indicated. α-Lactalbumin activity was determined as described under "Experimental Procedures." The data represent one of several similar experiments. Each value represents the mean ± S.E. of three separate determinations.

Table I

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>α-Lactalbumin activity (pmol lactose formed/mg wet wt tissue/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured control</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Insulin + prolactin + spermidine</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>4 × 10^{-9} M</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>4 × 10^{-5} M</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>4 × 10^{-4} M</td>
<td>41 ± 7</td>
</tr>
<tr>
<td>4 × 10^{-3} M</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>4 × 10^{-2} M</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>Insulin + hydrocortisone + prolactin</td>
<td>38 ± 6</td>
</tr>
</tbody>
</table>

Table II

Effect of putrescine, spermidine, Mg²⁺, and spermine on α-lactalbumin activity in cultured mammary gland

A final concentration of putrescine, spermidine, spermine, and Mg²⁺ was 4 mM. Other details are given in the legend to Table I. Each value represents the mean ± S.E. of six separate determinations on three separate experiments.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>α-Lactalbumin activity (pmol lactose formed/mg wet wt tissue/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured control</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Insulin + hydrocortisone + prolactin</td>
<td>120 ± 15</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>Insulin + prolactin + putrescine</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Insulin + prolactin + spermine</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Insulin + prolactin + Mg²⁺</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>Insulin + prolactin + spermidine</td>
<td>99 ± 8</td>
</tr>
</tbody>
</table>

The effect was maximal at 4 × 10^{-3} M. Similar results were obtained with casein synthesis. Table II shows that the stimulatory effect of spermidine is not simply due to its cationic property since putrescine, a precursor of spermidine, and Mg²⁺ had no effect. Spermine, another related polyamine, actually produced a decrease in α-lactalbumin. In similar experiments in which the effects of putrescine and spermine were examined throughout the concentration range 1 nM to 4 mM, no increase in α-lactalbumin was detected. Moreover, polycations such as polylysine and polyarginine had no stimulatory effect on synthesis of milk proteins at concentrations of 10 nM to 1 mM (not shown).

In view of the finding that spermidine could largely replace the requirement for hydrocortisone in augmentation of milk protein synthesis, experiments were undertaken to examine the concentration of spermidine in mammary epithelium cultured in various combinations of insulin, hydrocortisone, and prolactin. As shown in Fig. 2, the triple hormone combination produced a doubling of the initial spermidine concentration in mammary cells after 15 hours of culture. By 48 hours, the spermidine concentration nearly tripled to about 400 nmol per mg of tissue (11). The combination of insulin and prolactin or the combination of insulin and hydrocortisone also enhanced the polypeptide level, although to a smaller extent. Insulin alone maintained the initial concentration during the first 24 hours, whereas in the absence of the hormone, the spermidine concentration decreased rapidly. Hydrocortisone alone or prolactin by itself or the combination of the two hormones could not effectively maintain the initial level of the polypeptide during 3 days of culture (not shown). The concentration of putrescine was below the level of reliable detectability in these experiments. The concentration of spermine increased approximately 30% during the culture of mammary cells, but no appreciable difference was found among various combinations of the three hormones (11).

The cause and effect relationship between the increases in spermidine formation and milk protein synthesis can be studied by the use of a specific inhibitor of spermidine synthesis. Earlier reports showed (16, 17) that methylglyoxal bis(guanylhydrazone) prevents the increase in the cellular spermidine concentration that occurred during growth of lymphocytes in culture. Accordingly, this compound was employed to assess further the involvement of spermidine in milk protein synthesis. As shown in Table III, the addition of methylglyoxal bis(guanylhydrazone) to the triple hormone combination prevented the increase in the concentration of spermidine in mammary epithelium during 2 days of culture. Although this agent also prevented augmentation of milk protein synthesis, the inhibitory...
TABLE III

Effect of methylglyoxal bis (guanylhydrazone) on spermidine accumulation and milk protein formation in mammary epithelial cells in culture

Mammary gland explants from seven to eight midpregnant mice were cultured for 48 hours under the conditions indicated. A final concentration of spermidine, spermine, and putrescine was 4 mM. α-Lactalbumin activity and casein synthesis were determined as described under “Experimental Procedures.” Determination of spermidine concentration was also described under “Experimental Procedures.” The data represent one of four similar experiments. Each value is the average of two separate determinations.

| Culture conditions       | Spermidine concentration | α-Lactalbumin activity | Casein
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol/g wet wt</td>
<td>pmol lactose formed/mg wet wt/24 hr</td>
<td>cpm/mg wet wt/3 hr</td>
</tr>
<tr>
<td>Uncultured control</td>
<td>150</td>
<td>50</td>
<td>230</td>
</tr>
<tr>
<td>Insulin</td>
<td>40</td>
<td>20</td>
<td>155</td>
</tr>
<tr>
<td>Insulin + hydrocortisone</td>
<td>400</td>
<td>235</td>
<td>1100</td>
</tr>
<tr>
<td>+ prolactin</td>
<td>110</td>
<td>35</td>
<td>340</td>
</tr>
<tr>
<td>+ prolactin + 2 μM methylglyoxal bis (guanylhydrazone)</td>
<td>70</td>
<td>15</td>
<td>160</td>
</tr>
<tr>
<td>+ prolactin + 10 μM methylglyoxal bis (guanylhydrazone)</td>
<td>ND²</td>
<td>210</td>
<td>950</td>
</tr>
<tr>
<td>+ prolactin + spermidine</td>
<td>ND²</td>
<td>30</td>
<td>220</td>
</tr>
<tr>
<td>+ putrescine + 10 μM methylglyoxal bis (guanylhydrazone)</td>
<td>ND²</td>
<td>15</td>
<td>125</td>
</tr>
</tbody>
</table>

⁴ND, not determined because of the presence of exogenous amines.

The effect of the drug on milk protein synthesis was almost completely overcome by the concomitant presence of spermidine. The inhibition, however, could not be reversed by either spermine or putrescine. Moreover, the inhibitory effect of the drug on milk protein synthesis appeared to be relatively selective in the sense that this agent, at a concentration as low as 0.005 μg per ml, inhibited the rates of non-milk protein synthesis, RNA synthesis and DNA synthesis only about 20, 15, and 25%, respectively, as judged from the incorporation studies using [3H]leucine, [3H]uridine, and [3H]thymidine (not shown).

Since S-adenosyl-L-methionine decarboxylase plays a key role in the biosynthesis of spermidine by providing decarboxylated S-adenosyl-L-methionine, which, in turn, donates a propylamine moiety to putrescine to form spermidine (18–22), it was of interest to examine the effect of various hormones on the activity of S-adenosyl-L-methionine decarboxylase during the culture of mammary tissues. As shown in Fig. 3, at 15 hours of culture insulin increased the enzyme activity by 2-fold. The combination of insulin and prolactin produced little increase in enzyme activity. The presence of hydrocortisone together with insulin or with insulin and prolactin effected a further increase. At 48 hours of culture, the stimulatory effect of glucocorticoid became more evident. The presence of hydrocortisone alone with insulin or with insulin and prolactin increased enzyme activity by about 2.5- and 3-fold, respectively, whereas in the absence of the steroid, the activity decreased almost to the initial level.

As shown in Table IV, the stimulatory effect of hydrocortisone on the activity of S-adenosyl-L-methionine decarboxylase was observed at a concentration as low as 0.005 μg per ml. The effect was maximal at 0.5 μg per ml. Furthermore, it is clear that the steroid hormones with glucocorticoid activity such as aldosterone and corticosterone were equally effective in enhancing the enzyme activity, whereas 17β-estradiol, progesterone, and testosterone were inactive.

**FIG. 3.** Change in S-adenosyl-L-methionine decarboxylase activity in mammary epithelial cells cultured in the presence of various hormone combinations. Mammary gland explants pooled from 5 to 10 midpregnant mice were cultured for the indicated periods in the presence of various combinations of insulin (I), hydrocortisone (F), and prolactin (P). At the end of culture, the explants were treated with collagenase to obtain the epithelial cell preparations as previously described (9). The enzyme activity was determined on the 105,000 × g supernatant by measuring the production of 14CO2 from S-adenosyl-[carboxyl-14C]methionine as described under “Experimental Procedures.” Each point represents the mean of three separate experiments. Standard error is in the range of 7 to 12% for each point. Because of the variation in enzyme activity in uncultured control cells, the results are expressed as per cent of uncultured control. The enzyme activity in uncultured control varied from 1.50 to 3.70 pmol of 14CO2 per hour per g wet weight of tissue. Δ oo, no hormone (NH); o—o, insulin (I); ——, insulin plus hydrocortisone (IF); ▲—▲, insulin plus prolactin (IP); ○—○, insulin plus hydrocortisone plus prolactin (IFP).

48 hours of culture, the stimulatory effect of glucocorticoid became more evident. The presence of hydrocortisone along with insulin or with insulin and prolactin increased enzyme activity by about 2.5- and 3-fold, respectively, whereas in the absence of the steroid, the activity decreased almost to the initial level.

**DISCUSSION**

Under the influence of insulin, hydrocortisone, and prolactin, mammary epithelium undergoes marked developmental changes and synthesizes increasing amounts of milk proteins (1–8). In the present studies, it has been shown that the same triple hormone combination produces the largest increase in the concentration of spermidine in mammary epithelium. The cellular spermidine level begins to increase before the accelerated synthesis of milk proteins and it reaches a peak at 48 hours when synthesis of casein and α-lactalbumin is stimulated maximally...
three hormones (11). The effect of spermidine appears to be

In the presence of insulin and prolactin, small increases in milk protein synthesis occur, and the corresponding increase in spermidine concentration is small. Thus there is close correlation between milk protein synthesis and spermidine accumulation as a function of time and hormonal requirement. Recently, Russell and McVicker (10) reported that the spermidine concentration increased rapidly in the rat mammary gland during lactation. Thus the data presented here are consistent with their observation, and it may be concluded that enhancement of spermidine accumulation by insulin, hydrocortisone, and prolactin in vitro reflects physiological changes in mammary epithelium during lactogenesis.

The fact that addition of spermidine, in place of glucocorticoid, markedly stimulates milk protein synthesis in the presence of insulin and prolactin provides more direct evidence for the involvement of spermidine in lactogenesis. The minimal effective concentration of exogenous spermidine, $4 \times 10^{-8}$ M, is approximately in the range found in the cells that are actively engaged in milk protein synthesis under the influence of the three hormones (11). The effect of spermidine appears to be specific for glucocorticoid since this polyamine does not replace the requirement for insulin or prolactin.

The studies with the drug, methylglyoxal bis(guanylhydrazone), add further support to the view that spermidine is involved in the lactogenic response of mammary cells. This drug prevents an increase in both cellular spermidine concentration and milk protein synthesis when added to the triple hormone system. The inhibitory effect of the drug on milk protein synthesis is likely to be related to the inhibition of spermidine accumulation rather than to possible toxic side effects because concomitant presence of spermidine, but not putrescine or spermine, restores the milk protein synthesis to the level found in the triple hormone system.

$S$-Adenosyl-$L$-methionine decarboxylase is an enzyme which plays a fundamental role in the biosynthesis of spermidine (18-22). In the present studies, glucocorticoid, together with insulin, has been shown to produce a rapid and large increase in the activity of the enzyme in cultured mammary cells. The minimal effective concentration of hydrocortisone, 0.005 $\mu$g per ml, for the stimulation of enzyme activity is essentially the same as that for milk protein synthesis (8, 23). Other steroid hormones with glucocorticoid activity such as aldosterone and corticosterone were as effective in stimulating the activity of the enzyme as they were in augmenting casein synthesis (24). These data are consistent with the view that spermidine may mediate the effect of glucocorticoid in milk protein synthesis since stimulation of enzyme activity by the glucocorticoid may result in greater production of spermidine. A similar increase in the enzyme activity has been reported to occur during lactation in the rat mammary gland (10), and this may be ascribed largely to the effect of glucocorticoid. At present, it is not known whether hormonal stimulation of the enzyme activity is due to activation, increased synthesis, decreased breakdown of the enzyme, or a combination of any of the three possibilities.

Although spermidine does not meet the requirement for insulin, there are several lines of evidence to suggest that insulin may contribute to spermidine formation. Recently, it was reported (25) that in cultured mammary tissues insulin stimulates the activity of ornithine decarboxylase, an enzyme which catalyzes the formation of putrescine, a precursor of spermidine. We have obtained similar results. These results suggest that insulin may participate in the stimulation of spermidine synthesis, partly at the level of a precursor. In addition, the fact that the activity of $S$-adenosyl-$L$-methionine decarboxylase can be stimulated by putrescine (18) suggests the possibility that the stimulatory effect of insulin on the enzyme may result from the increased cellular level of putrescine. Although earlier studies have shown (18) that the increase in the activity of ornithine decarboxylase often occurs in association with cell proliferation, and insulin stimulates mammary cell proliferation in vitro (4), it is not clear whether the stimulation of ornithine decarboxylase activity by insulin is related to its mitogenic action on mammary cells. It has been shown earlier (10) that enzyme activity increases markedly in the rat mammary gland during lactation when very little cell proliferation occurs (20). Thus it is attractive to envision that insulin increases the activity of ornithine decarboxylase which plays a part in milk protein synthesis by facilitating the formation of the spermidine precursor.

The mechanism whereby spermidine, together with insulin and prolactin, stimulates milk protein synthesis is not well understood. Since spermidine simulates the action of glucocorticoid, and one of the proposed functions of the steroid is to facilitate the sustained accumulation of various cellular components in mammary epithelium (2, 8, 9, 27), it may be that spermidine functions, at least in part, by mediating such effects of the glucocorticoid. Indeed, it has been shown recently (9) that spermidine, in place of glucocorticoid, prevents decrease in glucose 6-phosphate dehydrogenase activity in mammary epithelium.

Earlier studies have shown that augmentation of milk protein formation requires RNA synthesis (23, 28) and that prolactin stimulates synthesis of all species of mammary RNA, as determined either by sucrose density gradient centrifugation (29) or by acrylamide gel electrophoresis (30). It has been shown further (8) that sustained stimulation of RNA synthesis by prolactin depends upon the presence of glucocorticoid. Glucocorticoid, by itself, does not stimulate the rate of RNA synthesis but prolongs the stimulation produced by prolactin. It will be of

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**Table IV**

Effect of various steroid hormones on $S$-adenosyl-$L$-methionine decarboxylase activity in cultured mammary epithelium

<table>
<thead>
<tr>
<th>Hormone system</th>
<th>$S$-Adenosyl-$L$-methionine decarboxylase activity (pmol $\text{CO}_2/hr$ per g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured control</td>
<td>2.00 ± 0.00</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.40 ± 0.10</td>
</tr>
<tr>
<td>Insulin + hydrocortisone</td>
<td>2.35 ± 0.15</td>
</tr>
<tr>
<td>Insulin + corticosterone</td>
<td>2.35 ± 0.15</td>
</tr>
<tr>
<td>Insulin + aldosterone</td>
<td>2.35 ± 0.15</td>
</tr>
<tr>
<td>Insulin + 17$\beta$-estradiol</td>
<td>2.35 ± 0.15</td>
</tr>
<tr>
<td>Insulin + progesterone</td>
<td>2.35 ± 0.15</td>
</tr>
<tr>
<td>Insulin + testosterone</td>
<td>2.35 ± 0.15</td>
</tr>
</tbody>
</table>

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1 T. Oks and J. W. Perry, unpublished observations.
interest to determine whether spermidine can mediate this action of glucocorticoid, particularly in view of the earlier hypothesis (10, 31, 32) that spermidine may be involved in the regulation of RNA synthesis.

Finally, we do not know the reason why exogenous addition of spermidine does not completely simulate the effect of hydrocortisone on milk protein synthesis. This is particularly evident in casein synthesis. This difference may be due to some unknown side effects of the polyamine. Alternatively, the steroid hormone may exert some critical effects which cannot be fulfilled by addition of the polyamine.

**Acknowledgment**—We would like to thank Dr. Y. J. Topper for his encouragement and continued support throughout this investigation.

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