

CHANGES IN INSULIN RESPONSIVENESS DURING DEVELOPMENT OF MAMMARY EPITHELIUM

TAKAMI OKA, JOHN W. PERRY, and YALE J. TOPPER. From the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

INTRODUCTION

Functional differentiation of mammary epithelium normally begins during pregnancy and culminates after parturition. A few weeks after the cessation of lactation the gland involutes and again becomes functionally dormant, and remains so until the onset of the next pregnancy. Previous studies on the development of mouse mammary gland *in vitro* revealed several critical roles of insulin (1-13). Insulin can promote epithelial cell proliferation and is required for conversion of nonsecretory mammary cells into secretory cells in the presence of glucocorticoid and prolactin. However, it was shown that mammary cells in mature virgin animals, cells which are developmentally dormant, lack responsiveness to insulin, whereas mammary cells in midpregnancy are responsive to insulin (14). These results suggest the possibility that change in responsiveness to insulin may be an important regulatory step in the differentiation of mammary epithelium.

In the present report we have extended our previous observations by examining the responsiveness to insulin of mammary epithelium in other developmental stages. It will be shown that responsiveness to the hormone changes in the various physiological states in a manner consistent with the view that these changes may be key factors in control of mammary gland development.

MATERIALS AND METHODS

Animals

C3H/HeN mice were used throughout the experiments. The immature virgin mice were 3-5-wk old; the mature virgin animals were 4-5 mo of age. The pregnant mice were in various stages of their first pregnancy. The 1st day of pregnancy was equated with the day when a vaginal plug was found. The lactating mice had been nursing litters of four to seven pups for 8-12 days. The postlactational period began when nursing was terminated by removal of the litter after 10 days of lactation. Maintenance of the mice required special care since the hormone responsiveness of mammary epithelium was affected by various environmental factors such as population density in the cages, bedding materials, and frequency of handling animals. A standard maintenance procedure employed was the following: constant lighting and temperature, i.e., lights off from 5 p.m. to 8 a.m., 25°C; housing of no more than five mice per cage (size 7 × 11 × 5 inches); odorless corncob bedding material; cleaning the cage twice a week. Mice were fed Purina rat chow and water *ad libitum*.

Experimental Procedures

Mammary gland explants were prepared from mice in various physiological stages and cultured in medium 199 (Microbiological Associates, Inc., Bethesda, Md.) containing penicillin G at 35 µg/ml as described previously (6). Insulin (a gift from Eli Lilly Co., Indianapolis, Ind.) was used at 5 µg/ml of medium. The medium was

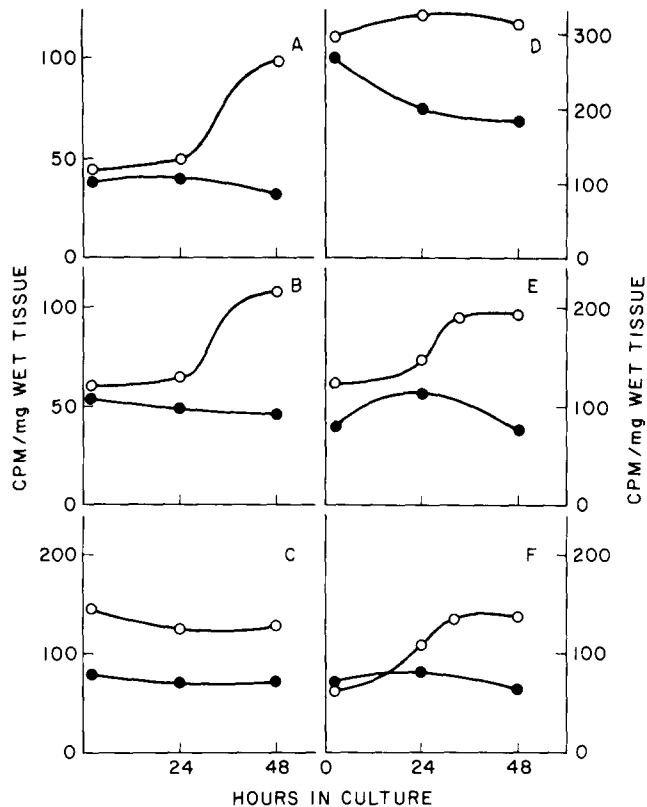


FIGURE 1 Time-course of accumulation of $[1-^{14}\text{C}]\text{AIB}$ in mammary gland explants from mice in various physiological stages. Incubations were carried out in the absence (\bullet — \bullet) or presence (\circ — \circ) of insulin. At the indicated times, accumulation of $[^{14}\text{C}]\text{AIB}$ was measured by addition of $[^{14}\text{C}]\text{AIB}$ at a concentration of $0.1 \mu\text{Ci/ml}$ of medium 3 h before termination of the experiment. The amount of isotope accumulated was determined as described previously (14). (A) immature virgin, (B) mature virgin, (C) midpregnancy, (D) midlactation, (E) postlactation (1 wk), (F) postlactation (6 wk). Each point represents the average of three determinations.

changed every 48 h. Accumulation of α - $[1-^{14}\text{C}]\text{aminoisobutyric acid}$ (AIB,¹ sp act 6.5 mCi/mmol, New England Nuclear, Boston, Mass.) was determined as described earlier (14). The combined activities of glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase in epithelial cells was assayed by the method of Glock and McLean (15) after removal of fat cells from explants by treatment with collagenase (type I, Worthington Biochemical Corp., Freehold, N. J.) as described (16). The data were expressed as the change in absorbance at 340 nm per min per mg wet weight of tissue. The extent of DNA synthesis was measured by allowing explants to incorporate $[\text{methyl-}^3\text{H}]\text{thymidine}$ (sp. act. 8 Ci/mmol, New England Nuclear) into trichloroacetic acid (TCA) precipitable

material for the indicated periods as previously described (6). That the incorporation of $[^3\text{H}]\text{thymidine}$ into TCA-insoluble material reflected epithelial DNA synthesis was established previously by autoradiographic and mitotic index studies (8).

RESULTS

Functionally dormant mammary epithelial cells from immature, mature virgin, and postlactational mice, and functionally active cells from pregnant and lactating mice were studied. Responsiveness of these cells to insulin was assessed kinetically in an organ culture system, by examining three parameters representing different levels of cellular activity: (a) the accumulation of the nonmetabolizable amino acid, AIB; (b) the activities of glucose-

¹Abbreviations used in the paper: AIB, α -aminoisobutyric acid, TCA, trichloroacetic acid.

6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase; and (c) DNA synthesis.

The effect of insulin on the accumulation of AIB was compared during 2 days of culture of mammary explants derived from mice in the various physiological stages. As shown in Fig. 1 A, B, F, insulin produced little increase in AIB accumulation during the first 24 h in the explants from immature, mature virgin, and 6-wk postlactational mice, respectively. During the 2nd day, however, all of those explants responded to insulin with an enhanced accumulation of AIB, and thereafter maintained their responsiveness during 96 h of culture (not shown). By contrast, in explants from midpregnant mice, stimulation of AIB accumulation by insulin was already evident in the first 4 h of culture, and the increment remained constant throughout the culture period (Fig. 1 C). In similar experiments the effect of insulin on AIB accumulation was detectable as early as the first 30 min of incubation (unpublished). In explants from lactating mice (Fig. 1 D), the initial accumulation of AIB was very high, and insulin did not enhance it. During culture, however, the effect of insulin became apparent since the rate declined in the absence of the hormone.

Physiologically, transition from an insulin-responsive to an unresponsive state appears to occur gradually during involution since explants from 1-wk postlactational mice exhibited some responsiveness to insulin in the first 4 h of culture (Fig. 1E), while the cells in 6-wk postlactational mice exhibited no responsiveness at this time (Fig. 1 F).

From the data presented above, it is apparent that a transition from an unresponsive to responsive state occurs some time during pregnancy. Additional studies were done to determine more precisely the time of this transition. Results shown in Table I indicate that insulin responsiveness, as determined by AIB accumulation, begins to develop at about the 2nd day of pregnancy. Thereafter responsiveness increases as pregnancy reaches midterm. At 12 days of pregnancy, the cells were maximally responsive, based on the fact that the increment in insulin-stimulated response was the same during the 1st day as during the 2nd day of culture. This was also true during the remainder of pregnancy.

Fig. 2 illustrates the kinetics of accumulation of the combined activities of glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase.

TABLE I
Insulin Responsiveness of Mouse Mammary Epithelium during Pregnancy in Terms of AIB Accumulation

Gestation <i>days</i>	Culture system	AIB			
		1-4 h	Increase	45-48 h	Increase
		<i>cpm/mg wet wt tissue</i>	%	<i>cpm/mg wet wt tissue</i>	%
1	NH	40 ± 5		35 ± 7	
	I	45 ± 7	12	72 ± 3	105
2	NH	80 ± 6		78 ± 8	
	I	114 ± 4	39	149 ± 11	81
4	NH	87 ± 8		79 ± 5	
	I	128 ± 6	47	151 ± 7	91
8	NH	123 ± 10		116 ± 12	
	I	182 ± 9	49	232 ± 15	100
12	NH	150 ± 15		125 ± 16	
	I	260 ± 18	73	213 ± 17	70
19	NH	250 ± 13		200 ± 17	
	I	450 ± 40	80	370 ± 16	85

Mammary explants were prepared from mice in the indicated stages of pregnancy and cultured in medium 199 containing either no hormone (NH) or insulin (I). Accumulation of [¹⁴C]AIB was determined by allowing explants to accumulate [¹⁴C]AIB (0.1 μCi/ml of medium) at indicated periods as described in Materials and Methods. At least three mice were examined in a given stage.

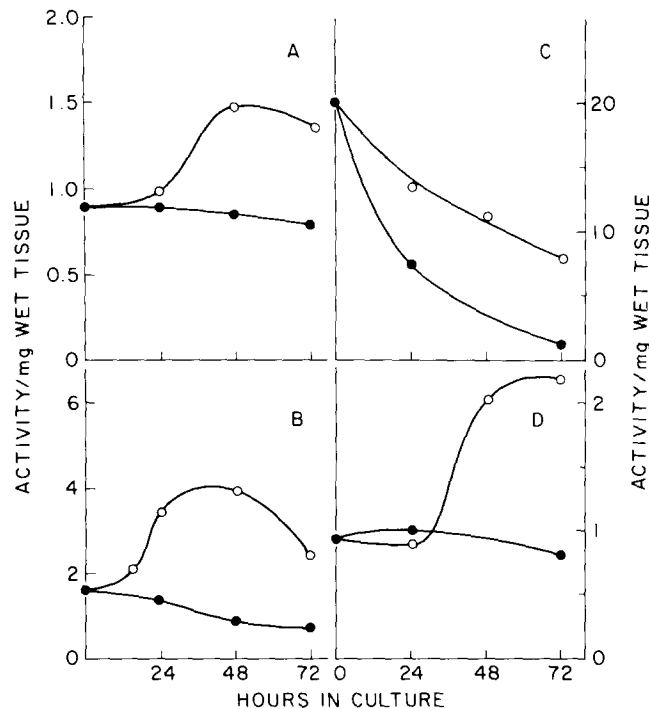


FIGURE 2 Time-course of accumulation of the combined activities of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase. Mammary explants from mature virgin (A), midpregnant (B), midlactational (C), and 6 wk postlactational (D) mice were cultured in the absence (●—●) or presence (O—O) of insulin. At the indicated times, the explants were treated with collagenase (12), and the epithelial cell fractions were assayed for the enzymes as described in Materials and Methods. The enzyme activities were expressed as the change in absorbance at 340 nm per mg wet weight of tissue. Each point represents the average of two determinations.

drogenase in the cultured epithelial cells from mature virgin, midpregnant, midlactational, and postlactational mice. The enzyme activities in cells from midpregnant mice increased rapidly to a near maximum after 24 h of incubation with insulin, whereas in the absence of the hormone, the activities declined steadily (Fig. 2 B). In contrast, the enzyme activities in the cells from mature virgin and postlactational mice changed very little in the first 24 h of culture despite the presence of insulin (Fig. 2 A, D). By the 2nd day, however, the activities increased in those cells which were incubated with insulin. In cells from midlactational mice, the enzyme activities were initially very high, and declined slowly in the presence of insulin (Fig. 2 C). In the absence of insulin, however, the decline was much faster than that which occurred in the presence of the hormone.

Fig. 3 depicts the time-course of DNA synthesis by mammary epithelium from mice in various physiological stages during a 4-day culture. As shown in Fig. 3 A, B, D, E, the explants from

immature, mature virgin, midlactational, and postlactational mice, respectively, were unresponsive to insulin during the first 24 h. During the 2nd day, however, DNA synthesis in those cells was markedly stimulated by insulin and reached a peak after 2-3 days of culture. Addition of larger amounts of the hormone did not shorten the 24-h lag period in any instance. It is noteworthy that a relatively small increase in DNA synthesis occurred even in the absence of insulin during a 4-day culture. The cells from midpregnancy, by contrast, showed a high initial rate, and responded markedly to insulin within the first 24 h (Fig. 3 C). In the absence of the hormone, the rate of DNA synthesis in these cells declined during culture.

DISCUSSION

Mammary epithelial cells begin their terminal differentiation during pregnancy and reach their developmental climax during lactation. The cells in other physiological states are developmentally dormant. The present studies demonstrate that mouse

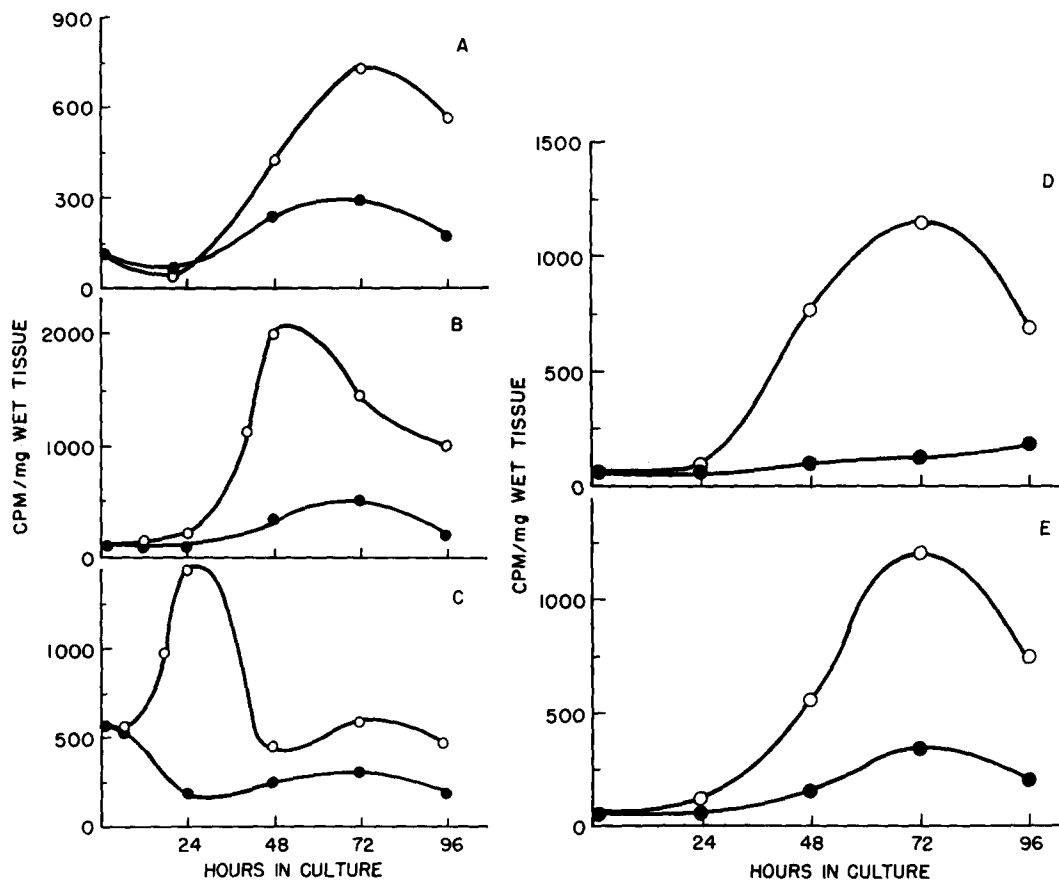


FIGURE 3 Time-course of DNA synthesis in mammary gland explants from mice in various physiological stages. Incubations were carried out in the absence (●—●) or presence (○—○) of insulin. The rate of DNA synthesis was measured by a 3 h labeling of explants with [*methyl*-³H]thymidine at a concentration of 1 μ Ci/ml of medium, at the indicated time. The incorporation of isotope into DNA was determined as described in Materials and Methods. (A) immature virgin, (B) mature virgin, (C) midpregnancy, (D) midlactation, (E) postlactation (6 wk). Each point represents the average of three determinations.

mammary cells during pregnancy and lactation are responsive to insulin, whereas cells in virgin and postlactational mice are unresponsive to the hormone. Such findings are consistent with the view (1-13, 17) that insulin may be required as one of the important physiological stimuli for the functional differentiation of mammary cells. Similar cyclical changes have been observed in responsiveness of mammary cells to epidermal growth factor (18) and serum factors (19-21), both of which stimulate DNA synthesis *in vitro* (unpublished). It is yet to be established whether or not insulin, epidermal growth factor, and/or serum factor(s) are the agents which promote cell proliferation during pregnancy. Nevertheless, these results, together with earlier findings (14, 20, 21), strongly

suggest that regulation of cellular responsiveness to insulin and other humoral factors may serve as a key determinant of the growth and development of mammary epithelium.

In general, the mammary cells appear to be either responsive or unresponsive to insulin in all respects studied, depending on the developmental state of the cells. Cells in lactation are an exception. In this instance, the cells are responsive to the hormone in terms of the AIB accumulation and the activities of glucose- and gluconate-6-phosphate dehydrogenase, but not in terms of DNA synthesis. This asynchrony may reflect a more general (22-24) but not universal (25-27) mutual exclusiveness between cell proliferation and specialized cellular activity.

The mechanisms whereby the cells acquire and lose responsiveness to insulin are not understood. In organ culture, responsiveness develops even in the absence of any added hormones about 1 day after culture² and this makes it possible for the cells to differentiate in vitro. In intact animals, development of the responsiveness appears to be hormone dependent. Recently, it has been found that the cells in nonpregnant mice and rats become responsive to insulin and to serum after administration of prolactin (20). Injection of insulin did not stimulate cell proliferation in mammary glands of these animals (unpublished data). These observations indicate that mammary cells in virgin mice and rats are indeed unresponsive to insulin, and that acquisition of responsiveness during pregnancy may, at least in part, be due to an increased level of serum prolactin or placental lactogen (28-30). Placental lactogen has been shown to simulate the several effects of prolactin on mammary cells (31). Furthermore, this effect of prolactin may also account for our occasional findings of insulin-responsive mammary cells in mature virgin animals, since the prolactin level increases during estrus (28). Moreover, in prolactin-treated mature virgin mice, the epithelium becomes unresponsive after 10 days of withdrawal of prolactin treatment (unpublished). Hence, it appears that the continuous presence of prolactin may be required for maintenance of insulin responsiveness in intact animals. Transition from lactation to involution, during which insulin responsiveness diminishes, may also be a consequence of a decrease in prolactin level in the circulation (28).

SUMMARY

Responsiveness of mouse mammary epithelium to insulin changes in various physiological states of the animal. Based on examination of several effects of insulin, responsive cells are found in the glands during pregnancy and lactation, states in which terminal differentiation of mammary epithelium takes place. Unresponsive cells are present in virgin and postlactational mice, states in which differentiation is arrested. It is suggested that insulin responsiveness may be an important factor

² We have observed some difference in the length of the unresponsive period, and also found that development of responsiveness in terms of AIB and DNA synthesis occurs asynchronously in time when we examined the virgin C3H/HeJ mice.

for regulation of mammary epithelial differentiation.

Received for publication 6 November 1973, and in revised form 20 March 1974.

REFERENCES

- ELIAS, J. J. 1959. *Proc. Soc. Exp. Biol. Med.* **101**:500.
- LASFARGUES, E. Y. 1960. *C. R. Seances Soc. Biol. Fil.* **154**:1720.
- RIVERA, E. M., and H. A. BERN. 1961. *Endocrinology.* **69**:340.
- PROP, F. J. A. 1961. *Pathol. Biol.* **9**:640.
- ICHINOSE, R. R., and S. NANDI. 1964. *Science (Wash. D. C.)* **14**:496.
- JUERGENS, W. G., F. E. STOCKDALE, Y. J. TOPPER, and J. J. ELIAS. 1965. *Proc. Natl. Acad. Sci. U. S. A.* **54**:629.
- STOCKDALE, F. E., and Y. J. TOPPER. 1966. *Proc. Natl. Acad. Sci. U. S. A.* **56**:1283.
- STOCKDALE, F. E., W. G. JUERGENS, and Y. J. TOPPER. 1966. *Dev. Biol.* **13**:266.
- VOYTOVICH, A. E., I. S. OWENS, and Y. J. TOPPER. 1969. *Proc. Natl. Acad. Sci. U. S. A.* **63**:213.
- MILLS, E. S., and Y. J. TOPPER. 1970. *J. Cell Biol.* **44**:310.
- MAYNE, R., and J. M. BARRY. 1970. *J. Endocrinol.* **46**:61.
- OKA, T., and Y. J. TOPPER. 1971. *J. Biol. Chem.* **246**:7701.
- VONDERHAAR, B. K., I. S. OWENS, and Y. J. TOPPER. 1973. *J. Biol. Chem.* **248**:467.
- FRIEDBERG, S. H., T. OKA, and Y. J. TOPPER. 1966. *Proc. Natl. Acad. Sci. U. S. A.* **56**:1283.
- GLOCK, G. E., and P. MCLEAN. 1954. *Biochem. J.* **56**:171.
- OKA, T., and J. W. PERRY. 1974. *J. Biol. Chem.* In press.
- TOPPER, Y. J. 1970. *Recent Prog. Horm. Res.* **26**:287.
- TURKINGTON, R. W. 1969. *Exp. Cell Res.* **57**:79.
- MAJUMDER, G. C., and R. W. TURKINGTON. 1971. *Endocrinology.* **88**:1506.
- OKA, T., and Y. J. TOPPER. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1693.
- HSUEH, H. W., and F. E. STOCKDALE. 1973. *J. Cell Biol.* **59**(2, Pt. 2):152. (Abstr.)
- STOCKDALE, F. E., and H. HOLTZER. 1961. *Exp. Cell Res.* **24**:508.
- YOUNG, R. W. 1962. *J. Cell Biol.* **14**:357.
- WESSELLS, N. K. 1964. *J. Cell Biol.* **20**:415.
- CAMPBELL, G. LEM., H. WEINTRAUB, B. H. MAYALL, and H. HOLTZER. 1971. *J. Cell Biol.* **50**:669.

26. COON, H. 1966. *Proc. Natl. Acad. Sci. U. S. A.* **55**:66.
27. CAHN, R. D., and M. B. CAHN. 1966. *Proc. Natl. Acad. Sci. U. S. A.* **55**:106.
28. AMENOMORI, Y., C. L. CHEN, and J. MEITES. 1970. *Endocrinology.* **86**:506.
29. LINKIE, D. M., and G. D. NISWENDER. 1972. *Endocrinology.* **90**:632.
30. MURR, S. M., G. E. BRADFORD, and I. I. GESHWIND. 1974. *Endocrinology.* **94**:112.
31. TURKINGTON, R. W., and Y. J. TOPPER. 1966. *Endocrinology.* **79**:175.