INHIBITION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE IN CULTURED L CELLS BY THE HYPOCHOLESTEROLEMIC DRUG CLOFIBRATE

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SUMMARY

The hypocholesterolemic drug clofibrate (ethyl-α-p-chlorophenoxyisobutyrate) was found to strongly suppress 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) activity in cultured mouse L cells at concentrations of 20 - 50 μg/ml. The half-life (t1/2) of the reductase (approximately 120 min) was strongly reduced when L cells were incubated with cycloheximide plus a maximal inhibitory concentration of clofibrate (50 μg/ml), resulting in a t1/2 value of 10 min. Preliminary kinetic analysis of the inhibition suggested that clofibrate increased the rate of inactivation (or degradation) of the reductase without affecting the rate of enzyme synthesis.

Since the hypolipidemic drug clofibrate (ethyl-α-p-chlorophenoxyisobutyrate) was introduced in 1962(1), various hypotheses to explain its hypocholesterolemic effect have been proposed from many laboratories, which include decreased absorption of cholesterol and enhanced excretion (2, 3), increased cholesterol oxidation (4), decreased synthesis of cholesterol (5-7), decreased release of hepatic lipoproteins (8), and redistribution of cholesterol between plasma and tissue compartments (9, 10). In the present communication we have studied effects of two hypolipidemic drugs clofibrate and simfibrate (1,3-propane-diol-bis-α-p-chlorophenoxyisobutyrate) (11) on the sterol synthesis and the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)* reductase (EC 1.1.1.34), the rate-limiting enzyme in cholesterogenesis, in cultured fibroblast cells. The results suggest that these drugs inhibit HMG-CoA reductase activity in cultured

* The abbreviation used is: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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L cells by increasing the rate of inactivation (or degradation) of the enzyme without affecting the rate of enzyme synthesis.

MATERIALS AND METHODS

Clofibrate and simfibrate were obtained from Chugai Seiyaku (Japan) and Yoshitomi Seiyaku (Japan), respectively, and 25-hydroxycholesterol was from Steraloids. [1-^{14}C]Acetate (60.1 Ci/mol) and DL-[3-^{14}C]HMG-CoA (18.8 Ci/mol) were purchased from New England Nuclear.

Stock cultures of mouse L cells (929) were grown at 37°C in Eagle's minimal essential medium (Nissui Co., Japan) supplemented with Kanamycin (60 μg/ml); 20 mM Tricine (Sigma), pH 7.4; 14.3 mM $NaHCO_3$; and 5% (v/v) fetal calf serum (GIBCO). For all experiments, cells from stock flasks (75 cm^2) were dissociated with 0.05% trypsin-0.01% EDTA, seeded (Day 0) at a concentration of approximately 3 x 10^5 cells per dish into plastic plates (60 x 15 mm, Falcon) containing 3 ml of fresh medium with 5% fetal calf serum and grown at 37°C in a humidified CO_2 incubator.

For the assay of HMG-CoA reductase cells were washed twice with Dulbecco's phosphate-buffered NaCl solution (GIBCO, Catalogue No. 419) and harvested by scraping with a rubber policeman. Preparation of cell extracts and assay of HMG-CoA reductase were carried out as described by Brown *et al.* (12), except that the detergent Kyro EOB was replaced by Brij 96 (Kao-Atlas Co., Japan).

For the experiments to determine sterol and fatty acid synthesis, 4 μCi of [^{14}C]acetate (20 μl)(60 Ci/mol) was added to the culture. After 60 min of incubation at 37°C in a humidified CO_2 incubator, 3 ml of 20% alcoholic KOH solution was added to the culture. The mixture was transferred to a test tube (1.7 x 13 cm) and then saponified at 75°C for 60 min. The digitonin-precipitable sterols and fatty acid formed were isolated and counted as described by Kandutsch and Chen (13). Protein was determined by the method of Lowry *et al.* (14).

RESULTS AND DISCUSSION

Fig. 1A shows the suppression of HMG-CoA reductase activity in L cells incubated for 60 min with varying concentrations of clofibrate and simfibrate. As indicated, both drugs, like 25-hydroxycholesterol, caused a strong suppression of the reductase activity. Concentrations required for 50% inhibition were 20, 4, and 0.5 μg/ml for clofibrate, simfibrate, and 25-hydroxycholesterol, respectively. The

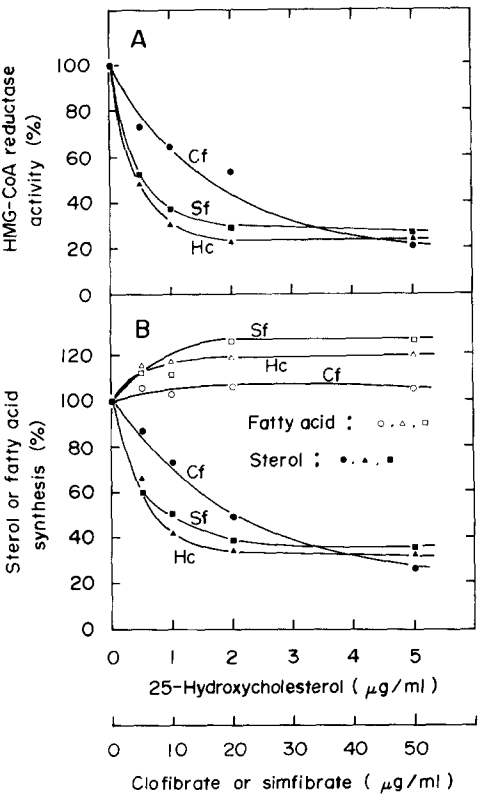

Fig. 1. Inhibitory effects of clofibrate, simfibrate and 25-hydroxycholesterol on HMG-CoA reductase activity (A) and on sterol and fatty acid syntheses (B) from [^{14}C]acetate in cultured L cells. Cells were grown in dishes as described in Materials and Methods. (A) On day 3 when cells were nearly confluent (average cell density 2 x 10^6 cells/dish), 30 μl of ethanol containing varying amounts of clofibrate (Cf), simfibrate (Sf), or 25-hydroxycholesterol (Hc) was added to the culture. After incubation at 37°C for 60 min in a humidified CO_2 incubator, cells were washed, harvested and assayed for HMG-CoA reductase. The results are expressed as percentage of the activity in control cells that received 30 μl of ethanol. The values represent the mean of duplicate dishes. The control activity was 56 pmol/min/mg protein. (B) On day 3, cells were incubated at 37°C for 60 min with 4 μCi of [^{14}C]acetate (60 Ci/mol) and 30 μl of ethanol containing varying amounts of clofibrate (Cf), simfibrate (Sf) or 25-hydroxycholesterol (Hc), and digitonin-precipitable sterols and fatty acid formed were isolated and counted. The results are expressed as percentage of incorporation in control cells. The values represent the mean of duplicate dishes. The control activities (dpm/min/mg protein) were 730 for sterols and 4,690 for fatty acid, respectively.

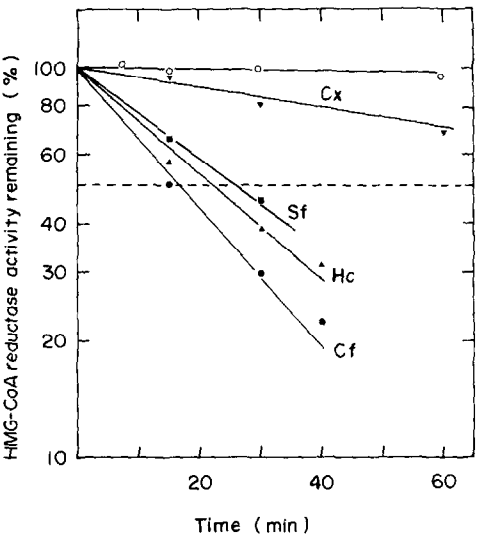

Fig. 2. Kinetics of decay in HMG-CoA reductase activity in L cells by clofibrate, simfibrate, 25-hydroxycholesterol and cycloheximide. Cells were grown in dishes as described in Materials and Methods. On day 3, cultures received 50 μg/ml of clofibrate (Cf), 20 μg/ml of simfibrate (Sf), 2 μg/ml of 25-hydroxycholesterol (Hc) or 100 μg/ml of cycloheximide (Cx) dissolved in 30 μl of ethanol and incubated at 37°C in a humidified CO_2 incubator. Control (o) received ethanol (30 μl) alone. At the indicated period cell extracts were prepared and assayed for HMG-CoA reductase. The activity at 0 time was 38 pmol/min/mg protein. The values represent the mean of duplicate dishes.

maximum inhibition caused by these agents was, however, approximately at the same level, ranging 70 - 80%. Sodium α-p-chlorophenoxyisobutyrate gave essentially the same results with slightly less potency. The effects of clofibrate and simfibrate on the [^{14}C]acetate incorporation into digitonin-precipitable sterols correlated well with changes in reductase activity (Fig. 1B).

At concentrations of 20-50 μg/ml, clofibrate and simfibrate showed no inhibitory effects both on fatty acid synthesis (Fig. 1B) and on the growth of L cells grown in a medium supplemented with whole serum. Further, high concentrations up to 400 μg/ml of these drugs added directly to

L-cell homogenate (cell-free extract) caused no detectable inhibition of HMG-CoA reductase activity.

As shown in Fig. 2, both clofibrate and simfibrate, like 25-hydroxycholesterol, caused a first order decay in reductase activity at their maximal inhibitory concentration. The t1/2 values (half-life) of reductase calculated from the decay curves were 17, 26 and 22 min with clofibrate (50 μg/ml), simfibrate (20 μg/ml) and 25-hydroxycholesterol (2 μg/ml), respectively. These values were far lower than that measured with cycloheximide (120 min at 100 μg/ml) (Fig. 2 and Table 1). Inactivating effects of clofibrate and simfibrate were potentiated by the presence of cycloheximide (Table 1). These data suggested that both hypocholesterolemic drugs as well as 25-hydroxycholesterol increased the rate of inactivation (or degradation) of reductase, and further that de novo synthesis of reductase was not completely suppressed by the drugs even at concentrations where reductase activity was maximally inhibited. The steady state kinetic analysis (15, 16) of inhibition of HMG-CoA reductase activity by varying concentrations of clofibrate and simfibrate suggested that the rate constant of synthesis of reductase was essentially unaffected by these drugs, whereas the apparent rate constant for degradation was increased about 10 fold by both drugs.

The results shown above indicate that the two hypocholesterolemic drugs, clofibrate and simfibrate, inhibit HMG-CoA reductase in cultured L cells by increasing the rate of inactivation or degradation of the enzyme. Since Thorp and Waring (1) reported that [^{14}C]acetate incorporation into sterols was reduced in liver from clofibrate-fed rats, many

Table 1. Effects of hypocholesterolemic drugs and 25-hydroxycholesterol plus and minus cycloheximide on half-life of HMG-CoA reductase in L cells.

Cells on day 3 were incubated at 37°C with 50 μg/ml of clofibrate, 20 μg/ml of simfibrate or 2 μg/ml of 25-hydroxycholesterol in the presence and absence of cycloheximide (100 μg/ml), or with cycloheximide alone. Half-life values were calculated from first order decay curves similar to the ones shown in Fig. 2.

	Concentration μg/ml	Half-life (t1/2), min	
		Alone	Plus cycloheximide
Clofibrate	50	17	10
Simfibrate	20	26	15
25-Hydroxycholesterol	2	22	13
Cycloheximide	100	-	120

investigators have studied the effects of the drug on hepatic cholesterogenesis (5-8). Some of them reported that HMG-CoA reductase activity was suppressed in liver slices and homogenates from the drug-treated rats (5, 6). The results of this study is consistent with those observations and further provide evidence that the inhibition of reductase results from the increased rate of inactivation (or degradation) of the enzyme.

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