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Development of Insulin-Sensitivity by Mouse Mammary Gland *In Vitro**

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Abstract. Explants from mammary gland of pregnant mice are sensitive to insulin, but corresponding explants from mature, virgin mice are insensitive to insulin in certain respects. Isolated virgin tissue can acquire insulin-sensitivity independent of exogenous insulin. Some endocrinological and developmental implications are discussed.

Epithelial cells in the mammary gland of the mature, virgin mouse undergo virtually no proliferation. In contrast, cell proliferation occurs extensively during pregnancy. The normal development of mouse mammary gland can be mimicked, *in vitro*, by the use of various hormones.[1] While mammary explants from virgin and pregnant mice require only insulin (endogenous in both animals) for stimulation of DNA synthesis,[2] the rapidity with which they respond to the hormone differs. Explants from pregnant mice respond rapidly; explants from virgin mice do so only after a considerable lag period.[2] Several questions are raised by these observations. Does the delayed response of the virgin tissue represent a constraint on DNA synthesis only, or is it a reflection of a more general initial lack of responsiveness to insulin? Furthermore, if the eventual initiation of DNA synthesis in virgin explants has a physiological counterpart, why does the endogenous hormone not promote DNA synthesis in mammary epithelial cells of the intact virgin animal?

The question of lack of responsiveness to endogenous hormones can also be raised in relation to the development of the rough endoplasmic reticulum (RER). There is, in fact, more extensive development of the RER in the gland of the pregnant mouse. Formation of RER can be stimulated in pregnant explants by combined treatment with insulin and glucocorticoid,[3] both of which are present in the virgin mouse. Why, then, does such development not occur in the virgin animal?

In this study, several insulin-dependent parameters, which represent various levels of cellular activity, have been compared in explants from virgin and pregnant mice. Certain more general aspects of hormone action, as they relate to problems of development, are also considered.

Materials and Methods. The previously described[4] organ culture technique employing mammary gland explants was used. Such explants contain epithelial and fat cells, predominantly. In order to ascertain whether or not the observed effects related to epithelial cells, the tissue was treated with collagenase and the epithelial

cells separated from the lysed fat cells by centrifugation.[5] In some instances, this separation was performed after the explants had been cultured. In other cases, fat cells were removed initially, and the residual epithelial cell–connective tissue complex was then cultured using the same technique employed with explants. By these means, it has been determined that these results probably reflect responses of the epithelial cells.

Insulin (crystalline beef insulin, a gift from the Eli Lilly Co.) was used at a calculated final concentration of 10^{-7} M. However, in the presence of bovine serum albumin (final concentration, 2.5%), which minimizes loss of insulin by adsorption on glassware, the same effects were manifested at a calculated insulin concentration of 10^{-9} M. Hydrocortisone was used at a final concentration of 10^{-5} M; we have previously reported that the minimal effective concentration of this hormone is 10^{-8} M.[6] Medium 199 (Microbiological Associates), containing fructose instead of glucose, was used in all experiments.

The pregnant mice used in these studies were in the middle of their first pregnancy. The virgin animals ranged from 3.5 to 5 months of age. All animals were of the C3H/HeN strain.

Results. Fig. 1 shows the initial time course of accumulation of the nonmetabolizable amino acid, α-amino isobutyric acid (AIB) by pregnancy- and virgin-

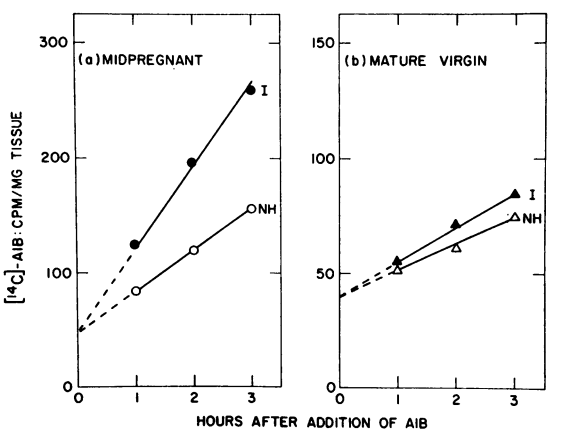


FIG. 1. Effect of insulin on the accumulation of α-[1-^{14}C]aminoisobutyric acid ([^{14}C]AIB) by mammary explants from midpregnant (*a*) and virgin (*b*) mice. The explants were incubated in the absence (NH) or in the presence (I) of insulin. After 1 hr of incubation [^{14}C]AIB (6.49 mCi/mmol, New England Nuclear) was added to each culture dish, to a final concentration of 0.1 μCi/ml. At the indicated times, accumulation of [^{14}C]AIB was measured as follows: explants were weighed, placed on glass filter paper, and washed 3 times with Medium 199 and 2 times with water by suction filtration. The explants and filter papers were transferred to scintillation vials and dissolved in 1 ml of NCS solubilizer (Amersham/Searle) at 50°C overnight. 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole, 0.4 g 1,4-bis[2-(4-methyl-5-phenyloazolyl]-benzene per liter toluene) was added to each vial and ^{14}C was measured in a liquid scintillation spectrometer. Each point represents the average of three determinations.

explants in the presence and absence of insulin. It can be seen that for the first 3 hr after the addition of AIB the rates are linear, and that during this time insulin exerts a much greater effect on the pregnancy- than on the virgin-explants. The hormone has no effect on the inulin space in either tissue.

In Fig. 2 the rate of accumulation of AIB at various times during 96 hr of culture is depicted. The rate of accumulation of the amino acid by pregnancy explants is constant over the entire 4-day culture period in the presence and absence of insulin. The increment in accumulation with and without insulin is, therefore, also constant during this time. The rate of accumulation by virgin explants in the absence of insulin is also constant during 4 days. During the first day, the rate of accumulation by virgin tissue in the presence of the hormone is constant, and is only slightly larger than in the absence of insulin. Thus, again we see a relatively

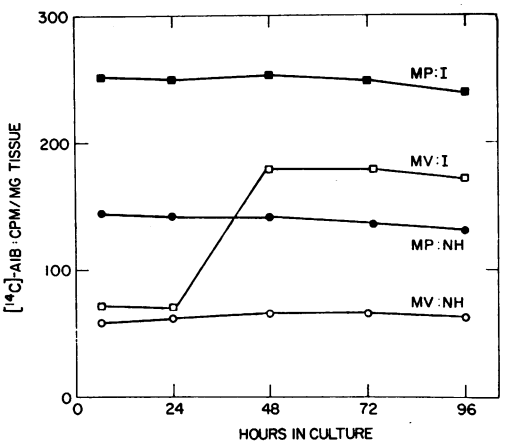


FIG. 2. The accumulation of [^{14}C] AIB during 96 hr of culture. Mammary gland explants prepared from mid-pregnant (*MP*) and virgin (*MV*) mice were cultured in the absence (*NH*) or in the presence (*I*) of insulin. [^{14}C] AIB (0.1 μCi/ml) was present for 3 hr ending at the times indicated, and uptake was measured as described in the legend to Fig. 1.

small response initially. By the end of the second day, however, insulin exerts a marked effect on the rate in the virgin explants. This enhanced rate of accumulation is maintained during the next 2 days. Similar results are obtained when corresponding epithelial cell–connective tissue complexes are used instead of explants. This indicates that these effects reflect responses of the epithelial cells, and that the development of insulin responsiveness in terms of this parameter does not require the presence of the fat cells.

The studies shown in Fig. 3 delineate more precisely the duration of the lag period in the virgin tissue. The lag period lasts 24–28 hr.

The experiments presented in Fig. 4 are designed to determine whether incubation of virgin tissue in the absence of insulin for different lengths of time has an effect on the kinetics of response to the hormone added at later time points. It can be seen that prior incubation for 12 or 24 hr in the absence of insulin has essentially no effect on these kinetics. The duration of the lag period, and the events which lead from hormone-insensitivity to sensitivity, are, therefore, independent of exogenous insulin. Moreover, once the tissue has traversed the insensitive period in the absence of the hormone (32 hr), the response to added insulin (32–36 hr) is very rapid (NH_{32}–I_{32-36} system).

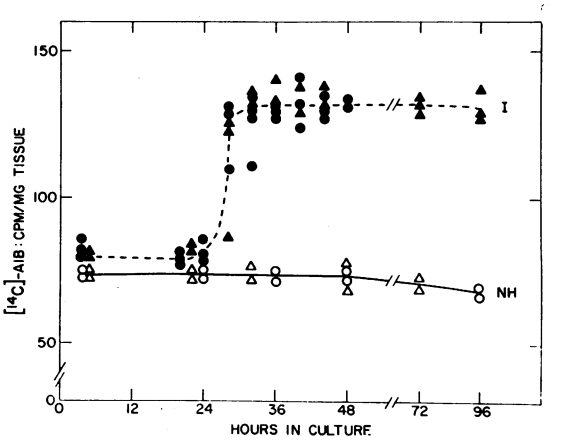


FIG. 3. Time course of AIB accumulation by mammary explants derived from mature virgin mice. Explants from two mice were cultured separately in the absence (*NH*) or in the presence (*I*) of insulin. The accumulation of AIB was measured after pulsing with [^{14}C]-AIB (0.1 μCi/ml) for 3 hr, ending at the times indicated. Each point represents a single determination. *Circles* represent values from one animal and *triangles* from another.

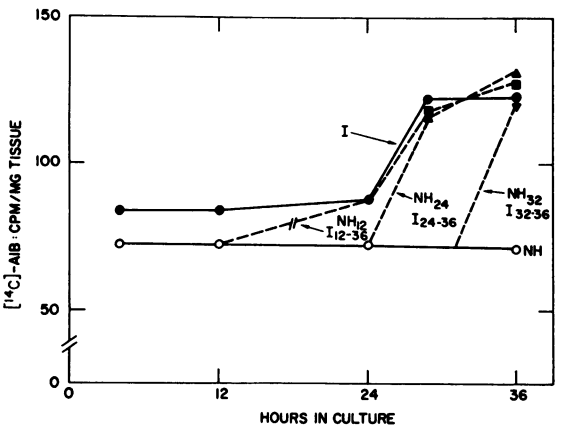


FIG. 4. Effect of culture in the absence of insulin on the subsequent insulin-stimulated accumulation of AIB by virgin explants. During culture in the absence (*NH*) of insulin, virgin explants were transferred to insulin (*I*) medium at the indicated times. AIB accumulation was measured after pulsing with [^{14}C]AIB (0.1 μCi/ml) for 3 hr, ending at the times shown. Each point represents the average of three determinations.

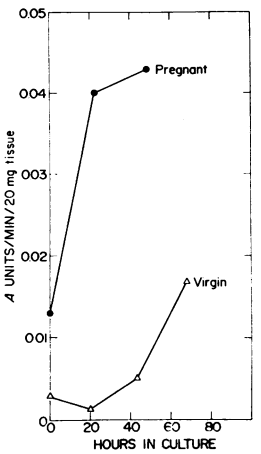
Formation of RER by alveolar cells in pregnancy explants requires both insulin and glucocorticoid. The experiments reported in Table 1 represent an attempt to understand why endogenous insulin and glucocorticoid do not promote RER in the mammary epithelial cells of the intact mature, virgin mouse. In these studies the membrane-associated enzyme, reduced-NAD dehydrogenase (EC 1.6.99.3), in epithelial cells of pregnancy- and virgin-explants, was assayed after 48 and 96 hr of culture under the conditions shown. The data reveal that insulin and hydrocortisone alone affect small increases in the enzyme activity in pregnancy tissue after 48 hr, but that the effect is much greater when both hormones are present. After 48 hr, virgin tissue shows no response to insulin, a small response to hydrocortisone, and the same small response to the combination of the two hormones. However, after 96 hr, at a time when the tissue does show some response to insulin alone, the two hormones together elicit a much greater stimulation than the single hormones. These results, considered in conjunction with those obtained with AIB, suggest that the absence of a synergistic effect on the virgin system after 48 hr is due, at least in part, to initial insensitivity to insulin. It is to be noted that sensitivity to insulin in terms of reduced-NAD dehydrogenase develops later than it does with respect to AIB uptake. A possible inference from these observations is that only little RER is formed in the mammary epithelial cells within the intact virgin animal, partly because this tissue is largely insensitive to insulin.

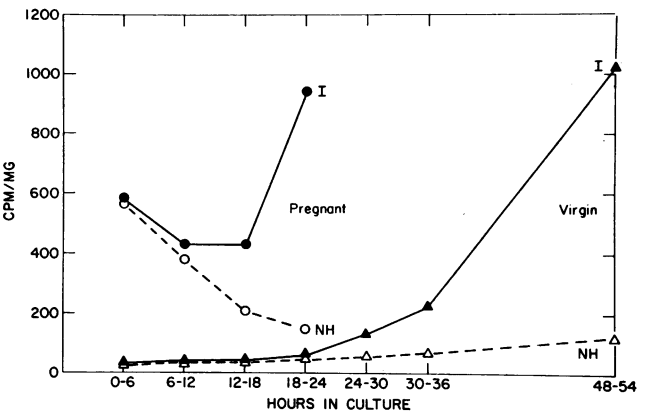
TABLE 1. *Changes in reduced-NAD dehydrogenase.*

	Mature virgin		Midpregnant
	Culture period		
Conditions	48 hr	96 hr	48 hr
	% of zero-time control		
No hormone	85	105	95
Insulin	100	135	125
Hydrocortisone	130	176	138
Hydrocortisone + insulin	130	420	310

40–60 mg of mammary explants was prepared from midpregnant or mature virgin mice for each hormone system. At the end of the culture, the explants from each system were weighed and treated with collagenase.[5] The resulting epithelial pellets were homogenized in 2 ml of cold 0.25 M sucrose, and the homogenates were centrifuged at 5500 × *g* for 20 min at 4°C. The supernatant was assayed.[7]

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) is an intracellular protein mainly present in the soluble fraction of the cytoplasm. Its formation in mammary gland explants has been shown to be dependent upon insulin.[8] Fig. 5





(*Left*) FIG. 5. Amount of the combined activities of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase as a function of time. Mammary explants from mid-pregnant and mature virgin mice were cultured in the presence of insulin. At the indicated times the tissue was treated with collagenase,[5] and extracts from the epithelial pellets were assayed for the enzymes.[9]

(*Right*) FIG. 6. DNA synthesis in mammary gland explants from pregnant and virgin mice. Incubations were carried out in the absence (*NH*) or presence (*I*) of insulin, and [methyl-^{3}H]-thymidine (20 Ci/mmol) was added (final concentration, 1 μCi/ml) for 6-hr periods during the culture, as indicated. The incorporation of isotope into DNA was determined by a modification of a method described previously;[2] no carriers were added. Each point represents the average of three determinations.

compares the time courses of the combined activities of this enzyme and phosphogluconate dehydrogenase (EC 1.1.1.43) found in pregnancy- and virgin-epithelial cells during culture of explants in the presence of insulin. Again, it is seen that the response of the virgin tissue to the hormone occurs 1–2 days later than the response of the pregnancy tissue. In the absence of insulin, the enzyme activity decreases progressively during culture in virgin tissue, and no increase occurs in pregnancy tissue.

The question as to why endogenous insulin does not promote DNA synthesis in mammary epithelial cells of the intact, mature virgin mouse will be considered next. The rate of incorporation of tritiated thymidine into trichloroacetic acid-insoluble material within the explants has been used to measure DNA synthesis by the epithelial cells, based on the observed correspondence between the time course of [^{3}H]thymidine incorporation and mitotic indices in the epithelium,[2] on autoradiographic studies[6] which demonstrated silver grains predominantly over epithelial nuclei (but not over fat-cell nuclei), and on a comparison of insulin-induced DNA synthesis by intact and deepithelialized mammary explants.[10]

Fig. 6 shows that DNA synthesis by pregnancy explants is relatively active during the first 6 hr in the presence (I) and absence (NH) of insulin, and that it declines more slowly in the next 6 hr with insulin than without it. Whereas with-

out insulin, DNA synthesis continues to decline, with insulin it plateaus between 12–18 hr, then rapidly increases to a maximum (ref 2, not shown here) between 18–24 hr. The very low initial extent of DNA synthesis by virgin explants is maintained during the first 24 hr both with and without insulin. The extent without insulin increases slightly during the second day, but the level of DNA synthesis with insulin increases markedly after 36 hr. Pregnancy tissue, then, responds to insulin, in terms of DNA synthesis, at least 18 hr before virgin tissue, and the corresponding maxima occur about 24 hr apart.[2]

To determine whether exogenous insulin is necessary during the first 24 hr in order that virgin tissue respond to the hormone during the second day, the experiments illustrated in Fig. 7 were performed. The rates of DNA synthesis

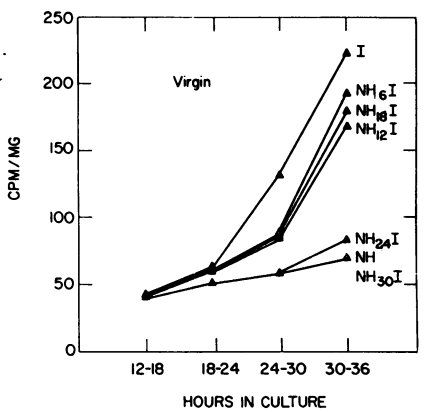


FIG. 7. Delay between the onset of insulin treatment and the DNA-synthesis response. Virgin mammary gland tissue was cultured for varying lengths of time in hormone-free (*NH*) medium before exposure to insulin (I). Cultures were pulsed with [methyl-^{3}H]thymidine and assayed as described in the legend to Fig. 6.

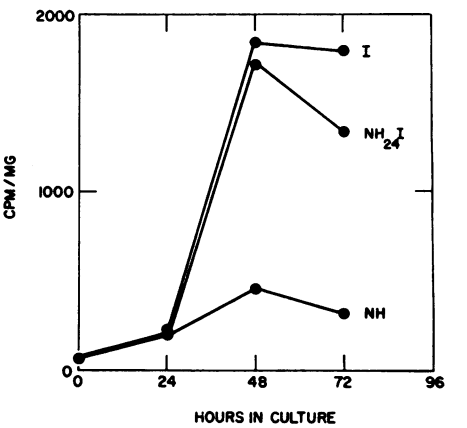


FIG. 8. Time courses of DNA synthesis. Virgin mammary explants were cultured for 72 hr in the absence (*NH*) of insulin, and in the presence (*I*) of insulin, or in the absence of insulin for 24 hr followed by exposure to insulin for 48 hr. Culutres were pulsed with [methyl-^{3}H]thymidine and assayed as described in the legend to Fig. 6.

are about the same in all systems for the first 24 hr. Between 24–30 hr, the rates in the NH_6I, $NH_{12}I$, and $NH_{18}I$ systems are the same, but a little slower than the rate in the system with insulin present throughout. However, between 24–36 hr, the slopes in all four cases are essentially identical. It appears that whereas virgin tissue may be slightly sensitive to insulin during the first 18 hr, as reflected by DNA synthesis (see also the results with AIB, Fig. 2) an important insulin-independent component is implicated in the eventual acquisition of greatly enhanced responsiveness to the hormone.

Between 30–36 hr, the $NH_{24}I$ system has a much slower rate than the others discussed above, but, as shown in Fig. 8, it can attain the same maximum, at the same time, as the system with insulin throughout. Note that the system without insulin also reaches a maximum, albeit a much lower one, at 48 hr. This demonstration that the time at which peak DNA synthesis occurs in isolated

virgin tissue is not a function of the time at which insulin is added fortifies the conclusion that the lag period in such tissue is, indeed, independent of exogenous insulin.

The delay in response of virgin mouse mammary gland to insulin does not necessarily mean that such tissue is totally incapable of being affected by the hormone initially. Although the final manifestation in each instance does not appear during the lag phase, one or more early effects, not detectable by the methods employed, may well be occurring during the so-called insensitive period.

Discussion. It has been shown that mammary epithelial cells from the pregnant mouse are initially sensitive to insulin *in vitro* in terms of several parameters, but that freshly explanted virgin tissue is either insensitive or only slightly sensitive. After one to several days in culture, the virgin tissue acquires sensitivity to the hormone comparable to that of the pregnancy tissue. Acquisition of the ability to respond to insulin is largely independent of exogenous insulin. Differences in the length of the lag period, as a function of particular responses, indicate that we may be dealing with mechanistically different manifestations of the hormone's action. The events which lead to insulin sensitivity *in vivo* during pregnancy are probably not identical to those which produce sensitivity in virgin tissue *in vitro*. Thus, altered levels of some hormones, such as estrogen, progesterone, and prolactin, and the *de novo* appearance of others, such as placental lactogen, may play a role in the intact animal during pregnancy. These factors clearly do not participate in the conversion of insulin-insensitive into sensitive virgin explants under the conditions employed. This conversion seems to be a consequence, particularly, of removal of the tissue from the animal.

If insulin is at least partially responsible for the initiation of epithelial DNA synthesis during pregnancy, the results could explain why this hormone does not perform a similar function in the mature, virgin animal. For some as yet unknown reason, the potentially sensitive tissue is, in fact, insensitive to insulin in this physiological state. Tissue explanation or pregnancy can lead to insulin-sensitivity.

These observations also have more general implications for interactions of hormones with tissues. If a given hormone has more than one target tissue, and each tissue is maximally sensitive at all times, modulations in the plasma level of the hormone would not be expected to lead to highly selective effects on a particular target. The selectivity of a hormone-response could be enhanced if the sensitivity of one or another target tissue varied under different physiological conditions. Furthermore, if this were true, the plasma level of hormones would not necessarily be an accurate reflection of hormone–tissue interaction.

Changes in sensitivity to various stimuli, such as hormones, also appear to impinge on fundamental problems of developmental biology. Responsiveness may be acquired, or lost, during maturation. Two interesting examples have recently been reported. Isolated human-fetal islets of Langerhans secrete insulin in response to glucagon, but not in response to glucose or tolbutamide. Sensitivity to the latter stimuli develops later.[11] Arginase activity in the liver of late-fetal rats is increased by the administration of thyroxine, but not by that of hydrocortisone. The liver of the 5-day-postnatal rat, on the other hand, no

longer responds to thyroxin in this way, but does so following injection of hydrocortisone.[12] Similarly, the mammary gland of the mature, virgin mouse is apparently not capable of responding to insulin, a potential stimulus present in the blood stream. Thus, the presence of a stimulus is no guarantee that a tissue–stimulus interaction will ensue. Furthermore, as shown with the mammary-explant system, development of the ability to respond to a signal, such as a hormone, is not necessarily dependent on the presence of the signal. In this respect, such development is not analogous to substrate-induced enzyme formation. The isolated tissue system described in this report may provide a useful model for studying such phenomena.

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Abbreviations: RER, rough endoplasmic reticulum; AIB, α-aminoisobutyric acid.

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