A Mevalonate Requirement for Maintenance of Fatty Acid and Protein Synthesis during Hormonally Stimulated Development of Mammary Gland in Vitro*

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The effect of compactin on hormonally induced lipogenesis and protein synthesis was studied in vitro in explants of mammary gland from mid-pregnant rabbits. Compactin blocks mevalonate synthesis by the specific inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase, and in this system, culture with 10 μM compactin for 24, 48, and 72 h inhibited incorporation of [1,14C]acetate (but not [2,14C]-mevalonate) into sterol by 98, 95, and 86%, respectively. Removal of compactin prior to assay rapidly reversed this effect and was associated with increased tissue 3-hydroxy-3-methylglutaryl-CoA reductase activity. Fatty acid synthesis (measured by incorporation of [1-14C]acetate or [4,5-3H]leucine) and protein synthesis (measured by incorporation of [4,5-3H]leucine) were both inhibited by about 50% after culture with compactin. This inhibition was not rapidly reversed by removal of compactin prior to assay, but it was prevented by inclusion of 1 mM mevalonolactone in the culture medium. After removal of compactin and continued culture in its absence for 24 h with hormones, the normal tissue capacity for fatty acid and protein synthesis was restored, indicating no permanent cell damage. The results suggest a specific requirement for mevalonate (or derived products) for the hormonal maintenance of the increased fatty acid and protein synthesis characteristic of the development of the mammary gland.

EXPERIMENTAL PROCEDURES

Animals—Primiparous New Zealand White rabbits were purchased from the Joint Animal Breeding Unit, Nottingham University School of Agriculture, Sutton Bonnington, Leicester, United Kingdom. The animals were at least 6 months old at time of mating.

Materials—[1,14C]Acetate, [2,14C]mevalonolactone, [3-14C]HMG-CoA, L-[4,5,3H]leucine, [1,2-14C]cholesterol, and [9,10,3H]palmitic acid were purchased from Amersham International, Little Chalfont, Buckinghamshire, United Kingdom. Mevalonic acid (triethylammonium salt) was purchased from New England Nuclear. Mevalonolactone (trileucammonium salt) was purchased from New England Nuclear. Mevalonolactone (trileucammonium salt) was purchased from New England Nuclear. 3-Hydroxy-3-methylglutaryl-CoA reductase, and in this system, culture with 10 μM compactin for 24, 48, and 72 h inhibited incorporation of [1,14C]acetate (but not [2,14C]-mevalonate) into sterol by 98, 95, and 86%, respectively. Removal of compactin prior to assay rapidly reversed this effect and was associated with increased tissue 3-hydroxy-3-methylglutaryl-CoA reductase activity. Fatty acid synthesis (measured by incorporation of [1-14C]acetate or [4,5-3H]leucine) and protein synthesis (measured by incorporation of [4,5-3H]leucine) were both inhibited by about 50% after culture with compactin. This inhibition was not rapidly reversed by removal of compactin prior to assay, but it was prevented by inclusion of 1 mM mevalonolactone in the culture medium. After removal of compactin and continued culture in its absence for 24 h with hormones, the normal tissue capacity for fatty acid and protein synthesis was restored, indicating no permanent cell damage. The results suggest a specific requirement for mevalonate (or derived products) for the hormonal maintenance of the increased fatty acid and protein synthesis characteristic of the development of the mammary gland.

The development of the mammary gland from the pregnant to the lactating state is associated with an increased capacity for expression of hormonal substrates as below.

Incubation of Explants—Explants (10–20 mg wet weight) were removed from the culture medium after 24, 48, or 72 h, blotted, weighed, and transferred to a scintillation vial containing 1 ml of incubation medium (gassed with O2 + CO2 (95% + 5%)). For measurement of incorporation of [14C]acetate and mevalonate into lipids, the medium consisted of Krebs bicarbonate buffer containing 1 mM glucose and either [1,14C]acetate (5 μCi; specific radioactivity 58 mCi/mmol) or [2,14C]-mevalonolactone (5 μCi; specific radioactivity 93 mCi/mmol). When leucine was the radioactive precursor, the incubation medium was the culture medium (medium 199 plus hormones) but containing [4,5-3H]leucine (10 μCi; specific radioactivity 76 mCi/mmol).

1 The abbreviation used is: HMC-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
In experiments where compactin or mevalonolactone was present in the culture medium, these were included in the incubation medium at the same concentration unless otherwise stated. Incubations were conducted at 37 °C for 1-2 h and were stopped by immersion in ice and washed with ice-cold phosphate-buffered saline prior to further processing (below).

Measurement of Lipid Synthesis from [1-14C]Acetate and [2-14C]Mevalonolactone—Explants were saponified, and digitonin precipitation of the nonsaponifiable lipid fraction was performed as previously described (1) but with [1,2-3H]cholesterol (20,000 dpm) added to all samples to correct for procedural losses. The saponifiable fraction was isolated following acidification of the saponification medium (1), and recovery of this fraction was monitored by inclusion of [9,10-3H]palmitic acid (200,000 dpm). After removal of solvent, the lipid fractions were counted for 14C and 3H radioactivity.

Measurement of the Incorporation of Radioactivity from [4,5-2H]Leucine into Lipid and Protein—After incubation with radioactive leucine, the explants were washed twice with 2 ml of ice-cold phosphate-buffered saline. Washed explants were extracted with chloroform:methanol (1:2, v/v) to extract lipid (17). The lipid extract was saponified, and digitonin precipitated. The insoluble residue after lipid extraction was dissolved in 0.2 ml of formic acid and assayed for radioactivity. This latter fraction measured incorporation of leucine into protein.

Preparation of Microsomal Fractions and Assay of HMG-CoA Reductase—Explants (20-40 mg wet weight) were homogenized manually in an all-glass homogenizer in 0.5 ml of 500 mM sucrose, 10 mM EDTA, and 10 mM mercaptoethanol, pH 7.0. The microsomal pellet was then obtained by centrifugation (1) and suspended in 0.15 ml of buffer containing 100 mM potassium phosphate, pH 7.5, 10 mM EDTA, and 5 mM dithiothreitol and used for the assay of HMG-CoA reductase activity. This was carried out as previously described (1) but modified to give a final volume of 0.15 ml and an incubation time of 3 h. Formation of [14C]mevalonate was found to be linear, with time up to 4 h and was proportional to protein. After lactonization, the product was isolated by thin-layer chromatography (18) and counted for radioactivity. Product recovery averaged 90%. Blanks were run without microsomes and with microsomes but without the NADPH-generating system.

Assay of Enzymes in Explant Homogenates—After culture, explants were suspended in 0.5 ml of 250 mM potassium phosphate buffer, pH 8.2, containing 1 mM EDTA and 1 mM dithiothreitol and then homogenized in an all-glass homogenizer for 2 min at maximum rotor speed. After centrifugation, the homogenate was centrifuged at 14,000 × g for 6 min at 4 °C in a microcentrifuge. The supernatant fraction was removed for enzyme assays. The following enzymes were determined according to previously published methods: citrate synthase (19), lactate dehydrogenase (20), and fatty acid synthase (21).

RESULTS

All experiments described below involved the culturing of mammary gland explants with hormones to stimulate development with or without compactin for 24 h or more. The rates of lipogenesis or protein synthesis were assayed at the end of the culture period in a short incubation (1-2 h) with the appropriate radioactive precursor in the presence or absence of compactin as described. In preliminary experiments (data not shown), we have confirmed that 10 μM compactin inhibited [1,14C]acetate incorporation into sterol when added directly to the assay system and that the same concentration of compactin had no significant effect upon incorporation of [1-14C]acetate into fatty acids or on [4,5-2H]leucine incorporation into protein when added directly to the assay system.

The Effect of Culture with Compaction on Sterol Synthesis—The stimulation of sterol synthesis from [1-14C]acetate which was seen after 24 h of culture with hormones (Fig. 1) was abolished by the presence of 10 μM compactin. The incorporation of [2-14C]mevalonolactone into sterol was unaffected by exposure to compactin for up to 72 h (Fig. 2), indicating the specificity of the effect. The residual rate of sterol synthesis from [1-14C]acetate in explants cultured with compactin was then obtained by centrifugation (1) and suspended in 0.15 ml of 250 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, and 5 mM dithiothreitol and used for the assay of HMG-CoA reductase activity. This was carried out as previously described (1) but modified to give a final volume of 0.15 ml and an incubation time of 3 h. Formation of [14C]mevalonate was found to be linear, with time up to 4 h and was proportional to protein. After lactonization, the product was isolated by thin-layer chromatography (18) and counted for radioactivity. Product recovery averaged 90%. Blanks were run without microsomes and with microsomes but without the NADPH-generating system.

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The effect of removal of compactin on sterol synthesis in hormonally stimulated explants cultured in the presence of compactin. Duplicate groups of 10 explants were cultured without hormones (C), with hormones (H), or with hormones plus compactin (2 μM) (A). They were then incubated with [1-14C]acetate for 1 h. A further group (Δ) was cultured in the presence of hormones plus compactin but was washed free of compactin for 1 h prior to incubation with [1-14C]acetate. Digitonin-precipitable sterol (DPS) was isolated as described under "Experimental Procedures." Results are expressed as the fold stimulation over the no-hormone control at a particular time point. The mean incorporation rates of [1-14C]acetate into sterol and compactin but was washed free of compactin for 1 h prior to incubation with [1-14C]acetate and compactin. The mean incorporation rates of precursors were for [1-'%]acetate into sterol and compactin but was washed free of compactin for 1 h prior to incubation with [1-14C]acetate into sterol (disintegrations/min. h" of tissue) were as follows: zero time, 454; 24 h, 60.

After culture, the control group (without compactin) was then incubated with [1-14C]acetate or [4,5-3H]leucine to determine rates of fatty acid, 1325

The Effect of Culture with Compactin on Fatty Acid and Protein Synthesis—In mammary gland explants, the hormonal stimulation of fatty acid synthesis can be determined by the rate of incorporation of [4,5-3H]leucine (Fig. 4a) as well as [1-14C]acetate (Fig. 4b). Culture with hormones stimulated incorporation of radiolabeled leucine and acetate by 12- and 64-fold, respectively. The lower response to hormones found with leucine as lipid precursor could reflect its major diversion into protein synthesis. The rate of incorporation of [3H]leucine into fatty acid was around 10% of the rate of incorporation into protein after hormonal stimulation of explants. In the presence of compactin, culture for more than 24 h resulted in a 40-60% inhibition (Fig. 4, a and b) of the rate of incorporation of both [3H]leucine and [14C]acetate into fatty acids. A similar result was observed when protein synthesis was measured. The hormonally stimulated incorporation of [4,5-3H]leucine into protein was inhibited by 40% when explants were cultured with compactin for 48 and 72 h (Fig. 5). As with fatty acid synthesis, little effect was noted after 24 h of culture with compactin. These effects of compactin on hormonally induced fatty acid and protein synthesis were prevented by the inclusion of mevalonolactone (1 mM) in the culture medium (Figs. 4 and 5). By itself, the mevalonolactone had no effect on either fatty acid synthesis or protein synthesis (data not shown), but it was taken up and rapidly metabolized by explants when it gave rise to digitonin-precipitable sterol (Fig. 2) and was associated with inhibition of sterol synthesis from [1-14C]acetate (Fig. 1).

Removal of compactin by a brief wash just before assay of protein or fatty acid synthesis caused no reversal of inhibition (Table I). This striking contrast with the response of sterol synthesis to removal of inhibitor confirmed that compactin had no direct reversible inhibitory action on protein or fatty acid synthesis and suggested that its effect was indirect and secondary to inhibition of mevalonate production. If this were so, then removal of compactin by washing followed by reculturing with hormones for a further 24 h should restore normal rates of protein and fatty acid synthesis. Fig. 6 shows the result of such an experiment. Fatty acid and protein synthesis rates were restored to 96 and 79%, respectively, of the control.

### Table I

| Conditions of incubation | % of hormonally stimulated incorporation rate (1-14C)acetate to Cholesterol | Fatty acid | Fatty acid | Protein |
|--------------------------|-----------------------------|------------|------------|---------|
| Compaction present (5)   | 14 ± 2                      | 49 ± 8     | 34 ± 5     | 60 ± 4  |
| Compaction removed (3)   | 131 ± 25*                   | 28 ± 11    | 31 ± 13    | 58 ± 9  |

* p < 0.01 with respect to group with compactin.

### Table II

| Additions to medium | Enzyme activities |
|---------------------|-------------------|
|                     | HMG-CoA reductase | Fatty acid synthase | Citrate synthase | Lactate dehydrogenase | FAS* x 10/LDH | HMGCoA x 10/LDH |
|                     | pmol/min·mg⁻¹ | nmol·min⁻¹·mg⁻¹ |                 |                      |            |                |
| None                | <0.02           | <30           | 7.8             | 47.9                | <0.6       | 0               |
| H                   | 0.69            | 230           | 6.9             | 62.5                | 8.5        | 1.4             |
| H + C               | 0.21            | 400           | 7.6             | 68.6                | 5.6        | 3.1             |
| H + C + M           | ND              | 500           | 7.7             | 73.1                | 8.1        | ND              |

*FAS, fatty acid synthase; HMGCoA, HMG-CoA reductase; LDH, lactate dehydrogenase; ND, not determined.
values when compactin was removed after 48 h and culture was continued in its absence for a further 24 h. Associated with these effects, there was considerable stimulation of sterol synthesis from [1-14C]acetate, resulting in an overshoot to 264% of control values. Thus, no permanent cell damage resulted from culture with 10 μM compactin.

**DISCUSSION**

These results show that, following inhibition of mevalonate and sterol synthesis by compactin in hormonally stimulated mammary explants, a significant reduction of protein and fatty acid synthesis occurred which can be prevented by supply of exogenous mevalonate and can be reversed by culture for 24 h (but not 1 h) in the absence of compactin.

Compactin is a powerful reversible inhibitor of HMG-CoA reductase, competing with HMG-CoA for this enzyme but being without effect on the other enzymes of HMG-CoA metabolism (13). In cultured fibroblasts, it has been shown to inhibit sterol synthesis but to be without inhibitory effect on fatty acid or protein synthesis (Refs. 25 and 16, respectively). In fact, its presence stimulates the appearance of new molecules of HMG-CoA reductase in isolated hepatocytes (25), and it causes increased numbers of low density lipoprotein receptors in the cell surface of fibroblasts (16). However, compactin does inhibit cell growth (2, 3), but this effect can be overcome by the supply of exogenous cholesterol, mevalonate, and products derived from mevalonate (3). Resistance to the growth-inhibiting effects of compactin is associated with
very high levels of HMG-CoA reductase (26), indicating that these effects of compactin are due to its specific action in depriving the cell of mevalonate.

Recent work has shown that mevalonate and its products play a complex role in the cell being required for the initiation (4, 5) and the continuation (27) of DNA synthesis in the cell cycle. Also by blocking mevalonate synthesis, compactin has been shown to inhibit differentiation in sea urchin (7) and mouse embryos (8). In both cases, this has been associated with the inhibition of protein glycosylations, and in the former case, the effect was reversed by the mevalonate-derived isoprene dolichol.

In view of this evidence for the specificity of effect but widespread and manifold repercussions of compactin treatment of cells, we interpret our results as indicating a specific requirement for mevalonate (or derived products) for the hormonal maintenance of the increased fatty acid and protein synthesis by depriving the cell of cholesterol.

We are currently investigating whether the inhibition of hormonally stimulated fatty acid and protein synthesis by compactin is related to a need for dolichol or other isoprenes.

REFERENCES

1. Middleton, B., Hatton, J., and White, D. A. (1981) J. Biol. Chem. 256, 4827–4831
2. Kaneko, I., Hazama-Shinada, Y., and Endo, A. (1978) Eur. J. Biochem. 87, 313–321
3. Brown, M. S., and Goldstein, J. L. (1980) J. Lipid Res. 21, 505–517
4. Habenicht, A. J. R., Glomset, J. A., and Ross, R. (1980) J. Biol. Chem. 255, 5134–5140
5. Yachnin, S., and Richman, D. P. (1982) Cell. Immunol. 72, 248–252
6. Menykovych, G., and Clowes, K. K. (1983) Biochim. Biophys. Acta 756, 138–143
7. Carson, D. D., and Lennarz, W. J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5709–5713
8. Surani, M. A. H., Kimber, S. J., and Osborn, J. C. (1983) J. Embryol. Exp. Morphol. 75, 205–223
9. Schmidt, G. H. (1971) Biology of Lactation, pp. 1–87, W. H. Freeman and Co., San Francisco
10. Forsyth, I. A., and Frey, R. P. (1971) J. Endocrinol. 51, 157–168
11. Forsyth, I. A., Strong, C. R., and Dils, R. (1972) Biochem. J. 129, 929–935
12. Deviny, E., Houdebine, L.-M., and Delouis, C. (1978) Biochim. Biophys. Acta 517, 360–366
13. Endo, A., Kuroda, M., and Tanzawa, K. (1976) FEBS Lett. 72, 323–326
14. Morgan, J. F., Morton, H. J., and Parker, R. C. (1950) Proc. Soc. Exp. Biol. Med. 73, 1–8
15. Al-Sarraj, K., Newbury, J., White, D. A., and Mayer, R. J. (1979) Biochem. J. 182, 837–845
16. Brown, M. S., Faust, J. R., Goldstein, J. L., Kaneko, I., and Endo, A. (1978) J. Biol. Chem. 253, 1121–1128
17. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 27, 911–917
18. Shapiro, D. J., Nordstrom, J. R., Mitschelen, J. J., Rodwell, Y. W., and Schimke, R. J. (1974) Biochim. Biophys. Acta 370, 369–377
19. Srere, P. A., Brazil, M., and Gonen, L. (1963) Acta. Chem. Scand. 17, S129–S134
20. Bergmeyer, H.-U., Bernt, E., and Hess, B. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed) pp. 736–743, Academic Press, New York
21. Lynen, F. (1969) Methods Enzymol. 14, 17–33
22. Endo, A., Tsujita, Y., Kuroda, M., and Tanzawa, K. (1979) Biochim. Biophys. Acta 575, 266–276
23. Edwards, P. A., Lemongello, D., Kane, J., Schechter, I., and Fogelman, A. M. (1980) J. Biol. Chem. 255, 3715–3725
24. Stange, E. F., Frecil, G., Schneider, A., Alavi, M., and Ditschuneit, H. (1981) Biochim. Biophys. Acta 663, 613–620
25. Bensch, W. R., Ingebritsen, T. S., and Diller, E. R. (1978) Biochim. Biophys. Res. Commun. 82, 247–254
26. Chin, D. J., Luskey, K. L., Anderson, R. G. W., Faust, J. R., Goldstein, J. L., and Brown, M. S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1185–1189
27. Quesney-Huneus, V., Galick, H. A., Siperstein, M. D., Erickson, S. K., Spender, T. A., and Nelson, J. A. (1983) J. Biol. Chem. 258, 378–385