THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 253, No. 4, Issue of February 25, pp. 1121-1128, 1978
Printed in U.S.A.

Induction of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Human Fibroblasts Incubated with Compactin (ML-236B), A Competitive Inhibitor of the Reductase*

(Received for publication, August 11, 1977)

MICHAEL S. BROWN,‡ JERRY R. FAUST, AND JOSEPH L. GOLDSTEIN

From the Department of Internal Medicine, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

ISAO KANEKO AND AKIRA ENDO

From Sankyo Company, Ltd., Tokyo, 140, Japan

The two compounds compactin and ML-236B are identical fungal metabolites isolated from strains of *Penicillium brevicompactum* and *Penicillium citrinum*, respectively. ML-236B has been shown to be a potent competitive inhibitor of rat liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-controlling enzyme in cholesterol biosynthesis (Endo, A., Kuroda, M., and Tanzawa, K. (1976) *FEBS Lett.* 72, 323–326). In the current studies we demonstrate that compactin is a potent competitive inhibitor of HMG-CoA reductase in extracts of human fibroblasts (K_i = 1.1 nM). Incubation of intact fibroblasts with 2.6 μM compactin produced a complete but readily reversible inhibition of [^{14}C]acetate incorporation into [^{14}C]cholesterol without affecting the incorporation of [^{14}C]mevalonate into [^{14}C]cholesterol. The compactin-mediated inhibition of HMG-CoA reductase activity in intact fibroblasts led to the production of large amounts of enzyme that was not active in the cell because it was inhibited by compactin. The presence of this enzyme could be demonstrated, however, by assays of HMG-CoA reductase activity in cell-free extracts under conditions in which the inhibitory effect of compactin was overcome by dilution. The appearance of this latent HMG-CoA reductase activity was prevented by cycloheximide. The latent HMG-CoA reductase that appeared in the presence of compactin could not be suppressed fully by the addition of low density lipoprotein to the culture medium. However, the enzyme could be completely suppressed by the addition of small amounts of mevalonate together with low density lipoprotein. From these data we suggest that incubation of intact fibroblasts with compactin may unmask a new regulatory system for HMG-CoA reductase that does not depend on exogenous cholesterol derived from low density lipoprotein but may depend on an endogenous compound generated from the metabolism of mevalonate.

* This research was supported by Grant HL 20948 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Established Investigator of the American Heart Association.

The activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase)[1] determines the rate of cholesterol synthesis in a wide variety of mammalian cells (1). In cultured human fibroblasts, the activity of this enzyme is regulated primarily by cholesterol that enters the cell bound to the plasma cholesterol transport protein, low density lipoprotein (LDL) (2). When LDL is available to fibroblasts, the lipoprotein binds to a specific LDL receptor on the cell surface, is taken up within coated endocytic vesicles, and is delivered to cellular lysosomes where its protein and cholesteryl ester components are degraded (for review, see Refs. 3 to 5). The resultant free cholesterol can be utilized by the cell for a number of structural and regulatory purposes, including synthesis of plasma membranes and suppression of HMG-CoA reductase activity and cholesterol synthesis. When fibroblasts are deprived of LDL, the activity of HMG-CoA reductase increases, enabling the cells to synthesize *de novo* the cholesterol required for plasma membrane synthesis and cell growth (6, 7).

Because of its importance in regulating cholesterol biosynthesis, the HMG-CoA reductase enzyme has been extensively studied, and enzyme inhibitors have been intensely sought. One class of such compounds consists of a variety of oxygenated sterols that have a potent ability to suppress HMG-CoA reductase activity when added to intact cells in tissue culture (6, 8–10). Inasmuch as these compounds have no inhibitory activity when incubated directly with HMG-CoA reductase in cell-free extracts (6, 9), their suppressive activity has been attributed either to an inhibition of the synthesis of the enzyme or to an enhancement in the rate of degradation of the enzyme within the cell or to a combination of both of these effects (6, 9, 10).

Recently, an important new class of true HMG-CoA reductase inhibitors has been isolated from the culture broth of the mold *Penicillium citrinum* by Endo and collaborators (11, 12). When incubated with microsomal HMG-CoA reductase from rat liver, the most potent of these compounds, designated ML-236B, behaved in a competitive fashion with respect to

[1] The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG, 3-hydroxy-3-methylglutarate; LDL, low density lipoprotein.

the substrate, HMG-CoA. The inhibitory constant (K_i) was in the range of 10 nM (12). The drug also inhibited the incorporation of [^{14}C]acetate into cholesterol in homogenates of rat liver. When given as a single oral dose to intact rats, ML-236B was able to lower plasma cholesterol levels acutely (11). The structure of ML-236B was shown to be identical with that of a drug called compactin, which was independently isolated from extracts of *Penicillium brevicompactum* by Brown *et al.* (13), who were studying its antifungal activity.

In the current studies, we have used compactin as a tool to explore the regulation of HMG-CoA reductase in cultured human fibroblasts. The data demonstrate that compactin is an extremely potent and readily reversible competitive inhibitor of HMG-CoA reductase in these cells. An unexpected result was obtained when intact fibroblasts were treated with compactin. At the same time that the drug completely inhibited HMG-CoA reductase activity inside the cell (as revealed by a comparison of the incorporation of [^{14}C]acetate and [^{14}C]mevalonate into cholesterol in intact monolayers), it also caused a marked increase in the cellular content of HMG-CoA reductase activity (as measured in cell-free extracts).

EXPERIMENTAL PROCEDURES

Materials – Sodium ^{125}I (carrier free in 0.05 N NaOH), [1-^{14}C]oleic acid (58 mCi/mmol), and DL-[2-^{14}C]mevalonic acid lactone (13 mCi/mmol) were purchased from Amersham/Searle. DL-3-Hydroxy-3-methyl[3-^{14}]glutaryl coenzyme A (26.2 mCi/mmol) and [1,2-^{3}H]cholesterol (50 Ci/mmol) were obtained from New England Nuclear Corp. [2-^{14}C]Acetic acid, sodium salt (51 to 59 mCi/mmol) was obtained from either Amersham/Searle or New England Nuclear Corp. DL-Mevalonic acid lactone, 3-hydroxy-3-methylglutaric acid (HMG), and cycloheximide were purchased from Sigma Chemical Co. DL-2-Methylbutyric acid was purchased from Eastman. Compactin (M_r = 390) was the gift of Dr. R. B. Fears of Beecham Pharmaceuticals. 25-Hydroxycholesterol was obtained from Steraloids, Inc. Other chemicals, thin layer chromatographic supplies, and tissue culture supplies were obtained from sources as previously reported (2, 6).

Solutions – Compactin in the lactone form was converted to the sodium salt by heating at 50° for 1 h in 0.1 N NaOH. The final solution (0.24 mg/ml, 0.62 mM) was adjusted to pH 7.7 with HCl and stored in multiple aliquots at −20° until use. Aliquots of HMG (0.82 M) were made up in water, adjusted to pH 6.9 with NaOH, and stored at −20° until use. Aliquots of mevalonic acid lactone (0.77 M) were made up in 10 mM potassium phosphate, pH 4.5, and stored at −20° until use. 25-Hydroxycholesterol was dissolved in ethanol (2 mg of sterol/ml) just prior to each experiment. The sterol was greater than 99% pure as judged by gas-liquid chromatography.

Cells – Cultured fibroblasts were derived from skin biopsies obtained from normal subjects. Cells were grown in monolayer and used between the 5th and 20th passage. Stock cultures were maintained in a humidified incubator (5% CO_2) at 37° in 250-ml flasks containing 10 ml of Medium A [Eagle's minimum essential medium (Gibco, Catalogue No. F-11) supplemented with penicillin (100 units/ml); streptomycin (100 μg/ml); 20 mM Tricine, pH 7.4; 24 mM $NaHCO_3$; 1% (v/v) nonessential amino acids] and 10% (v/v) fetal calf serum. Unless otherwise noted, all experiments were done in a similar format. On Day 0, confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin-0.02% EDTA solution, and 1 × 10^5 cells were seeded into each Petri dish (60 × 15 mm) containing 3 ml of Medium A with 10% fetal calf serum. On Day 3 the medium was replaced with 3 ml of fresh Medium A containing 10% fetal calf serum. On Day 5, when the cells were in late logarithmic growth, each monolayer was washed with 3 ml of phosphate-buffered saline, after which 2 ml of fresh Medium A containing 5% or 10% (v/v) human lipoprotein-deficient serum were added (final protein concentration, 2.5 or 5 mg/ml). Experiments were initiated on Day 6 or 7 after the cells had been incubated for 24 or 48 h in the presence of lipoprotein-deficient serum.

Lipoproteins – Human LDL (density 1.019 to 1.063 g/ml) and lipoprotein-deficient serum (density > 1.215 g/ml) were obtained from the plasma of individual healthy subjects and prepared by differential ultracentrifugation (2). Fetal calf lipoprotein-deficient serum was prepared as previously described (6). The concentration of LDL is expressed in terms of its protein content. The mass ratio of total cholesterol to total protein in LDL was 1.6:1. ^{125}I-labeled LDL (specific activity, 200 to 350 cpm/ng of protein) was prepared as previously described (14). For each experiment, the ^{125}I-LDL was diluted with unlabeled LDL to give the final specific activity indicated in the legends.

Incorporation of [^{14}C]Acetate and [^{14}C]Mevalonate into ^{14}C-labeled Nonsaponifiable Lipids and [^{14}C]Cholesterol and Incorporation of [^{14}C]Acetate into ^{14}C-labeled Fatty Acids by Intact Fibroblasts – Monolayers were incubated at 37° in 2 ml of Medium A containing 10% human lipoprotein-deficient serum and supplemented with either 1 mM [2-^{14}C]acetate or 0.4 mM [2-^{14}C]mevalonate. After the indicated interval, the medium from each dish was transferred to a glass tube, each cell monolayer was dissolved in 1 ml of 0.1 N NaOH, and the dissolved cell suspension was pooled with the medium. To each 3-ml sample of medium plus cells was added 3 ml of ethanol, 0.5 ml of 50% KOH, and 10^5 cpm of [1,2-^{3}H]cholesterol (50 Ci/mmol) as an internal standard. Each sample was then saponified and extracted with petroleum ether, and the [^{14}C]cholesterol was isolated and quantified by thin layer chromatography as previously described (15, 16). The value for total ^{14}C-labeled nonsaponifiable lipids was obtained by fractionating and counting the entire chromatogram. The data are expressed as the picomoles or nanomoles of precursor incorporated into ^{14}C-labeled nonsaponifiable lipids or [^{14}C]cholesterol per mg of total cell protein.

To measure fatty acid synthesis, the aqueous phases from the petroleum ether extractions were pooled together, acidified with concentrated HCl, and extracted three times with petroleum ether. The pooled organic phases were then evaporated to dryness, resuspended in chloroform containing 100 μg of linoleic acid as carrier, and subjected to thin layer chromatography on Silica Gel G with a solvent system consisting of heptane/diethyl ether/acetic acid (90:30:1). The fatty acid spot (R_f 0.30) was visualized by iodine vapor and quantified as previously described for the measurement of cholesteryl ^{14}C-esters (17). The data are expressed as the picomoles of [^{14}C]acetate incorporated into ^{14}C-fatty acids per mg of total cell protein.

Incorporation of [^{14}C]Oleate into Cholesteryl [^{14}C]Oleate by Intact Fibroblast Monolayers – Monolayers were incubated at 37° in 2 ml of Medium A containing 5% human lipoprotein-deficient serum and supplemented with 0.1 mM [^{14}C]oleate bound to albumin (17). The cells were then washed, harvested, and extracted with chloroform/methanol (2:1) (17). The cholesteryl [^{14}C]oleate was isolated by thin layer chromatography and quantified as previously described (17). Esterification activity is expressed as the picomoles of cholesteryl [^{14}C]oleate formed per h per mg of total cell protein.

Assay of HMG-CoA Reductase Activity in Cell-free Fibroblast Extracts – The rate of conversion of 3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA (10,000 cpm/nmol) to [^{14}C]mevalonate was measured in detergent-solubilized extracts as previously described (2). HMG-CoA reductase activity is expressed as picomoles of [^{14}C]mevalonate formed per min per mg of detergent-solubilized protein.

Measurement of Amounts of Cell-bound ^{125}I-LDL and Degraded ^{125}I-LDL in Fibroblast Monolayers – Monolayers were incubated in a CO_2 incubator at 37° with ^{125}I-LDL in 2 ml of Medium A containing 5% human lipoprotein-deficient serum. After the indicated interval, measurements of the cell-bound ^{125}I-LDL (*i.e.* receptor-bound plus internalized) and degraded ^{125}I-LDL were made as previously described (18). In all experiments, the data are expressed as high affinity ^{125}I-LDL binding and degradation. To obtain these data, values for the low affinity (or nonspecific) processes were subtracted from the observed total ^{125}I-LDL values. The low affinity values were determined by parallel incubations of monolayers with ^{125}I-LDL in the presence of a 30-fold excess of unlabeled LDL. In each case where this correction was made, the values for the low affinity processes were less than 10% of the observed total values.

Other Assays – The content of free and esterified cholesterol in fibroblasts was determined by a previously described gas-liquid chromatographic method (7). The protein content of extracts and whole cells was determined by a modification of the method of Lowry *et al.* (19), with bovine serum albumin as a standard.

RESULTS

As shown in the double reciprocal plot of Fig. 1, compactin is an extremely potent competitive inhibitor of HMG-CoA reductase in extracts of human fibroblasts. The affinity of the

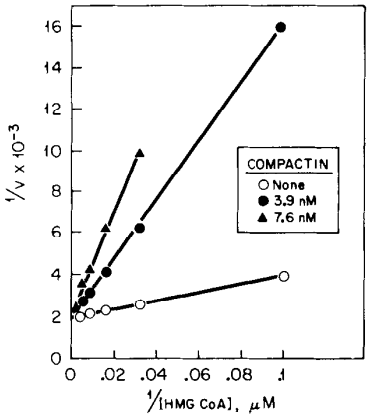

FIG. 1. Competitive inhibition of HMG-CoA reductase activity in cell-free fibroblast extracts by compactin. Detergent-solubilized extracts were prepared from fibroblasts that had been grown under standard conditions and incubated in 2 ml of Medium A containing 5% human lipoprotein-deficient serum for 48 h prior to harvest. Aliquots (25 μl) containing 34 μg of protein were incubated at 37° for 2 h under standard conditions for the HMG-CoA reductase assay except that the concentration of [^{14}C]HMG-CoA was varied from 10 to 250 μM and the reaction tubes contained the indicated concentration of compactin (○, none; ●, 3.9 nM; and ▲, 7.6 nM). The data are plotted as the reciprocal of the reaction velocity (picomoles of [^{14}C]mevalonate formed/tube × 10^{-3}) *versus* the reciprocal of the concentration of HMG-CoA (μM). Each value represents the average of duplicate assays.

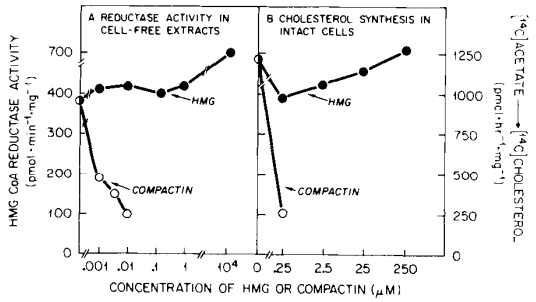

FIG. 2. Inhibition of HMG-CoA reductase activity in cell-free fibroblast extracts (*A*) and of cholesterol synthesis from [^{14}C]acetate in intact fibroblasts (*B*) by compactin and lack of inhibition of either process by HMG. Experiment A, detergent-solubilized extracts were prepared from fibroblasts that were grown under standard conditions and incubated in 2 ml of Medium A containing 5% human lipoprotein-deficient serum for 48 h prior to harvest. The HMG-CoA reductase assays were conducted at 37° for 2 h under standard conditions except for the addition of the indicated concentration of either compactin (○) or HMG (●). Each value represents the average of duplicate assays. Experiment B, on Day 7 each monolayer received 2 ml of Medium A containing 10% human lipoprotein-deficient serum and the indicated concentration of either compactin (○) or HMG (●). After incubation at 37° for 2 h, each monolayer was pulse-labeled for 2 h with 1 mM [^{14}C]acetate (59 cpm/pmol), after which the medium and cells were harvested for determination of their content of [^{14}C]cholesterol. Each value represents the average of duplicate incubations.

enzyme for compactin (K_i = 1.1 nM) is 10,000-fold higher than its affinity for HMG-CoA (K_m = 11 μM).

Compactin contains a side chain whose structure is similar to that of 3-hydroxy-3-methylglutarate (HMG) and mevalonate (13). However, as shown in Fig. 2*A*, HMG failed to inhibit HMG-CoA reductase activity in fibroblast extracts when added at concentrations more than 10^6-fold higher than the effective inhibitory concentrations of compactin. Similarly, sodium mevalonate failed to inhibit the enzyme at concentrations as high as 1 mM (data not shown). Compactin also contains a 2-methylbutyrate side chain that has been shown to contribute to its inhibitory action (12). Free 2-methylbutyrate also failed to inhibit HMG-CoA reductase activity in fibroblast extracts when added at concentrations as high as 1 mM (data not shown).

As expected from its ability to inhibit HMG-CoA reductase activity in cell-free extracts, compactin was a potent inhibitor of cholesterol synthesis when added acutely to intact fibroblasts. The rate of [^{14}C]acetate incorporation into [^{14}C]cholesterol was inhibited by more than 80% at 0.25 μM compactin (Fig. 2*B*). In contrast, HMG failed to inhibit this incorporation at concentrations as high as 250 μM (Fig. 2*B*).

The inhibition of cholesterol synthesis by compactin was sustained as long as the compound was kept in the culture medium. As shown in Fig. 3, when fibroblasts were incubated in the absence of lipoproteins, [^{14}C]acetate was incorporated into cholesterol at a nearly linear rate for 72 h. This incorporation was almost completely suppressed by compactin at 0.26 μM and was completely suppressed when the drug was present at a concentration of 2.6 μM. The inhibition was sustained throughout the 72-h duration of the experiment. In experiments not shown, cholesterol synthesis from [^{14}C]acetate in mutant fibroblasts from subjects with the homozygous form of familial hypercholesterolemia was also inhibited at the same concentrations of compactin as were normal fibroblasts.

The specificity of the compactin effect on HMG-CoA reductase is indicated by the experiment in Table I which shows that in intact fibroblasts compactin at a concentration of 2.6 μM produced nearly complete inhibition of [^{14}C]acetate incorporation into total ^{14}C-labeled nonsaponifiable lipids and [^{14}C]cholesterol, but did not affect the incorporation of [^{14}C]mevalonate, the product of the HMG-CoA reductase reaction. It should be noted that the absolute rate of [^{14}C]acetate incorporation was greater than that of [^{14}C]mevalonate because the concentration of the latter substrate was not saturating. Compactin also failed to affect the incorporation of [^{14}C]acetate into ^{14}C-labeled fatty acids in intact cells (Fig. 4*B*). The experiment shown in Fig. 4 also revealed that under

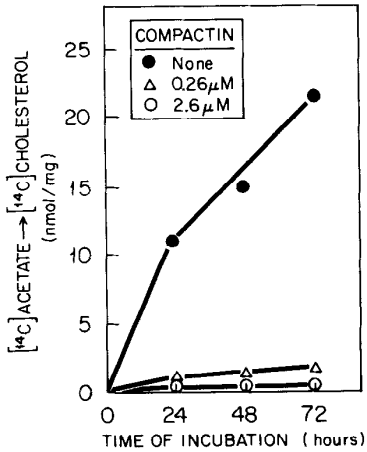

FIG. 3. Inhibition of cholesterol synthesis from [^{14}C]acetate by compactin in intact fibroblasts. On Day 6 each monolayer received 2 ml of Medium A containing 10% human lipoprotein-deficient serum, 1 mM [^{14}C]acetate (5000 cpm/nmol), and the indicated concentration of compactin. After incubation at 37° for the indicated interval, the medium and cells were harvested for determination of their content of [^{14}C]cholesterol. Each value represents the average of duplicate incubations.

TABLE I

Inhibition by compactin of [^{14}C]acetate, but not [^{14}C]mevalonate incorporation into ^{14}C-labeled nonsaponifiable lipids and [^{14}C]cholesterol by intact fibroblasts

On Day 7 each monolayer received 2 ml of Medium A containing 10% human lipoprotein-deficient serum, the indicated concentration of compactin, and either 1 mM [^{14}C]acetate (51 cpm/pmol) or 0.4 mM [^{14}C]mevalonate (13 cpm/pmol). After incubation for 4 h at 37°, the medium and cells were harvested for determination of their content of total ^{14}C-labeled nonsaponifiable lipids and [^{14}C]cholesterol. Each value represents the average of duplicate incubations.

Compactin	[^{14}C]Acetate incorporation into		[^{14}C]Mevalonate incorporation into	
	Total nonsaponifiable lipids	Cholesterol	Total nonsaponifiable lipids	Cholesterol
μM	$pmol \cdot h^{-1} \cdot mg^{-1}$		$pmol \cdot h^{-1} \cdot mg^{-1}$	
0	1080 (100%)[a]	775 (100%)	79 (100%)[a]	53 (100%)
0.26	157 (15%)	110 (14%)	75 (95%)	57 (108%)
2.6	25 (2.3%)	12 (1.6%)	86 (108%)	51 (96%)

[a] The numbers in parentheses represent the percentage of the values obtained in cells receiving no compactin.

conditions in which compactin completely inhibited [^{14}C]acetate incorporation into cholesterol in intact cells (Fig. 4*A*), the activity of HMG-CoA reductase, as measured in cell-free extracts, actually increased progressively (Fig. 4*C*).

The increase in cellular content of HMG-CoA reductase activity that occurred at the same time that [^{14}C]acetate incorporation into cholesterol was inhibited by compactin differed from previous results obtained with 25-hydroxycholesterol and other cholesterol analogues. Like compactin, these oxygenated derivatives of cholesterol suppress [^{14}C]acetate incorporation into cholesterol when added to intact cells, but in doing so they caused a marked decrease in HMG-CoA reductase activity as measured in cell-free extracts (6, 8–10). This difference in behavior of compactin and 25-hydroxycholesterol is illustrated by the experiment shown in Table II. Intact fibroblasts exposed to compactin exhibited a reduction in the rate of [^{14}C]acetate incorporation into cholesterol (22% of the control value), and a similar reduction (14% of the control value) was achieved by 25-hydroxycholesterol. Despite the similar inhibition of cholesterol synthesis achieved by the two compounds in intact cells, extracts of the compactin-treated cells showed a much higher HMG-CoA reductase activity (120% of control) as compared with the 25-hydroxycholesterol-treated cells (4.5% of the control). The increased HMG-CoA reductase activity of the compactin-treated cells appeared to be a latent activity in that it was inhibited within the cell but was unmasked when the cells were solubilized and the compactin was diluted in the solubilization and assay mixture.

The enhanced latent HMG-CoA reductase activity that developed in cells treated with compactin led to a marked overshoot in the rate of [^{14}C]acetate incorporation into cholesterol when compactin was removed from the culture medium. Fig. 5 shows the rate of cholesterol synthesis from [^{14}C]acetate in cells that had been exposed to compactin for 19 h (Fig. 5, zero time). When compactin was removed from the culture medium, the rate of cholesterol synthesis increased by more than 6-fold within 1 h, indicating rapid reversibility of the compactin-mediated inhibition. Because compactin had elicited an increase in HMG-CoA reductase activity, the rate of synthesis of cholesterol from [^{14}C]acetate was 2-fold higher immediately after removal of compactin than it was in cells that were never exposed to the drug. Thereafter, the rate of

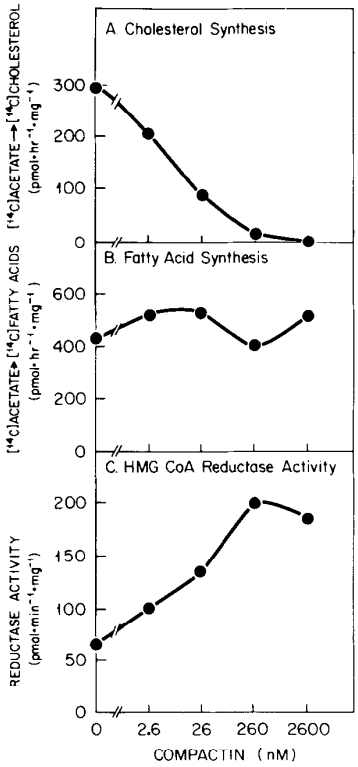

FIG. 4. Effect of varying concentrations of compactin on the incorporation of [^{14}C]acetate into [^{14}C]cholesterol (*A*) and ^{14}C-fatty acids (*B*) by intact fibroblasts and on HMG-CoA reductase activity as measured in cell-free extracts (*C*). On Day 7 each monolayer received 2 ml of Medium A containing 5% human lipoprotein-deficient serum and the indicated concentration of compactin. After incubation for 30 min at 37°, the monolayers were divided into two sets. For one set (*A* and *B*), each monolayer was pulse-labeled for 3 h with 1 mM [^{14}C]acetate (59 cpm/pmol), after which the medium and cells were harvested for determination of their content of [^{14}C]cholesterol (*A*) and ^{14}C-fatty acids (*B*). Cells from the second set of monolayers were harvested at the same time for measurement of HMG-CoA reductase activity (*C*). Each value represents the average of duplicate incubations.

TABLE II

Differential effects of compactin and 25-hydroxycholesterol on [^{14}C]acetate incorporation into [^{14}C]cholesterol by intact fibroblasts and on HMG-CoA reductase activity as measured in cell-free extracts

On Day 7 each monolayer received 2 ml of Medium A containing 5% human lipoprotein-deficient serum, 1 μl of ethanol, and the indicated addition. After incubation at 37° for 2 h, the monolayers were divided into two sets. For one set, each monolayer was pulse-labeled for 2 h at 37° with 1 mM [^{14}C]acetate (39 cpm/pmol), after which the medium and cells were harvested for determination of their content of [^{14}C]cholesterol. Cells from the second set of monolayers were harvested at the same time (after 4 h at 37°) for measurement of HMG-CoA reductase activity. Each value represents the average of triplicate incubations.

Addition to medium	[^{14}C]Acetate → [^{14}C]cholesterol by cells	HMG-CoA reductase activity in extracts
	$pmol \cdot h^{-1} \cdot mg^{-1}$	$pmol \cdot min^{-1} \cdot mg^{-1}$
None	1170 (100%)[a]	299 (100%)[a]
Compactin, 0.25 μM	254 (22%)	360 (120%)
25-Hydroxycholesterol, 2.5 μM	160 (14%)	14 (4.5%)

[a] The numbers in parentheses represent the percentage of the values obtained in cells receiving no additions.

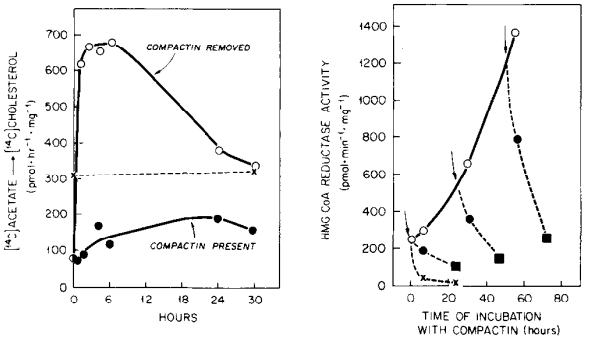

FIG. 5 (*left*). Increase in rate of cholesterol synthesis from [^{14}C]acetate in intact fibroblasts after removal of compactin from the culture medium. On Day 7 each monolayer received 2 ml of Medium A containing 10% human lipoprotein-deficient serum and 1 μM compactin. After incubation at 37° for 19 h (zero time), the medium was removed and each monolayer was washed with 3 ml of phosphate-buffered saline. Each monolayer then received 2 ml of fresh medium with (●) or without (○) 1 μM compactin, and the incubations were continued at 37°. After the indicated interval, each monolayer was pulse-labeled for 1 h at 37° with 1 mM [^{14}C]acetate (39 cpm/pmol), after which the medium and cells were harvested for determination of their content of [^{14}C]cholesterol. ×- - -×, the rate of incorporation of [^{14}C]acetate into cholesterol in parallel incubations in which the monolayers were treated in the same manner except that they never were exposed to compactin. Each value represents the average of duplicate incubations.

FIG. 6 (*right*). Induction of HMG-CoA reductase activity in intact fibroblasts incubated with compactin and reduced suppression by LDL added after varying intervals. Cell monolayers were prepared as described under "Experimental Procedures." On Day 5 the cells received 2 ml of Medium A containing 10% human lipoprotein-deficient serum, and all cells were harvested at the same time on Day 8. At the indicated time before harvest, groups of dishes received 1 μM compactin. LDL (50 μg of protein/ml) was added to groups of dishes at varying intervals after the addition of compactin. The times of LDL addition are indicated by the *arrows*. The schedule was arranged in such a way that at the time of harvest each dish had been incubated either in the absence of LDL (○) or in the presence of LDL for 6 h (●) or 24 h (■). HMG-CoA reductase activity was also measured in cells that never received compactin but were incubated with LDL for 6 or 24 h (×). Each value represents the average of duplicate incubations.

cholesterol synthesis began to decline, and by 30 h it had reached the level observed in cells that had not been exposed to compactin.

The increase in latent HMG-CoA reductase activity was even more striking in extracts of cells exposed to compactin for longer periods. In the experiment shown in Fig. 6, HMG-CoA reductase activity was more than 7-fold higher in extracts of cells that had been exposed to compactin for 60 h as compared with cells that had never been exposed to the drug. The level of HMG-CoA reductase attained (1400 pmol·min^{-1}·mg^{-1}) was more than 3-fold higher than has been observed previously under any conditions in human fibroblasts in this laboratory (20). Not only was the HMG-CoA reductase activity increased by compactin, but the susceptibility of this latent enzyme activity to suppression by LDL was also blunted. Thus, when LDL was added to cells that had never been exposed to compactin, the lipoprotein achieved an 89% suppression of HMG-CoA reductase activity within 6 h. However, when cells were exposed to compactin for varying periods and then LDL was added back to the cells, the percentage suppression of HMG-CoA reductase activity over the first 6 h was reduced to the range of 40%. After exposure to LDL for 24 h, the activity of HMG-CoA reductase

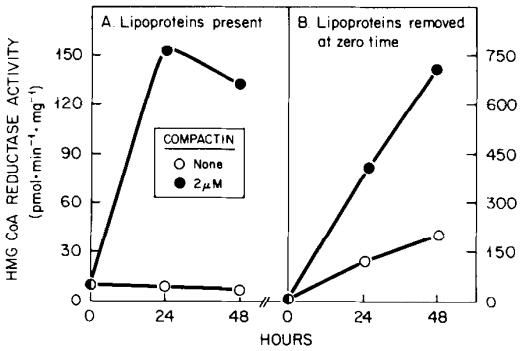

FIG. 7. Increase in HMG-CoA reductase activity in intact fibroblasts incubated with compactin in the presence (*A*) or absence (*B*) of lipoproteins. Cell monolayers were prepared as described under "Experimental Procedures." On Day 3, each monolayer received 3 ml of Medium A containing 10% fetal calf serum. On Day 5 (zero time), each monolayer received 3 ml of Medium A containing either 10% fetal calf serum (*A*) or 10% fetal calf lipoprotein-deficient serum (*B*) in the absence (○) or presence (●) of 2 μM compactin. After incubation at 37° for the indicated time, cells were harvested for measurement of HMG-CoA reductase activity. Each value represents the average of triplicate incubations.

in compactin-treated cells was still more than 20-fold higher than enzyme activity in the LDL-treated cells that had not been exposed to compactin (Fig. 6).

Compactin had the ability to increase the activity of HMG-CoA reductase to high levels even in cells that were grown continuously in the presence of lipoproteins. As shown in Fig. 7*A*, the addition of compactin to cells growing in whole serum containing lipoproteins was followed by a 15-fold increase in enzyme activity within the next 24 h. When compactin was added to cells growing in serum from which the lipoproteins were removed, the drug also caused a marked increase in enzyme activity (Fig. 7*B*). However, in the absence of lipoproteins the relative difference between the activity in the presence and absence of compactin (3.5-fold) was not as great as it was when lipoproteins were present since the baseline value also rose due to the removal of lipoproteins.

The induction of high levels of HMG-CoA reductase activity by compactin required protein synthesis. When compactin was added to cells growing in the presence of LDL, the increase in HMG-CoA reductase activity was completely blocked by cycloheximide (Fig. 8).

Although LDL was able to suppress HMG-CoA reductase activity only partially in cells grown in the presence of compactin, the additional presence of small amounts of mevalonolactone permitted complete suppression. As shown in Fig. 9*A*, when cells were grown in the absence of lipoproteins but in the presence of compactin, the addition of increasing amounts of mevalonolactone led to a progressive suppression of HMG-CoA reductase activity. This suppression presumably occurs because mevalonate, which is the product of HMG-CoA reductase, can be converted into sterols and other products by the cell at a normal rate in the presence of compactin. In the absence of lipoproteins, relatively high levels of mevalonolactone (*i.e.* levels above 1 mM) were required to produce a marked suppression of HMG-CoA reductase activity. In the presence of LDL, however, the addition of much smaller amounts of mevalonolactone (*i.e.* less than 0.1 mM) was able to achieve virtually complete suppression of HMG-CoA reductase activity (Fig. 9*B*). The experiment of Fig. 9 was a chronic one in which the cells were incubated for 72 h in the presence

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.