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LACK OF CORRELATION BETWEEN THE RATE OF CHOLESTEROL BIOSYNTHESIS AND THE ACTIVITY OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE IN RATS AND IN FIBROBLASTS TREATED WITH ML-236B

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SUMMARY: ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, was studied in the rat and cultured human fibroblasts. A marked increase in liver reductase was found in fed and fasted rats; there was no effect on cholesterol-7α-hydroxylase. ML-236B did not alter tissue sterol levels in fed or fasted rats. In fibroblasts from normal or familial hypercholesterolemic individuals, ML-236B caused a marked increase in reductase. In contrast, the incorporation [2-^{14}C]-acetate into sterol was decreased while the incorporation into fatty acids was increased. Cycloheximide prevented the increase in fibroblast reductase.

INTRODUCTION: Cholesterol is an essential component of all mammalian cell membranes as well as a precursor for steroid hormones and bile acids. On the other hand, cholesterol in excessive amounts can be lethal as in atherosclerosis. 3-Hydroxy-3-methylglutaryl Coenzyme A reductase (EC 1.1.1.34) is the rate limiting step for cholesterol biosynthesis (1). Because of the decisive role of this enzyme in sterol biosynthesis, the regulation of this enzyme has been under intensive investigation. Recently, it was reported that ML-236B, a fungal metabolite, is a competitive inhibitor (K_I - 10 nM) of rat liver microsomal HMG-CoA reductase[1] (2). With the availability of a competitive inhibitor of HMG-CoA reductase, the regulation of this enzyme and the subsequent effect on sterol biosynthesis, may be more carefully evaluated.

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[1] Abbreviations: DTT, dithioerthyritol; G-6-P, glucose-6-phosphate; HMG-CoA, DL-3-hydroxy-3-methylglutaryl-CoA; LDHS, lipoprotein deficient human serum (density >1.25 g/ml); FCS, fetal calf serum; FHC, familial hypercholesterolemic, receptor negative fibroblast; NSL, non-saponifiable lipids (sterols).

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In this communication we report on the effect of ML-236B on HMG-CoA reductase and tissue sterol levels in the rat as well as on HMG-CoA reductase and sterol and fatty acid synthesis in cultured human fibroblasts[2].

MATERIALS AND METHODS: Animals - Male Sprague-Dawley rats weighing ca. 150 g were housed in an air-conditioned, light-cycled (dark 7:00 pm - 7:00 am) room and had free access to Purina Rat Chow and water. Food was removed after administration of the compound. ML-236B[3] was suspended in 1% Methocel using a Potter-Elvehjem homogenizer and was administered orally at a dose of 20 mg/kg. A control group received an equal volume of 1% Methocel. Animals were killed by decapitation either 3 hours (fed) or 24 hours (fasted) after administration of the compound. Liver Microsome Preparation - Microsomes were prepared by a modification of the method of Linn (3) using a buffer that was 0.1 M in Tris·HCl (pH 7.4 at 5°), 0.02 M in EDTA and 0.002 M in DTT. Microsomes were resuspended in a volume of this buffer equivalent to the original liver weight. Microsomal protein was determined by the method of Yonitoni (4) modified by elimination of H_2O_2 and addition of 0.1% deoxycholic acid. Absorbance was measured at 330 nm (5) using bovine serum albumin as a standard; a turbidity blank was included. HMG-CoA Reductase Assay - Duplicate determinations were made in 0.1 ml incubation mixture containing 50 µmoles Tris·HCl (pH 7.4 at 37°) 10 µmoles EDTA, 1 µmole DTT, 30 µmoles G-6-P, 30 µmoles NADP, 1 unit of G-6-P dehydrogenase, 0.3 µmoles of [3-^{14}C]-HMG-CoA (2130 dpm/nmol) and microsomes equivalent to 0.02 g of liver. Components were preincubated at 37° for 10 minutes before initiating the reaction by adding the HMG-CoA. After incubation in a shaking water bath at 37° for 15 minutes, the reaction was terminated by the addition of 0.01 ml of 12 N HCl. Further work-up was as described by McNamara et al (6). Cholesterol-7α-hydroxylase Assay - Duplicate determinations were made utilizing the method of Nicolau et al (7), modified by the addition of α-amino-ethanethiol (10mM) to the assay mixture (8). Determination of Tissue Sterols - Sterols were extracted with alcohol-acetone (1:1) from the serum, liver and liver microsomes (9). In order to correct for losses during the sterol isolation steps [4-^{14}C]-cholesterol and [4-^{14}C]-cholesterol oleate were used as internal standards. Further work-up was as described by Salen, et al (10). Leucine Incorporation - Cells were incubated for 3 hour with 0.4 mM L-[4,5-^{3}H]-leucine (0.83 µCi/µmole), then separated from the medium by centrifugation and dissolved in 1N KOH. Protein was precipitated by 6.25% trichloroacetic acid in the presence of bovine serum albumin and unlabeled leucine. The pellet was

[2] While this manuscript was in preparation, Brown et al (21) reported on a study in which effects of ML236B on fibroblast HMG-CoA reductase, similar to those reported here, were noted.

[3] Generously supplied by Dr. Ko Arima, Sankyo Co., Ltd., Tokyo, Japan.

dissolved in 1N KOH, precipitated with trichloroacetic acid and then washed three times with 5% trichloroacetic acid. This pellet was dissolved in Soluene 100 (Packard Instrument Company., Downers Grove, IL) and assayed by liquid scintillation counting. Fibroblasts - Human fibroblast cell lines were obtained from the Institute for Medical Research, Camden, N. J. Normal (GM179) and familial hypercholesterolemic, receptor negative (GM488) fibroblasts were grown in monolayer and used between the seventh and twentieth passages. Tissue culture conditions were as described by Brown et al (11). The medium utilized was developed at the Rockefeller University (12); water was prepared using the Milli-Q System (Millipore, Corp., Bedford, Mass.). Enzyme extracts were prepared and assayed as described by Brown et al (11). [2-^{14}C]-acetate incorporation into lipids was as described by Miller et al (13). Fibroblast protein was determined utilizing the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). A minimum of five plates were used for each assay. LDHS was obtained by sequential centrifugation (14). Statistics - Group means were tested for homogeneity using one-way analysis of variance. Significance of individual differences between means was tested using Duncan's multiple range test (15).

RESULTS AND DISCUSSION: While Endo et al have reported that ML-236B reduced serum cholesterol levels in the acutely treated rat (16), we found no effect on serum sterol levels compared with fed (66.9 vs. 63.1 mg/dl) or fasted (71.2 vs. 76.5 mg/dl) controls. Furthermore, we did not observe a change in either liver or liver microsomal cholesterol concentration (19).

A marked increase in HMG-CoA reductase activity was observed in fed rats three hours following the administration of ML-236B (Table 1). Although HMG-CoA reductase was reduced in the fasted control rats as compared to the control fed rats (17), the elevated reductase activity with ML-236B persisted through the 24 hour fasting period. It has been previously reported that ML-236B reduced the incorporation of [^{14}C]-acetate into sterol in rat liver slices (2,16,18). These observations taken together suggest a disparity in quantitating sterol biosynthesis as determined by either HMG-CoA reductase activity or the incorporation of acetate into sterol.

In order to explore this effect of ML-236B on sterol biosynthesis, studies were made using cultured human fibroblasts.

TABLE 1. Effect of ML-236B on HMG-CoA Reductase and Cholesterol-7α-Hydroxylase in Fed and Fasted Rats

Treatment	HMG-CoA Reductase[1]	Cholesterol-7α[2] Hydroxylase
	Fed	
Control	12.2 ± 2.6[5]	54.0 ± 18.7
ML-236B	20.1 ± 1.3[3]	87.4 ± 4.2[4]
	Fasted	
Control	6.8 ± 2.9	29.73 ± 6.8
ML-236B	54.7 ± 12.2[3]	18.59 ± 1.3

Conditions were as outlined in Materials and Methods.

[1] p moles of mevalonic acid·mg^{-1} microsomal protein·hr^{-1}.

[2] p moles of 7α-OH cholesterol·mg^{-1} microsomal protein·hr^{-1}.

[3] Statistically different from respective control: P= <0.05.

[4] Statistically different from respective control: P= <0.1.

[5] Values are mean ± standard error of the mean.

Results from normal or FHC fibroblasts maintained in either FCS or LDHS supplemented medium are found in Table 3. In all cases assayable HMG-CoA reductase activity was markedly increased by ML-236B. In contrast, the rate of acetate incorporation into NSL was dramatically reduced. Mevalonate incorporation into NSL was only slightly reduced indicating that the inhibition of sterol synthesis was at the level of HMG-CoA reductase. Treatment with ML-236B resulted in an increased incorporation of acetate into fatty acids.

The increased HMG-CoA reductase activity found in rats and fibroblasts treated with ML-236B suggested that HMG-CoA reductase may be induced and, therefore, represents an in-

TABLE 2. Effect of ML-236B on HMG-CoA Reductase, Acetate and Mevalonate Incorporation into Non-Saponifiable Lipids, and Acetate into Fatty Acids in Human Fibroblasts.

Fibroblast	Additions	HMG-CoA Reductase[1]	Acetate Incorporation[2] NSL	Acetate Incorporation[2] Fatty Acids	Mevalonate Incorporated[2] NSL
			FCS		
Normal	Ethanol	3.9 ± 0.2[5]	5.9 ± 0.5	58.2 ± 1.5	3.5 ± 0.4
	ML-236B	44.7 ± 2.1[3]	4.5 ± 0.8	54.6 ± 2.1	2.4 ± 0.1[3]
FHC	Ethanol	25.0 ± 0.5	36.7 ± 3.3	84.0 ± 4.5	3.2 ± 0.1
	ML-236B	148.0 ± 7.2[3]	14.8 ± 1.3[3]	158.3 ± 15.4[3]	2.6 ± 0.1[4]
			LDHS		
Normal	Ethanol	69.3 ± 2.4	40.0 ± 0.7	22.3 ± 0.5	3.7 ± 0.2
	ML-236B	147.2 ± 8.3[3]	4.5 ± 0.1[3]	52.6 ± 9.0[3]	3.1 ± 0.6[4]
FHC	Ethanol	43.3 ± 3.5	50.8 ± 1.2	25.2 ± 2.1	3.0 ± 0.2
	ML-236B	220.0 ± 29.0[3]	10.2 ± 1.0[3]	71.1 ± 7.1[3]	2.4 ± 0.2[4]

Conditions were as described in Materials and Methods. Ethanol or ethanol containing ML-236B was added to give a final concentration of 0.1% and 2.7 μM, respectively Additions to medium did not alter cell viability as determined by trypan blue exclusion.

[1] pmol of mevalonic acid·mg^{-1} detergent soluble protein·Min^{-1}.

[2] pmol·mg^{-1} cell protein·min^{-1} incorporated into respective fractions.

[3] Statistically different from respective control: P = <0.01.

[4] Statistically different from respective control: P = <0.05.

[5] Values are mean ± standard error of the mean.

crease in enzyme protein synthesis. To test this hypothesis, studies were made in fibroblasts with ML-236B in the presence or absence of cycloheximide. When only cycloheximide was present in the medium, HMG-CoA reductase was reduced from control levels (Table 3). Furthermore, ML-236 again increased the assayable HMG-CoA reductase activity. However, in those cells which were first treated with cycloheximide and subsequently with ML-236B, the assayable HMG-CoA reductase activity was reduced to the cycloheximide control levels. The results obtained with [4-^{3}H]-leucine clearly show that cycloheximide blocked protein synthesis and, furthermore, that ML-236B did not act as a general stimulus for cellular protein

TABLE 3. Effect of Cycloheximide on the ML-236B Mediated Increase of HMG-CoA Reductase in FHC Fibroblasts

Group	Medium Additions 0 Hours	Medium Additions 3 Hours	HMG-CoA-Reductase[1]	Leucine Incorporation[2]
1	Growth Medium	Ethanol	20.6 ± 2.2[5]	15.0 ± 0.6
2	Cycloheximide	Ethanol	11.0 ± 2.4[3]	0.9 ± 0.1[3]
3	Growth Medium	ML-236B	48.6 ± 9.7[3]	14.2 ± 0.5
4	Cycloheximide	ML-236B	16.0 ± 3.4[4]	0.6 ± 0.6[4]

Conditions were as outlined in Materials and Methods. FHC cells growing in 10% FCS medium were treated on day 7. At 0 hrs. 100 µl of growth medium or growth medium containing 14.4 mM cycloheximide was added. At 3 hrs. 100 µl of 20% ethanol or 20% ethanol containing 86 µM ML-236B was added. Leucine incorporation was assayed starting at 3 hrs. Cells were harvested at 6 hrs.

[1] pmol of mevalonic acid·mg^{-1} detergent soluble protein·min^{-1}.

[2] pmol of leucine·mg^{-1} cell protein·min^{-1} .

[3] Statistically different from Group 1: $P<0.01$ using Duncans Multiple range algorithm on log transformed data.

[4] Statistically different from Group 3: $P<0.01$ using Duncans Multiple range algorithm on log transformed data.

[5] Values are mean ± standard error of the mean.

synthesis. From these results, we conclude that ML-236B induced HMG-CoA reductase protein synthesis.

The striking and unexpected finding in these studies was the induction of HMG-CoA reductase by the competitive inhibitor, ML-236B. Since the incorporation of acetate into sterol was inhibited in the intact cell, it may be concluded that HMG-CoA reductase was inhibited intracellularly. Upon disruption of the cells, there was a dramatic increase in assayable HMG-CoA reductase, which may be attributed to the dilution of intracellular ML-236B during preparation for the assay. Thus, the inhibition of HMG-CoA reductase with ML-236B is readily reversible.

The induction of this enzyme indicates that as the number of active molecules of HMG-CoA reductase was reduced by the

inhibitor, the cell responded by increased synthesis of the enzyme protein in order to maintain a basic rate of sterol biosynthesis. It is generally accepted that HMG-CoA reductase in the cultured human fibroblast is primarily regulated by the low density lipoprotein cholesterol which enters the cell via high-affinity binding sites (20). In this regard, it is of interest that in the normal fibroblasts with a full completment of LDL receptors and grown in the presence of FCS, ML-236B increased the assayable HMG-CoA reductase. Perhaps there is a basic cellular requirement for de novo synthesized cholesterol even in the presence of adequate exogenous cholesterol. The lack of correlation between HMG-CoA reductase activity and the incorporation of acetate into sterol indicates that caution should be exercised in relating HMG-CoA reductase activity to a cholesterol biosynthetic rate.

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REFERENCES

1. Rodwell, V. W., Nordstrom, J. L. and Mitschelen, J. J. (1976) Adv. in Lipid Res. 14, 1-74.
2. Endo, A., Kuroda, M. and Tanzawa, K. (1976) FEBS Letters 72, 323-326.
3. Linn, T. C. (1967) J. Biol. Chem. 242, 984-989.
4. Yonitoni, T. (1961) J. Biol. Chem. 236, 1680-1688.
5. Koch, A. L., and Putnam, S. L. (1971) Biochem. 44, 239-245.
6. McNamara, D. J., Quackenbush, F. W., Rodwell, V. W. (1972) J. Biol. Chem. 247, 5805-5810.
7. Nicolau, G., Shefer, S., and Salen, G. (1974) J. Lipid Res. 15, 146-151.
8. Milton, J. R., Scholan, N. A., and Boyd, G. S. (1971) Eur. J. Biochem. 20, 569-579.
9. Sperry, W. and Webb, M. (1950) J. Biol. Chem. 187, 97-106.
10. Salen, G. S., Ahrens, E. H., and Grundy, S. M. (1970) J. Clin. invest. 49, 952-967.
11. Brown, M. S., Dana, S. E., and Goldstein, J. L. (1974) J. Biol. Chem. 249, 789-796.
12. McNamara, D. J. and Poria, A., Personal Communication.

13. Miller, N. E., Weinstein, D. B., and Steinberg, D. (1977) J. Lipid Res. 18, 438-450.
14. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) J. Clin. Invest. 34, 1345-1353.
15. Kirk, R. E., Experimental Design: Procedures for the Behavioral Sciences (1968) Brooks/Cole, Belmont, CA.
16. Endo, A., Kuroda, M., and Tsujida, Y. J. Antibiotics (1976) 29, 1346-1348.
17. Shapiro, D. J. and Rodwell, V. W. (1972) Biochemistry 11 1042-1044.
18. Endo, A., Tsjita, Y. and Tanazawa, K. (1977) Eur. J. Biochem. 77, 31-36.
19. Ingebritsen, T. S., Bensch, W. R., and Diller, E. R., unpublished observations.
20. Brown, M. S., and Goldstein, J. L. (1976) Science 191, 150-154.
21. Brown, M.S., Faust, J. R., Goldstein, J. L., Kaneko, I. and Endo, A. (1978) J. Biol. Chem. 253 1121-1128.