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Regulation of Fatty Acid Biosynthesis by Intermediates of the Cholesterol Biosynthetic Pathway

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The biosynthesis of cholesterol and fatty acid (FA) proceeds by independent pathways. Information is lacking on potential interaction that could provide feedback regulation between these pathways. In an attempt to search for a new approach to produce a dual effect on regulation of these two processes, we have identified mevalonate-5-diphosphate (MevPP) decarboxylase, an enzyme of the cholesterol biosynthesis pathway, the inhibition of which leads not only to the suppression of cholesterol but also FA biosynthesis. Mechanistic studies with Hep G2 cells using specific inhibitors for MevPP decarboxylase and related enzymes reveal that the inhibitory effect on FA biosynthesis is mainly due to the accumulation of MevPP, resulting from MevPP decarboxylase inhibition. The present study proposes a new mechanism through which interpathway regulation could take place between the cholesterol and FA cascades.

Recent advance in the development of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors for treatment of hypercholesterolemia has demonstrated the value of targeting pharmacological intervention at the rate-limiting site of a biosynthetic pathway (1,2). Since growing evidence has indicated that hypertriglyceridemia may also be a risk factor for the development of coronary heart disease, particularly in patients with combined hyperlipidemia (3-5), there is an apparent need for new agents with a dual effect on suppression of the plasma cholesterol and TG levels, and possibly by a new mechanism.

In search for such an approach we have chosen to intervene at the site of MevPP decarboxylase (EC 4.1.1.33.) of the cholesterol pathway. This is based on the consideration that such inhibition may lead not only to inhibition of cholesterol biosynthesis, but also to the accumulation of MevPP. As the first of a series of diphosphate-containing intermediates in the cholesterol pathway, MevPP has the potential to produce a secondary effect which may have an impact on FA biosynthesis. This study presents evidence showing that accumulation of MevPP resulting from MevPP decarboxylase inhibition plays a regulatory role on FA biosynthesis in a cellular system.

MATERIALS AND METHODS

All materials were purchased from Sigma Chemical Co. unless otherwise specified. 6-fluoromevalonolactone (6-FMev lactone), 4,4-dimethylmevalonolactone (4,4-DiMeMev lactone), 5-acetoxy-6-fluoromevalonate methyl ester (6-FMevMe) and 5-acetoxymevalonate methyl ester (MevMe) were synthesized in our Chemistry laboratories following the published methods (6-8). R-[3-^{14}C]MevPP was purchased from NEN Research Products. Lovastatin was a gift of the Merck Company.

Inhibition of MevPP decarboxylase. The activity of MevPP decarboxylase was assayed by a modification of the

Abbreviations used: FA, fatty acid; TG, triglyceride; MevP, mevalonate-5-phosphate; MevPP, mevalonate-5-diphosphate; 6-FMev, 6-fluoromevalonic acid; 6-FMevMe, 5-acetoxy-6-fluoromevalonate methyl ester; MevMe, 5-acetoxymevalonate methyl ester; 6-FMev lactone, 6-fluoromevalonolactone; 4,4-DiMeMev lactone, 4,4-dimethylmevalonolactone; DMEM, Dulbecco's modified Eagle medium.

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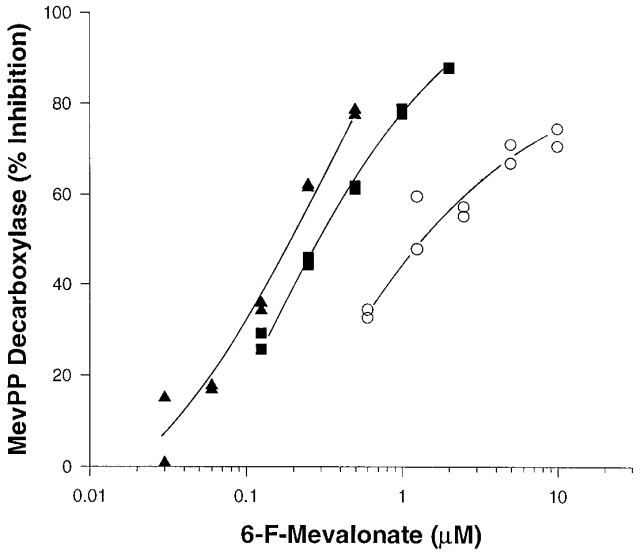

FIG. 1. Inhibition of MevPP decarboxylase by 6-FMev. Sodium salt of 6-FMev was preincubated with the crude human MevPP decarboxylase preparation, in the presence of ATP (2 mM) and Mg^{2+} (3 mM), at 37°C for the time periods: 0 (○), 10 min (■) and 30 min (▲). Enzyme activity was assayed as described in Methods. Percent inhibition was calculated in reference to control (without inhibitor) for the respective treatment condition.

procedure described by Alvear et al. (9). The reaction mixture (0.1 ml final volume) contained 0.05 M BisTris-HCl buffer, pH 7.0, 2 mM ATP, 3 mM $MgSO_4$. 1 mM dithiothreitol and human Hep G2 cell cytosolic preparation (100,000 *g* supernatant) serving as enzyme source for MevPP decarboxylase. After a preincubation period as designated at 37°C, [^{14}C]MevPP (1.4 μM) was added to start the reaction. Samples were incubated for 20 min at 37°C, 25 μl of a solution containing Tris base (1 M) and alkaline phosphatase (8 mg/100 μl) was added to stop the reaction (pH $>$ 8). The mixture was incubated at 37°C for 60 min; extracted with ethylacetate (500 μl), and counted in a liquid scintillation counter for the formation of isoprenoid products.

Cholesterol and FA biosynthesis in Hep G2 cells. Human hepatoma cells, Hep G2, obtained from American Type Culture Collection (Rockville, MD) were cultured in 6-well dishes (Corning, NY) in Dulbecco's modified Eagle medium (DMEM) containing 8% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin and 0.1 mM nonessential amino acids (Gibco, Grand Island, NY) in an atmosphere of 5% CO_2 in a humidified incubator at 37°C. Cells of 3 days old were washed with phosphate-buffered saline (PBS) and incubated at 37°C with DMEM (without FBS) in the presence or absence of special agent as designated for 24 h. Cells were then washed with PBS and incubated with DMEM (without FBS) containing the test compound for 2 h at 37°C. [^{14}C]Sodium acetate (2 μCi/well) was added and the samples were incubated for 1 h at 37°C. Cell samples were washed 2 times with cold PBS and, with the aid of cold PBS and a rubber policeman, transferred into 13 $\times$ 100 mm glass tubes. After centrifugation (2,000 g for 10 min), the sedimented cells were recovered and hydrolyzed with 2 M NaOH at 60°C for 30 min. Aliquots of samples were taken for protein analysis (10); the remaining was hydrolyzed for an additional 1 h at 95°C. The samples were lyophilized overnight under vacuum. After acidification (18 M H_2SO_4), the samples were extracted with 1 ml of chloroform-toluene (9:1); aliquots (200 μl) were taken for determination of [^{14}C]acetate incorporation into cholesterol and FA by thin-layer chromatography (TLC). TLC plates were developed initially in hexane/2-propanol/acetone/diethylamine (90:5:5:0.5, v/v) for 1 inch; and, after drying, in hexane/chloroform/diethylamine (50:50:0.5 v/v) for 4.5 inches. Radioactive zones corresponding to cholesterol and free FA were counted in a liquid scintillation counter.

RESULTS

Inhibition of MevPP decarboxylase. In an enzyme assay using crude MevPP decarboxylase preparation from human Hep G2 cells, 6-FMev (free acid) inhibited the conversion of [^{14}C]-MevPP to isopentenyl diphosphate with an IC_{50} of about 0.2-2 μM (Fig. 1). The inhibition was time-dependent on preincubation of the compound with the enzyme preparation (in the presence of ATP and Mg^{++}).

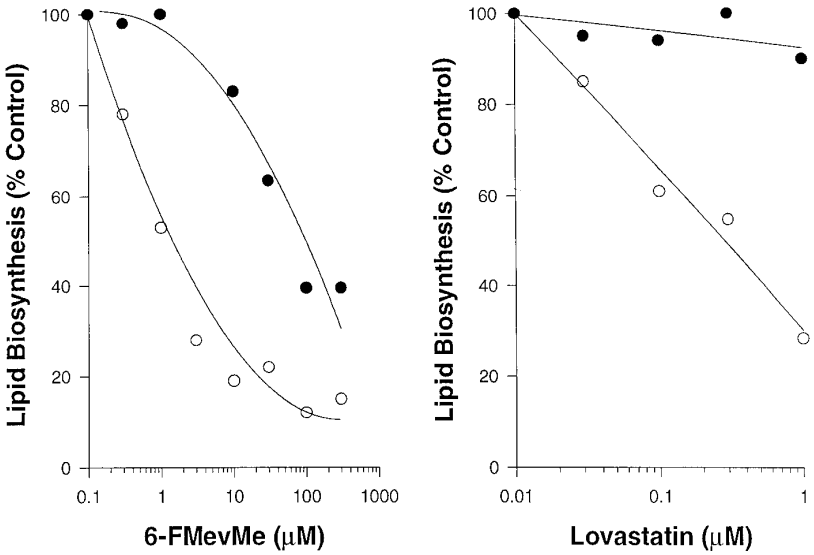

FIG. 2. Effects of 6-FMevMe and lovastatin on cholesterol and FA biosynthesis in Hep G2 cells. Hep G2 Cells (3 days old) in C6 wells were incubated in DMEM for 24 h. Cells were incubated with test compound (varying concentrations) for 2 h prior to the addition of [^{14}C]acetate (1 h) to measure the cholesterol (○) and FA (●) biosynthesis. Percent inhibition was calculated in reference to control (without test compound). The results of a typical experiment are shown; each point represents a single measurement. The same pattern of inhibition was consistently noted in other experiments.

Activity profiles of MevPP decarboxylase inhibition on cholesterol and FA biosynthesis in Hep G2 cells. We studied the effect of 6-FMevMe, a derivative of 6-FMev with improved cell-bioavailability, on cholesterol biosynthesis in Hep G2 cells. Although weaker in effect than the reference HMG-CoA reductase inhibitor, lovastatin (IC_{50} about 0.1 μM), 6-FMevMe inhibited cholesterol biosynthesis with an IC_{50} of about 1 μM (Fig. 2). While lovastatin was ineffective on FA biosynthesis, 6-FMevMe inhibited with an IC_{50} of about 100 μM, 100 times its IC_{50} for cholesterol biosynthesis, indicating a potential role in FA biosynthesis inhibition.

Mechanistic studies probing the fatty acid-inhibitory effect of MevPP decarboxylase inhibition in Hep G2 cells. To show the link between FA biosynthesis inhibition and MevPP accumulation, we used exogenous mevalonate to enhance the cholesterol cascade and the intracellular level of MevPP. With MevMe (1 mM) as a source of exogenous mevalonate (Fig. 3), the inhibitory effect of 6-FMevMe, while remaining unchanged on cholesterol biosynthesis, was greatly enhanced on FA biosynthesis (IC_{50} = 1 μM). Relative to the control condition, addition of exogenous mevalonate reduced [^{14}C]acetate incorporation into both cholesterol and FA.

Enhanced production of endogenous mevalonate produced a similar effect. Using Hep G2 cells pretreated with lovastatin (0.1 μM, 24 h), a condition known to cause enhanced expression of HMG-CoA reductase in cellular systems (11), the inhibitory effect of 6-FMevMe on FA biosynthesis was again enhanced (IC_{50} = 1 μM), matching its effect on cholesterol biosynthesis (Figure 3).

To obtain further support, we tested for the effect of totally eliminating MevPP accumulation on FA biosynthesis. Using lovastatin-treated Hep G2 cells as described above, lovastatin, at elevated concentrations (0-30 μM), was added to terminate the cholesterol pathway. Lovastatin addition, while exacerbating the cholesterol inhibition caused by 6-FMevMe (Fig. 4, left panel), concentration-dependently attenuated the inhibitory effect on FA biosynthesis produced by 6-FMevMe (right panel).

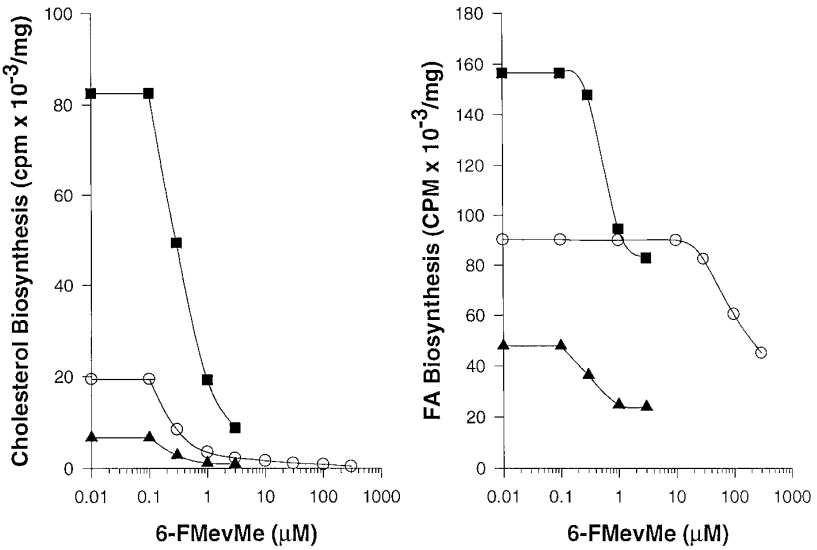

FIG. 3. Effects of exogenous mevalonate and lovastatin pretreatment on inhibitory activity of 6-FMevMe on cholesterol and FA biosynthesis in Hep G2 cells. Cells (3 days old) in C6 wells were preincubated in DMEM with (■) or without lovastatin (0.1 μM) for 24 h. All samples received 6-FMevMe (varying concentration) in DMEM for 2 h (as described in Fig. 2). Samples without lovastatin preincubation were subdivided into two groups: with (▲) and without (○) the addition of MevMe (1 mM in DMEM) for 1 h prior to the addition of [^{14}C]acetate. The data for lipid biosynthesis were expressed as cpm incorporation per mg of cell protein. The results of a typical experiment are shown; each point represents a single measurement. The same pattern of inhibition was consistently noted in other experiments.

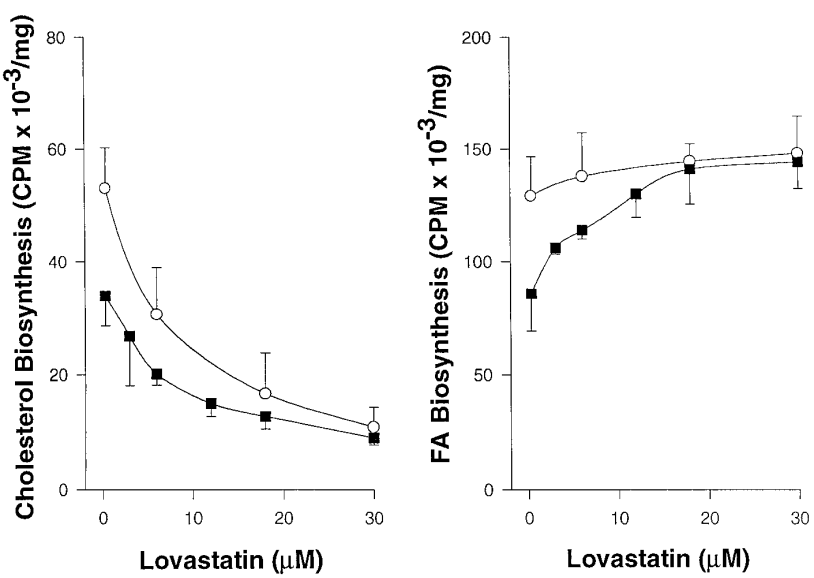

FIG. 4. Effects of lovastatin on the inhibition of cholesterol and FA biosynthesis produced by 6-FMevMe in Hep G2 cells. Cells (3 days old) in C6 wells were preincubated with lovastatin (0.1 μM in DMEM) for 24 h. Samples were treated with (■) or without (○) 6-FMevMe (1 μM in DMEM) for 3 h. Lovastatin (varying concentration) was added 2 h prior to the addition of [^{14}C]acetate. The data for lipid biosynthesis were averages of triplicate measurements and expressed as cpm incorporation per mg of cell protein (average±SEM).

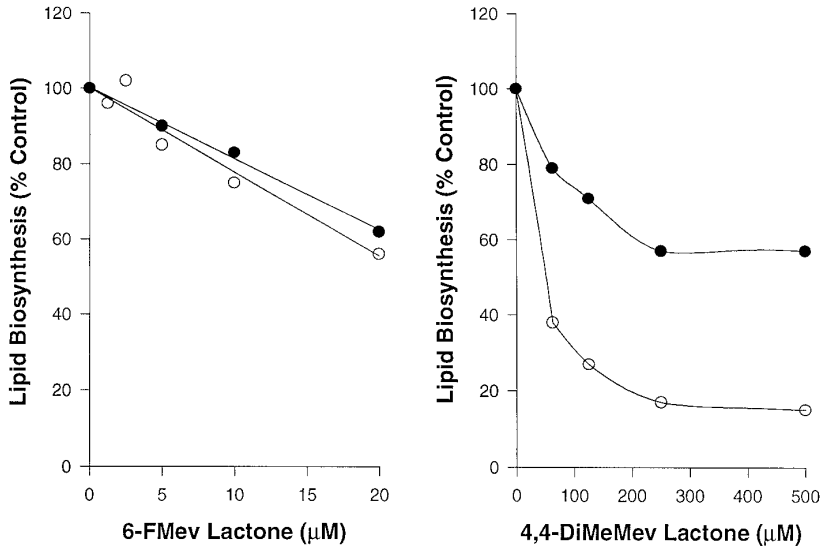

FIG. 5. Effects of 6-FMev lactone and 4,4-dimethylmevalonolactone on cholesterol and FA biosynthesis in Hep G2 cells. Cells (3 days old) in C6 wells were preincubated with lovastatin (0.1 μM in DMEM) for 24 h. Samples were incubated with test compound (varying concentrations) for 2 h prior to the addition of [^{14}C]acetate (1 h) for the measurement of cholesterol (○) and FA (●) biosynthesis. The results of a typical experiment are shown; each point represents a single measurement. The same pattern of inhibition was consistently noted in other experiments.

Effect of MevP kinase inhibition on cholesterol and FA biosynthesis in Hep G2 cells. Since MevPP decarboxylase inhibition may also produce accumulation of mevalonate-5-phosphate (MevP), substrate for MevPP biosynthesis, we studied the effect of inhibition of the MevP-metabolizing enzyme, mevalonate kinase, using a published compound, 4,4-DiMeMev lactone (16). Using Hep G2 cells pretreated with lovastatin as described above, results in Fig. 5 show that while 6-FMev lactone inhibited both cholesterol and FA production equipotently (IC_{50} about 20 μM) as seen earlier with 6-FMevMe under similar conditions (Fig. 3), 4,4-DiMeMev lactone exhibited only a weak effect on FA ($IC_{50} \sim 600$ μM) relative to its effect on cholesterol biosynthesis ($IC_{50} \sim 30$ μM). Given that the accumulated levels of MevP and MevPP that were produced by these two agents for a set percentage of cholesterol biosynthesis inhibition may be quite similar, MevP clearly has only a marginal effect on FA biosynthesis modulation relative to MevPP.

DISCUSSION

We chose to intervene at the site of MevPP decarboxylase to explore the hypothesis that the accumulation of MevPP could potentially produce a secondary effect on FA biosynthesis. MevPP could mimic inorganic pyrophosphate in inhibiting protein phosphatases involved in reversible phosphorylation of specific types of proteins (12). It is important to note that acetyl-CoA carboxylase and HMG-CoA reductase, the rate-limiting enzymes for the FA and cholesterol biosynthesis respectively, are regulated by reversible phosphorylation, with the dephosphorylated forms being the active enzyme versions (13-15). We hypothesize that MevPP accumulation could have a special effect in regulating cholesterol and FA biosynthesis in biological systems.

The present study demonstrates that MevPP decarboxylase inhibition indeed can lead to a dual effect on inhibition of cholesterol and FA biosyntheses in cellular and animal (data not shown) systems. While the cholesterol effect is the obvious result of a direct inhibition of the

cholesterol pathway, the effect on FA biosynthesis appears to be indirect, resulting mainly from the accumulation of MevPP. Hence, the FA effect is more pronounced when the cholesterol pathway is enhanced. We arrive at this conclusion based on several lines of evidence. In Hep G2 cells, under conditions when the cholesterol pathway is upregulated, either by exogenous supply of mevalonate methyl ester as the exogenous source of mevalonate or by enhanced endogenous production (via HMG-CoA reductase expression enhancement by pretreatment with lovastatin), the effect of MevPP decarboxylase inhibition, as produced by 6-FMevMe, on FA biosynthesis inhibition is more pronounced (IC_{50} for 6-FMevMe changed from about 100 to 1 μM). Addition of high concentrations of lovastatin to terminate cholesterol production attenuates such effect. Due to the dependency of this effect on the level of cholesterol biosynthesis, the finding rules out the direct involvement of the MevPP decarboxylase inhibitor (6-FMevPP produced in situ) or metabolites derived from it, and favoring the accumulation of certain pathway products of the cholesterol cascade, up to the step of MevPP formation, as mediators of the FA effect. Additional information on the differing effects of the MevP kinase and MevPP decarboxylase inhibition confirms the role of MevPP as the major mediator of the FA-inhibitory effect. As for the remaining PP-containing intermediates of the cholesterol pathway: isopentenyl diphosphate, geranyl diphosphate and farnesyl diphosphate, the lack of suitable inhibitors for comparative study keeps their roles unclear.

Despite clear indication of the MevPP effect on FA biosynthesis modulation, its molecular basis remains unclear. If the effect is indeed mediated by modulating the reverse phosphorylation of acetyl-CoA carboxylase as originally hypothesized, it could have a similar effect on HMG-CoA reductase activity. Acetyl-CoA carboxylase and HMG-CoA reductase are affected by reversible phosphorylation through similar mechanisms (13-15). However, further study is needed to examine the effect of MevPP on enzyme systems involved in reversible phosphorylation of these two enzymes.

The finding that the addition of exogenous mevalonate *per se* reduces [^{14}C]acetate incorporation into both cholesterol and FA in Hep G2 cells (Fig. 3) is of interest. Since mevalonate is not a direct substrate for FA biosynthesis (16), the effect can only be interpreted as the result of inhibition of FA biosynthesis, as indicated in here, by its conversion to MevPP. Collectively, these findings point to a basic role of the cholesterol pathway in regulating FA biosynthesis. MevPP decarboxylase inhibition, in causing MevPP accumulation, may serve to enhance this natural feedback role of the cholesterol pathway. Thus, when cholesterol production is excessive, it will turn off the cascade through gene regulation by suppressing the expression of HMG-CoA reductase (17,18). In the mean time, a more direct, short term effect may take place by the down-regulation of FA biosynthesis resulting from the accumulation of MevPP. Supporting this latter conjecture, an earlier report showed the extreme hypertriglyceridemic effect produced by prolonged dosing of lovastatin in Syrian Golden hamsters was reversed by co-administration with mevalonate (19). This effect could be partly mediated by affecting the endogenous level of MevPP. On this basis, MevPP decarboxylase inhibition appears to have a distinct advantage over that produced by the other approaches for not suppressing the endogenous level of MevPP.

Given the unstable nature of MevPP, its steady state level under natural environment will fluctuate depending on such conditions as the cholesterol pathway activity, and the rate of MevPP degradation by the specific phosphatases. As for the advantages of MevPP decarboxylase inhibition, it awaits further evaluation of compounds with improved potency and pharmacological profiles in animal systems.

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