Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5682–5684, September 1981
Cell Biology

Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells

(epidermal growth factor/somatomedin C)

FRANKLYN F. BOLANDER, JR.*†, KEVIN R. NICHOLAS*, JUDSON J. VAN WYK‡, AND YALE J. TOPPER*

*Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205; and ‡Department of Pediatrics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Communicated by G. Gilbert Ashwell, May 28, 1981

ABSTRACT **In the presence of cortisol and prolactin, insulin at concentrations as low as 1 ng/ml significantly stimulates casein synthesis in mammary explants from midpregnant mice; maximal synthesis occurs at 10 ng/ml. However, in the absence of insulin, no detectable immunoprecipitable casein is produced. Insulin also supports enhanced accumulation of casein mRNA in the presence of cortisol and prolactin; neither epidermal growth factor nor somatomedin C has this effect. These inductive actions of insulin are not secondary to a general maintenance effect on the mammary epithelial cell; insulin, epidermal growth factor, and somatomedin C can support the accumulation of RNA in rough endoplasmic reticulum equally well. In addition, these effects do not reflect a specific insulin requirement for prolactin sensitivity; epidermal growth factor can support prolactin-induced total RNA synthesis as well as insulin can. The results demonstrate that, although insulin, epidermal growth factor, and somatomedin C can all function as cell maintenance agents, only insulin, together with cortisol and prolactin, can induce casein mRNA accumulation.**

Casein can be induced in mouse mammary explants in the presence of insulin, cortisol, and prolactin. Placental lactogen (1) and, in some instances, growth hormone (2, 3), can substitute for prolactin in this regard. Other glucocorticoids (4) can substitute for cortisol in this induction. On the other hand, other agents tested as possible substitutes for insulin have been found ineffective. Neither serum (5) nor epidermal growth factor (6) supports the induction of casein synthesis when used in the presence of cortisol and prolactin, although both are active mitogenic agents for mammary epithelial cells. Despite these findings, insulin has not been regarded as essential physiologically for phenotypic expression of casein genes in mammary epithelial cells (for review, see ref. 7). Rather, it has been considered to function in cell maintenance *in vitro*. In more recent studies, it has been reported that prolactin (8) and cortisol (9, 10) are required for accumulation of casein mRNA ($mRNA_{csn}$) in rodent mammary explants. Although insulin was routinely added to these cultures, its possible contribution was ignored. The intent of the present study is to determine whether or not insulin has a unique function in the accumulation of mouse mammary $mRNA_{csn}$, besides its role in cell maintenance.

MATERIALS AND METHODS

Materials. Ovine prolactin (NIH-P-S-14) was kindly provided by the Hormone Distribution Program, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, and crystalline porcine insulin (lot 615-08E-220) was donated by Eli Lilly. Epidermal growth factor (lot 1076 28) was purchased from Collaborative Research (Waltham, MA), and cortisol was obtained from Calbiochem. The somatomedin C used was derived from the final high-pressure liquid chromatography purification step described previously (11) and was free of both insulin and insulin-like growth factor II. This material was 33% pure, as determined by radioimmunoassay; the amounts given below take this factor into account. Medium 199/Hanks' salts was purchased from GIBCO, Hepes was obtained from Sigma, and rabbit anti-sheep IgG antiserum was from Miles-Yeda. $^{33}P_i$, carrier-free, and [5-^{3}H]uridine (30 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were purchased from New England Nuclear.

Organ Culture. The abdominal mammary glands from C_3H/HeN mice, 10–12 days into their first pregnancy, were removed under sterile conditions and explants were prepared as described (12). The culture medium (medium 199/Hanks' salts/20 mM Hepes, pH 7.6) contained cortisol (1 μg/ml), prolactin (1 μg/ml), and insulin, epidermal growth factor, or somatomedin C at 50 ng/ml, unless otherwise stated. The explants were incubated in 35-mm Corning culture dishes at 37°C under air, and the medium was changed daily.

Assays. Casein was determined by immunoprecipitation (13) with sheep anti-mouse antiserum except that a 4-hr pulse with $^{33}P_i$ (10 μCi/ml) was used instead of with [^{3}H]amino acids. $mRNA_{csn}$ was measured by RNA excess hybridization with a [^{3}H]cDNA probe complementary to purified 15S $mRNA_{csn}$ (14); using this probe, the $R_0t_{1/2}$ [initial concentration of RNA (moles of nucleotide/liter) × time (sec) for 50% hybridization] of purified $mRNA_{csn}$ was 2.8×10^{-3} mol·sec/liter. Total RNA isolated from each experimental group except the cortisol/prolactin system did not vary significantly and averaged 0.58 ± 0.06 μg/mg; total RNA isolated from the cortisol/prolactin system was 0.38 ± 0.03 μg/mg. The amount of RNA in the rough endoplasmic reticulum (RER) was determined in a mammary-epithelial cell-enriched fraction as described (15). The assays were performed after 48 hr of incubation.

Total RNA synthesis was measured in explants cultured first with insulin or epidermal growth factor for 96 hr and then for 12 hr with insulin and prolactin or epidermal growth factor and prolactin, respectively. During the final 4 hr, the explants were labeled with [^{3}H]uridine (0.5 μCi/ml); the amount of RNA synthesized was determined by the method of Green and Topper (16). Results are expressed as percent stimulation over the respective control values; the baseline in the two systems did not vary significantly. These values reflect RNA synthesis in epithelial cells; cleared fat pads do not respond to prolactin in regard to RNA synthesis (16).

RESULTS

The studies shown in Fig. 1 were designed to determine whether or not physiological levels of insulin, like the phar-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: $mRNA_{csn}$, casein mRNA; RER, rough endoplasmic reticulum.

† Present address: Dept. of Biology, Univ. of South Carolina, Columbia, SC 29208.

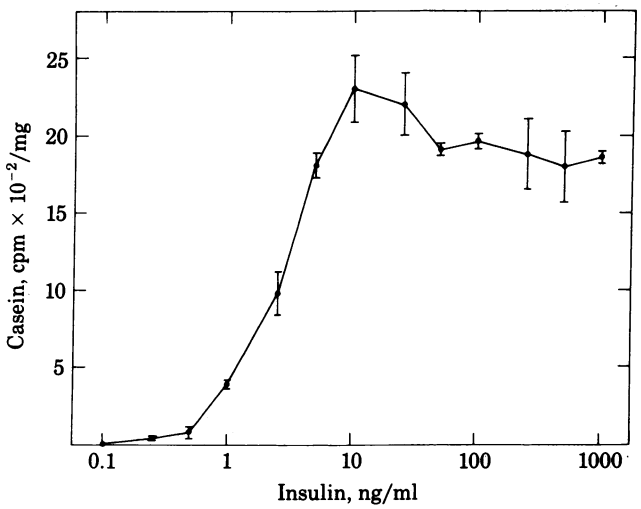

FIG. 1. Insulin dose–response relationship for casein synthesis in mammary gland explants from midpregnant mice. Explants were cultured for 44 hr in medium 199 containing cortisol (1 μg/ml), prolactin (1 μg/ml), and insulin and then pulsed for 4 hr with $^{33}P_i$ (10 μCi/ml). Casein was determined by immunoprecipitation; results (radioactivity per mg of tissue) are mean ± SEM of three pools of tissue.

macological doses (5 μg/ml) used previously (12), can satisfy the insulin requirement for induction of casein synthesis by mouse mammary explants. It is clear that, in the presence of cortisol and prolactin, no detectable induction occurs in the absence of insulin; a significant response occurs at concentrations as low as 1 ng/ml, and a maximal response is evoked by insulin at 10 ng/ml.

The experiments presented in Table 1 compare the abilities of insulin, epidermal growth factor, and somatomedin C to support the accumulation of $mRNA_{csn}$ in the presence of cortisol and prolactin and the formation of RNA in RER in the presence of cortisol. Serum somatomedin C levels in the midpregnant mouse are ≈100 ng/ml by radioimmunoassay (17). However, due to limited supplies of somatomedin C, a concentration of 50 ng/ml was used in these experiments. Serum epidermal growth factor levels in the pregnant mouse are not known, but basal levels are 1.5 ng/ml (18). As serum levels in humans increase at least 10-fold during gestation (19), an epidermal growth factor concentration of 50 ng/ml was felt to mimic the levels in the pregnant mouse reasonably. Insulin was also used at 50 ng/ml.

Table 1. Effect of growth factors on $mRNA_{csn}$ accumulation and RER formation

Addition	$mRNA_{csn}$, %	RNA in RER, μg/100 mg
None (t_0)	0.099 ± 0.011	2.2 ± 0.2
None (cultured)	0.020 ± 0.004	2.0 ± 0.4
Insulin	1.39 ± 0.16	9.9 ± 0.5
Epidermal growth factor	0.093 ± 0.008	8.8 ± 0.5
Somatomedin C	0.103 ± 0.011	9.1 ± 0.3

Mammary gland explants from midpregnant mice were either assayed immediately (t_0) or cultured for 48 hr in medium 199 containing growth factor (50 ng/ml) and cortisol (1 μg/ml). When $mRNA_{csn}$ was to be assayed, prolactin (1 μg/ml) was also present. $mRNA_{csn}$ was determined by RNA excess hybridization (10) and RNA in RER was measured in an epithelial cell-enriched fraction (15). Results are mean ± SEM of three pools of tissue, except for somatomedin C which, because of limited supplies, was assayed twice and is average ± range.

Table 1 shows that the concentration of $mRNA_{csn}$ in fresh mammary glands from midpregnant mice is almost 0.1% of total RNA. In the presence of only cortisol and prolactin, this level decreases to 0.02% in 48 hr. When insulin is also present, a 14-fold increase in $mRNA_{csn}$ accumulation occurs. Both epidermal growth factor and somatomedin C maintain the initial concentration, but they do not support enhanced accumulation of $mRNA_{csn}$. By contrast, all three factors support a 4- to 5-fold increase in RER-associated RNA in the presence of cortisol. Insulin is not a specific requirement for other functions of prolactin; epidermal growth factor is as effective as insulin in supporting prolactin-induced total RNA synthesis (26 ± 3% and 24 ± 3%, respectively).

DISCUSSION

Prolactin (8) and glucocorticoid (9, 10) are required for the accumulation of $mRNA_{csn}$ in rodent mammary gland explants. Although insulin has also been added to these systems consistently, its role has been considered only in terms of cell maintenance, despite the fact that serum can support epithelial cell replication in these explants (5) but cannot substitute for insulin in regard to casein synthesis. Perhaps the exclusive emphasis on cell maintenance is due to the use of insulin at high concentrations in the mammary system and to the fact that, in general, insulin has rarely been considered to be intimately associated with gene expression. To determine whether or not insulin has a fundamental role in mouse casein gene expression, other than as a cell maintenance factor *in vitro*, two questions have been asked: (*i*) Can physiological concentrations of insulin operate effectively in the system and (*ii*) can other agents, if shown to support vital cell functions, substitute for insulin in regard to $mRNA_{csn}$ accumulation?

Physiological concentrations of insulin (1–10 ng/ml) can indeed stimulate casein synthesis in mouse mammary gland explants (Fig. 1). This effect is not related to stimulation of glucose transport, as insulin can support casein synthesis when fructose replaces glucose in the medium (unpublished observations). Nor is this effect related merely to cell maintenance as reflected by fabrication of RER. When cortisol alone is added to the explant system, no increase in RER occurs (Table 1). Insulin, in addition to cortisol, promotes a 4-fold increase, and both epidermal growth factor and somatomedin C are as effective as insulin. Furthermore, both epidermal growth factor (5) and somatomedin C (20) can stimulate proliferation of mouse mammary epithelial cells *in vitro* although neither can substitute for insulin in regard to $mRNA_{csn}$ accumulation. Insulin makes possible a 14-fold increase in messenger concentration over the original level, while epidermal growth factor and somatomedin C do not promote an increment in $mRNA_{csn}$ although they can maintain the initial level. However, as the somatomedins have a low affinity for insulin receptors (21), it is possible that somatomedin C at a higher concentration than that used in this study could induce $mRNA_{csn}$ accumulation via activation of the insulin receptor.

Evaluation of the mechanism of action of insulin should also include examination of pretranscriptional events. For example, as diabetic rats have reduced lactogenic receptors in both the liver (22) and mammary tumors (23), it was conceivable that insulin may only be required to maintain prolactin receptors or some postreceptor apparatus related to prolactin. This seems unlikely as epidermal growth factor can support prolactin-induced total RNA synthesis as well as insulin.

It appears, then, that insulin plays a vital role in the expression of mouse casein genes independent of its cell maintenance functions. Although insulin, cortisol, and prolactin are all required for the induction of $mRNA_{csn}$ accumulation in mouse

mammary explants, this combination may not be needed in mammary tissue from some other species. It has been reported that prolactin can induce $mRNA_{csn}$ accumulation in rabbit explants (24) and casein synthesis in human explants (25) in the absence of exogenous insulin or cortisol.

Insulin has been shown to be important in gene expression in at least two other systems. By itself, it can induce δ-crystallin mRNA in cultures of chicken lens epithelial cells (26), and the administration of insulin to adrenalectomized rats induces the mRNA for tyrosine aminotransferase in the liver (27). However, in the latter case, insulin is not unique; glucocorticoids can induce this enzyme in rat hepatoma cells cultured in the absence of insulin (28). It is not clear whether insulin is an absolute requirement for δ-crystallin mRNA induction in lens epithelial cells; although serum can substitute for insulin in this regard (26), the active component in the serum remains to be identified. Furthermore, there may be an as yet untested factor capable of substituting for insulin in the mouse mammary system. However, the previous studies, together with the present report, suggest that insulin may be indispensable for the expression of certain genes related to cell differentiation, in addition to serving as a metabolic and growth factor.

Note Added in Proof. While this manuscript was in press, it was reported (29) that the estrogen-mediated induction of ovalbumin gene transcription requires a somatomedin-like peptide hormone. Insulin, proinsulin, or multiplication-stimulating activity can satisfy this requirement. It was also reported that insulin exerts a selective effect on pancreatic amylase gene expression (30).

We gratefully acknowledge Dr. M. E. Svoboda for her work in purifying the somatomedin C used in this study. This work was supported, in part, by National Institutes of Health Grant AM 01022-25 to J.J.V.W., who is also the recipient of Career Research Award AM 1411518.

1. Turkington, R. W. (1968) *Endocrinology* **82**, 575–583.
2. Wang, D. Y., Hallowes, R. C., Smith, R. H., Amor, V. & Lewis, D. J. (1972) *J. Endocrinol.* **52**, 349–358.
3. Markoff, E. & Talamantes, F. (1980) *Endocr. Res. Commun.* **7**, 269–278.
4. Turkington, R. W., Juergens, W. G. & Topper, Y. J. (1967) *Endocrinology* **80**, 1139–1142.
5. Majumder, G. C. & Turkington, R. W. (1971) *Endocrinology* **88**, 1506–1510.
6. Turkington, R. W. (1969) *Exp. Cell Res.* **57**, 79–85.
7. Banerjee, M. R., Ganguly, R., Mehta, N. M. & Ganguly, N. (1982) in *Hormone Regulation of Experimental Mammary Tumors*, ed. Leung, B. (Eden, Los Angeles), in press.
8. Matusik, R. J. & Rosen, J. M. (1978) *J. Biol. Chem.* **253**, 2343–2347.
9. Ganguly, R., Ganguly, N., Mehta, N. M. & Banerjee, M. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6003–6006.
10. Nagaiah, K., Bolander, F. F., Nicholas, K. R., Takemoto, T. & Topper, Y. J. (1981) *Biochem. Biophys. Res. Commun.* **98**, 380–387.
11. Svoboda, M. E., Van Wyk, J. J., Klapper, D. G., Fellows, R. E., Grissom, F. E. & Schleuter, R. J. (1980) *Biochemistry* **19**, 790–797.
12. Juergens, W. F., Stockdale, F. E., Topper, Y. J. & Elias, J. J. (1965) *Proc. Natl. Acad. Sci. USA* **54**, 629–634.
13. Ono, M. & Oka, T. (1980) *Cell* **19**, 473–480.
14. Takemoto, T., Nagamatsu, Y. & Oka, T. (1980) *Dev. Biol.* **78**, 247–257.
15. Oka, T. & Topper, Y. J. (1971) *J. Biol. Chem.* **246**, 7701–7707.
16. Green, M. R. & Topper, Y. J. (1970) *Biochim. Biophys. Acta* **204**, 441–448.
17. D'Ercole, A. J. & Underwood, L. E. (1980) *Dev. Biol.* **79**, 33–45.
18. Cohen, S. & Savage, C. R. (1974) *Rec. Prog. Horm. Res.* **30**, 551–574.
19. Ances, I. G. (1973) *Am. J. Obstet. Gynecol.* **115**, 357–362.
20. Ptashne, K., Hsueh, H. W. & Stockdale, F. E. (1979) *Biochemistry* **18**, 3333–3339.
21. King, G. L., Kahn, C. R., Rechler, M. M. & Nissley, S. P. (1980) *J. Clin. Invest.* **66**, 130–140.
22. Baxter, R. C. & Turtle, J. R. (1978) *Biochem. Biophys. Res. Commun.* **84**, 350–357.
23. Smith, R. D., Hilf, R. & Senior, A. E. (1979) *Cancer Biochem. Biophys.* **3**, 117–121.
24. Devinoy, E., Houdebine, L.-M. & DeLouis, C. (1978) *Biochim. Biophys. Acta* **517**, 360–366.
25. Gaffney, E. V., Polanowski, F. P., Blackburn, S. E., Lambrase, J. T. & Burke, R. E. (1976) *Cell Differ.* **5**, 69–81.
26. Milstone, L. M. & Piatigorsky, J. (1977) *Exp. Cell Res.* **105**, 9–14.
27. Hill, R. E., Lee, L.-L. & Kenney, F. T. (1981) *J. Biol. Chem.* **256**, 1510–1513.
28. Tomkins, G. M., Thompson, E. B., Hayashi, S., Gelehrter, T., Granner, D. & Peterkofsky, B. (1966) *Cold Spring Harbor Symp. Quant. Biol.* **31**, 349–360.
29. Evans, M. I., Hager, L. J. & McKnight, G. S. (1981) *Cell* **25**, 187–193.
30. Korc, M., Owerbach, D., Quinto, C. & Rutter, W. J. (1981) *Science* **209**, 351–353.