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short communications

Control of sterol synthesis and of hydroxymethylglutaryl CoA reductase in skin fibroblasts grown from patients with homozygous type II hyperlipoproteinemia

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Abstract In skin fibroblasts grown from four children with a homozygous form of type II hyperlipoproteinemia, the feedback control of sterol synthesis and the inhibitory effect on hydroxymethylglutaryl (HMG) CoA reductase activity by serum or low density lipoprotein were present, though diminished compared with the effects in normal fibroblasts. Stimulation of HMG CoA reductase by insulin and inhibition of acetyl CoA carboxylase by serum lipids were not impaired in these type II cells, indicating a degree of specificity in the abnormal response of the reductase. A rapid and convenient method for isolation of mevalonolactone in the course of the assay of HMG CoA reductase is described.

Supplementary key words feedback inhibition of cholesterol synthesis · low density lipoproteins · mevalonic acid assay

In cultured human skin fibroblasts, the rate of cholesterol synthesis and the activity of HMG CoA reductase (EC 1.1.1.34), an enzyme limiting the above process, are influenced by serum lipoproteins in a manner consistent with negative feedback regulation of cholesterol synthesis in these cells. It has been reported that in fibroblasts derived from persons homozygous for type II hyperlipoproteinemia (familial hypercholesterolemia) (referred to below as type II fibroblasts), such regulation is absent (1) or diminished (2). We have investigated this abnormality in fibroblasts from four patients clinically diagnosed as having the homozygous form of type II hyperlipoproteinemia.

Abbreviations: HMG, hydroxymethylglutaryl; LDL, low density lipoprotein; TLC, thin-layer chromatography.

METHODS

Cells

Skin fibroblasts were cultured from three apparently healthy donors (N1, N2, and N3) and from four patients with type II hyperlipoproteinemia. The latter were children with phenotypic characteristics of the homozygous form of this syndrome (3). All had xanthomata (tendinous or cutaneous, or both), serum cholesterol concentrations higher than 500 mg/dl, and triglyceride concentrations less than 200 mg/dl. Both parents of siblings J.P. and T.P. and of L.H. had hypercholesterolemia. The father of V.C. was not available for testing, though the mother had hypercholesterolemia. Fibroblasts were grown to confluency in Eagle's minimum essential medium (which contains 5 mM glucose) (4) with 10% (v/v) fetal calf serum. Falcon flasks, 75 cm^2, were used for enzyme studies and, 100 × 20 mm plastic petri dishes were used for assays of lipid synthesis. The cells were washed three times with Dulbecco's phosphate-buffered saline (5) and incubated, usually for 24 hr, in the minimal essential medium with or without glucose and other additions as indicated. The medium was then removed and the cells were washed three times with phosphate-buffered saline.

Studies of incorporation of [1-^{14}C]acetate into nonsaponifiable lipids and fatty acids were carried out as described earlier (6). HMG CoA reductase activity was assayed according to the method of Brown, Dana, and Goldstein (7), which includes TLC of the radioactive mevalonolactone. In some experiments the procedure for isolation of mevalonolactone was modified as follows. After a

TABLE 1. Effect of serum fractions on nonsaponifiable lipid and fatty acid synthesis in normal and type II fibroblasts

Incubation Conditions		Nonsaponifiable Lipids		Fatty Acids	
24 hr	Additional 6 hr	Normal	Type II	Normal	Type II
		cpm × 10⁻³/mg cell protein			
Whole serum		5.1 ± 0.3	26.0 ± 4.1	11.8 ± 0.9	14.5 ± 0.5
Extracted serum		144.4 ± 12.8	163.5 ± 2.3	43.9 ± 10.7	55.3 ± 3.3
Extracted serum	Whole serum	17.1 ± 0.1	58.1 ± 2.0	24.4 ± 0.7	39.3 ± 1.8
Extracted serum	LDL	9.4 ± 1.0	98.0 ± 8.0		

Cells from N1 and V.C. were incubated for 24 hr in medium without glucose, containing 10% fetal calf serum or 10% solvent-extracted serum. Where indicated, LDL (final concentration of cholesterol, 100 μg/ml) was added or the medium was replaced with one containing 10% serum, and the incubation was continued for 6 hr before the assay. Data presented are the means of values from duplicate samples of cells ± one-half the range.

1-hr incubation, [5-^{3}H]mevalonic acid (ca. 10^4 cpm) and 25 μl of 5 N HCl were added; the samples were further incubated for 30 min at 37°C and applied to 70 × 10 mm columns of AG-1-X8 formate (200–400 mesh; Bio-Rad Laboratories, Richmond, Calif.). Mevalonolactone was eluted with 8 ml of water, and 0.1 mg of carrier mevalonolactone was added to the eluate. Samples were dried on a steam bath and the residue was dissolved in 2 ml of methanol for radioassay. Reductase activity is reported as ^{14}C cpm in mevalonolactone (corrected for recovery of [^{3}H]mevalonate and for ^{14}C isolated after incubation with boiled enzyme) per milligram of protein. The ratio of ^{14}C to ^{3}H in the column eluate was equal to or slightly lower than the same ratio in a mevalonate fraction prepared by TLC of another aliquot of the same sample. This indicated that the radiopurity of the biosynthesized [^{14}C]mevalonate isolated by the ion exchange method was at least as high as that separated by TLC in a system that was previously demonstrated to yield a radiochemically pure product (8). The column method was found by us to be rapid, convenient, and accurate. The values for boiled enzyme were usually less than 10% of the lowest assayed activities. Acetyl CoA carboxylase was determined according to Jacobs, Sly, and Majerus (9). Protein was determined by the method of Lowry et al. (10). Human LDL and d 1.21 infranate were isolated by standard methods from freshly obtained sera of normal donors. Solvent-extracted human serum was prepared as described earlier (6). The procedure consisted of treating serum with a cold mixture of ethanol–ether 1:1 followed by multiple washings of the precipitated proteins with ether, dissolving the air-dried residue in 0.14 M NaCl, and exhaustively dialyzing against saline. The preparation was sterilized by ultrafiltration.

MATERIALS

HMG CoA was purchased from P-L Biochemicals, Inc., Milwaukee, Wis., and sodium [1-^{14}C]acetate (50 Ci/mole), [3-^{14}C]HMG CoA (13.7 Ci/mole), and DL-[5-^{3}H]mevalonic acid (2.0 Ci/mole) were purchased from New England Nuclear Corp., Boston, Mass. The radioactive HMG CoA was diluted before use with unlabeled material to a specific radioactivity of 3.3 Ci/mole.

RESULTS AND DISCUSSION

Sterol synthesis

The synthesis of sterols and fatty acids was greatly increased after incubation with solvent-extracted serum compared with that in cells incubated with whole serum (**Table 1**). For fibroblasts treated with extracted serum, the rates of nonsaponifiable lipid synthesis were similar in normal and type II cell lines; however, when incubated with serum, the synthesis in the latter was less depressed. There was a small difference between these two cell lines with respect to the inhibitory effects on fatty acid synthesis.

The effects of incubation with d 1.21 infranate and of the addition of LDL on the synthesis of nonsaponifiable lipids and digitonin-precipitable sterols were studied in three normal and four type II cell lines (**Table 2**). Because of some variation in the precursor specific radioactivity within this experimental series, the results are presented relative to those in samples incubated with fetal calf serum. The d 1.21 infranate did not increase sterol synthesis as much as extracted serum (compare Tables 1 and 2), possibly because the ultracentrifugal procedure was less likely to remove serum lipids completely. Furthermore, ether can be expected to extract also substances other than the main lipid components of lipoproteins. In spite of these potential differences, both preparations influenced sterol synthesis in a similar fashion. An additional 6-hr incubation, with LDL or serum, of cells that had been previously treated with a lipid-deficient serum preparation suppressed sterol synthesis relative to that in the "stimulated" cells but, again, more so in normal than in

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TABLE 2. Effects of d 1.21 infranate and LDL on nonsaponifiable lipid synthesis in normal and type II fibroblasts

Cell Line	Nonsaponifiable Lipids: d 1.21 Infranate	Nonsaponifiable Lipids: LDL[a]	Digitonides: d 1.21 Infranate	Digitonides: LDL[a]
	relative activity[b]			
N1	571	150	805	169
N2	467	194	540	268
N3	389	185	490	241
V.C.	220	144	250	165
L.H.	310	260	356	306
J.P.	225	183	168	164
T.P.	204	171	241	186

Cells were incubated for 24 hr in medium containing 5 mM glucose and 5% fetal calf serum or 5% d 1.21 human serum infranate.

[a] LDL (final concentration of cholesterol 50 μg/ml) was added and the incubation was continued for an additional 6 hr before the assay.

[b] Radioactivity in cpm/mg of cell protein relative to that in sample incubated with fetal calf serum taken as 100.

type II fibroblasts (Tables 1 and 2). These results are consistent with those of Khachadurian and Kawahara (2), who have also shown an inhibition of sterol synthesis by serum lipids in type II homozygous cells.

HMG CoA reductase activity

After incubation for 24 hr in medium with whole serum, reductase activity in type II fibroblasts was several times higher than in control cells (**Table 3**). Incubation for 24 hr in medium with extracted serum increased activity in all cells, and the levels observed in type II cells were not significantly different from those in normal cells. These results are at variance with those reported by Brown, Dana, and Goldstein (1), whose type II homozygous cell lines did not show any inhibition of HMG CoA reductase by the same concentrations of LDL cholesterol as used in this study.

In the experiment shown in **Table 4,** 5 mM glucose was included, which caused lower enzyme activities compared with those shown in Table 3, consistent with our previous observation (11). The lowering effect of glucose was demonstrated in the presence of serum, and it was undiminished in the homozygous cells (results not shown). In normal cells incubated for 24 hr with extracted serum or with d 1.21 infranate, reductase activity was, respectively, 11 or 6 times that in the same cells incubated with whole serum. When medium was replaced with one containing whole serum or when LDL was added and the incubation was continued for 8 hr, reductase activity declined considerably. Similar to the above-shown differences in sterol synthesis, reductase activity did not rise as much in normal fibroblasts incubated with d 1.21 infranate as it did in cells incubated with extracted serum. In

TABLE 3. Effects of whole serum and extracted serum on HMG CoA reductase in normal and type II fibroblasts

Cell Line	HMG CoA Reductase Activity: Whole Serum	HMG CoA Reductase Activity: Extracted Serum
	cpm × 10⁻³/mg protein	
N1	1.4 ± 0.4	86.3 ± 8.0
N2	2.2 ± 0.4	63.5 ± 1.8
V.C.	12.8 ± 0.2	58.9 ± 0.3
L.H.	21.9 ± 0.8	58.9 ± 1.8
J.P.	11.1 ± 0.4	68.1 ± 1.2
T.P.	7.9 ± 0.4	50.7 ± 2.4

Cells were incubated for 24 hr in medium without glucose, containing whole serum or solvent-extracted serum (10% v/v). Data are presented as in Table 1.

type II cells, on the other hand, activities in cells incubated with extracted serum and with d 1.21 infranate were quite similar. Perhaps these findings are due to the presence of sufficient residual lipid in the d 1.21 infranate to depress reductase activity in the normal cells but not in the type II cells. It was further demonstrated by us that insulin stimulates HMG CoA reductase to a similar degree in type II fibroblasts and normal cells (results not shown) (11); the insulin effect appears, therefore, to be unimpaired.

It was previously reported by Jacobs et al. (9) that in normal skin fibroblasts acetyl CoA carboxylase activity was higher after incubation with extracted serum than it was in cells incubated with whole serum. We have observed that a 20 to 40% difference between the activities in cells treated by the two media occurred to a similar degree in type II fibroblasts and in normal ones and that, in both groups, the activities were similarly depressed when cells that had been previously incubated with extracted

TABLE 4. Effect of serum fractions on HMG CoA reductase in normal and type II fibroblasts

Incubation Conditions: 24 hr	Incubation Conditions: Additional 8 hr	HMG CoA Reductase Activity: Normal Cells	HMG CoA Reductase Activity: Type II Cells
		cpm × 10⁻³/mg protein	
Whole serum		1.0 ± 0.0	3.4 ± 0.5
Extracted serum		11.7 ± 0.3	9.3 ± 0.5
Extracted serum	Serum	0.9 ± 0.1	6.6 ± 0.3
Extracted serum	LDL	2.0 ± 0.1	6.9 ± 0.5
d 1.21 infranate		5.9 ± 0.6	8.1 ± 0.1
d 1.21 infranate	Serum	0.9 ± 0.1	5.9 ± 0.1
d 1.21 infranate	LDL	1.1 ± 0.1	5.4 ± 0.3

Cells from N1 or V.C. were incubated for 24 hr in medium containing 5 mM glucose and either whole serum, solvent-extracted serum, or d 1.21 infranate (each equivalent to 10% [v/v] whole serum). Where indicated, the medium was replaced with one containing 10% serum or LDL (final concentration of cholesterol, 50 μg/ml) was added, and the incubation was continued for 8 hr before assay of HMG CoA reductase. Data are presented as in Table 1.

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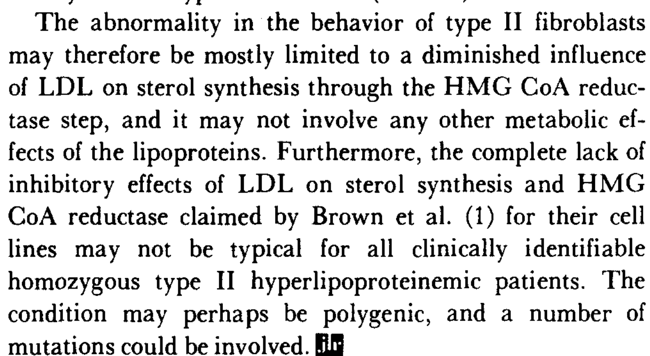
serum were exposed for an additional 8 hr to a new medium containing 10% serum. Fatty acid synthesis was stimulated to a similar extent in both types of cells; in activated samples it was inhibited by serum by 44% for normal and by 29% for type II fibroblasts (Table 1).



The abnormality in the behavior of type II fibroblasts may therefore be mostly limited to a diminished influence of LDL on sterol synthesis through the HMG CoA reductase step, and it may not involve any other metabolic effects of the lipoproteins. Furthermore, the complete lack of inhibitory effects of LDL on sterol synthesis and HMG CoA reductase claimed by Brown et al. (1) for their cell lines may not be typical for all clinically identifiable homozygous type II hyperlipoproteinemic patients. The condition may perhaps be polygenic, and a number of mutations could be involved.

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