REGULATION OF FATTY ACID SYNTHESIS BY FARNESYL PYROPHOSPHATE

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Fatty acid biosynthesis is transcriptionally regulated by liver X receptor (LXR) and its gene target, sterol regulatory element binding protein-1c (SREBP-1c). LXR activation is induced by oxysterol end products of the mevalonate pathway and is inhibited by the upstream non-sterol isoprenoid, geranylgeranyl pyrophosphate (GGPP). Whether isoprenoids play a role in regulating the transcription of genes involved in fatty acid biosynthesis is unknown. In CaCo-2 colon epithelial cells, depletion of mevalonate and its derivatives, including oxysterol ligands for LXR, increased fatty acid synthesis. Addition of mevalonate or its isoprenoid derivative, farnesyl pyrophosphate (FPP), prevented this increase. The effects of FPP were likely due to itself or its degradation products, as none of its downstream derivatives, GGPP, ubiquinone or cholesterol, were effective. Moreover, the effects of FPP could not be accounted for by protein prenylation, as inhibition of farnesylation did not alter fatty acid synthesis in mevalonate-depleted cells incubated with the isoprenoid. Neither was fatty acid synthesis in these cells altered by inhibition of β-oxidation. Mevalonate depletion increased fatty acid synthase (FAS) mRNA by transcriptional mechanisms, without increasing gene expression of other enzymes involved in fatty acid biosynthesis or of SREBP-1c. The abundance of mature SREBP-2 but not SREBP-1 was increased following mevalonate depletion. FPP prevented the increase in FAS mRNA in mevalonate-depleted cells without altering SREBP-2 activation. Thus, FPP regulates fatty acid synthesis by a mechanism that is likely independent of the SREBP pathway.

The biosynthesis of fatty acids and cholesterol are closely related. Enzymes catalyzing the synthesis and metabolism of both lipids are coordinately regulated by the same transcription factors, the sterol regulatory element binding proteins (SREBP) and the liver X receptors (LXR) (1-3). LXR belong to a superfamily of nuclear hormone receptors that are ligand-activated transcription factors (3). They form obligate heterodimers with retinoid X receptors (RXR), another member of this receptor family, and subsequently bind to specific response elements in regulatory regions of their target genes (3,4). Targets of LXR/RXR activation include several genes involved in cholesterol metabolism (3). In addition, the nuclear receptor heterodimer also induces the transcription of genes involved in fatty acid synthesis by activating another transcription factor, sterol regulatory element binding protein-1c (SREBP-1c) (2). Three SREBPs have been described, SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are products of a single gene through alternative splicing, whereas SREBP-2 is a product of separate gene (1). While SREBP-1c activates genes involved in the synthesis of fatty acids, SREBP-2 preferentially activates genes for cholesterol synthesis and the LDL receptor (1). Ligands for LXR include various oxidized derivatives of cholesterol, which is an end-product of the mevalonate pathway (Figure 1) (5-7). Depletion of cholesterol and its oxysterol derivatives, decreases transcriptional activity of the LXR/RXR heterodimer (8). Addition of mevalonate and oxysterol ligands for LXR restores transcription mediated by the nuclear receptor complex. In contrast, the upstream non-sterol isoprenoid derivative of mevalonate, geranylgeranyl pyrophosphate (GGPP), and its alcohol derivative,
geranylgeraniol (GGOH), inhibit the activity of the LXR/RXR heterodimer by interfering with LXR activation (8,9). As a result, GGPP decreases transcription of an LXR target gene, the ATP binding cassette protein A1 (ABCA1), that is involved in cholesterol efflux from cells (9). It is not known whether other target genes of LXR, such as enzymes involved in fatty acid biosynthesis, are similarly altered by non-sterol isoprenoid derivatives of mevalonate.

Depletion of both sterol and non-sterol derivatives of mevalonate by inhibition of HMG-CoA reductase has previously been observed to enhance fatty acid and triglyceride synthesis (10,11). Although this was accompanied by increased expression of some of the genes involved in fatty acid synthesis, it is unclear whether the underlying mechanism involved increased activation of SREBP-1c. Because inhibition of mevalonate synthesis would limit the availability of cholesterol and its oxidized derivatives, enhanced lipogenesis under such conditions cannot be due to increased ligand-mediated activation of LXR. In fact under such conditions LXR activation and transcription of SREBP-1c would be decreased. We, therefore, postulated that the effect of HMG-CoA reductase inhibition on increasing fatty acid synthesis is independent of SREBP-1c and is due to limited availability of non-sterol isoprenoid derivatives of mevalonate.

It has been suggested that isoprenoids affect cellular processes by multiple mechanisms. Isoprenoids such as farnesyl pyrophosphate (FPP) and GGPP are utilized for prenylating membrane-bound small GTPases, such as members of the Ras superfamily, which play critical roles in cell proliferation and cytoskeletal maintenance (12,13). Isoprenylation of these proteins is necessary for their translocation to the plasma membrane and subsequent activation. Besides serving as substrates for post-translational modification, FPP and GGPP have been reported to regulate the transcription of some of these proteins (14). The mechanisms by which isoprenoids may directly or indirectly affect transcription of proteins are not well understood. In this respect, GGPP has been shown to inhibit the transcription of ABCA1 by acting as a direct antagonist of ligand-mediated activation of LXR (9). However, the mechanism/s by which FPP could alter transcriptional events is/are not defined.

CaCo-2 colon epithelial cells have active lipogenic pathways and thus serve as a model for examining the interaction between isoprenoids and fatty acid synthesis. Using these cells we demonstrate that depletion of mevalonate and all its derivatives, causes a transcriptional-dependent increase in fatty acid synthesis and fatty acid synthase (FAS) mRNA levels. Exogenous mevalonate and its isoprenoid derivative, FPP, prevent this increase whereas other derivatives of mevalonate do not. Gene expression of SREBP-1c and of its other lipogenic targets, acetyl-CoA carboxylase (ACC) and ATP-citrate lyase (ACL) and the abundance of mature SREBP-1 protein remain unaltered. In contrast, the abundance of mature SREBP-2 and the expression of its lipogenic targets genes are enhanced following mevalonate depletion. FPP prevents the increase in fatty acid synthesis and FAS mRNA without altering the activation of SREBP-2. Thus, FPP regulates fatty acid synthesis by a transcriptional mechanism that is likely mediated by FAS and is independent of SREBP.

**METHODS**

**Materials:** Lovastatin, mevalonolactone, isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), FPP, farnesol (FOH), GGPP, MTT, squalene, actinomycin D, and etomoxir were obtained from Sigma-Aldrich (St. Louis, MO). Cholesterol was purchased from Steraloids (NewPort, RI). Lovastatin and mevalonolactone were dissolved in 0.1N NaOH to generate their respective active open acid forms and the pH was adjusted to 7.4 with 0.1N HCl. FTI-277 and ubiquinone were from EMD Biosciences (San Diego, CA). [14C]-Sodium acetate (56.5mCi/mmol) was from Perkin Elmer Life and Analytical Sciences (Boston, MA) and [1-14C]-palmitic acid (50 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Silica gel thin layer chromatography plates (250 microns) were purchased from Analtech (Newark, DE). Monoclonal antibodies to human SREBP-1 and SREBP-2 were purchased from BD Biosciences (San Diego, CA). Monoclonal antibody to human caspase-3 and rabbit polyclonal antibody to lamin A (H102) were from Santa Cruz.
Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody raised against the carboxy terminus of prelamin A was a generous gift from Dr. Scott Kaufmann (Mayo Clinic, Rochester, MN). Anti-mouse IgG-HRP, anit-rabbit IgG-HRP and ECL Western Blotting detection reagents were purchased from Amersham Biosciences (Buckinghamshire, England). SuperSignal West Pico chemiluminescent substrates were from Pierce (Rockford, IL). All other materials were reagent grade.

Cell Culture: CaCo-2 cells, obtained from Dr. F.J. Field (University of Iowa), were maintained at 37°C in 5% CO₂ in T-75 or T-150 cm² flasks in DMEM (Sigma-Aldrich, St. Louis, MO) containing 4.5g/L glucose, 1.5mg/L sodium bicarbonate, 1mM sodium pyruvate, 2mM L-glutamine and 0.1mM non-essential amino acids and supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100µg/mL streptomycin and 0.1mg/mL gentamycin. Cells were sub-cultured in 6-well plates at a density of 0.08 x 10⁶ cells/well. Near confluent monolayers obtained after 5 days in culture were incubated for 24 hours with serum-free M199 containing the various treatments. Appropriate vehicle controls for every treatment were included. Serum was excluded from the incubation media to avoid the effect of exogenous sterols and fatty acids on de novo fatty acid synthesis.

Fatty Acid Synthesis: Cells were pulsed for the last 2 hours of a 24-hour incubation with 1µCi/well of [14C]-sodium acetate. After the incubation, cells were rinsed with PBS and cellular lipids were extracted and saponified as previously described (15). Labeled sterols and free fatty acids were sequentially extracted, with 2 X 3mL of hexane, under alkaline and acidic conditions, respectively. Incorporation of labeled acetate into each fraction was determined by liquid scintillation counting. To ensure that there was no cross-contamination of counts between the two lipid fractions, fatty acid and sterol extracts from a few separate experiments were isolated as above. Total extracts of each lipid fraction were dried under nitrogen, taken up in 100µL chloroform and spotted on thin layer chromatography plates together with fatty acid and cholesterol standards (Nuchek, Elysian, MN). The plates were then eluted in a solvent system of hexanes:ethyl ether:acetic acid, 75:25:1, v/v, and visualized under iodine vapor. Incorporation of labeled acetate into bands corresponding to free fatty acids and cholesterol was determined by scanning the plates using the AMBIS radioisotope detection scanner (Scanalytics, Billerica, MA). Over 95% of counts incorporated into the total sterol or fatty acid extracts was recovered in bands corresponding to the respective lipid.

β-oxidation of palmitic acid: Cells were collected from T-150cm² flasks by trypsinization and counted. One million cells were pre-incubated with 10µM etomoxir in Krebs Ringer bicarbonate buffer in flasks fitted with central wells. After 30 minutes 0.1µCi of 2µM [1-14C]-palmitic acid was added to each flask and 400µL of hyamine hydroxide (Perkin Elmer Life Sciences, Boston, MA) was added to each central well. The total volume of the incubation mixture per flask was 1 mL. Hyamine hydroxide was added to trap released [14C]-CO₂. Flasks were made air-tight with non-vented caps. Following incubation for one hour at 37°C, hyamine hydroxide was transferred to 10mL of Emulsifier Safe liquid scintillation cocktail (Perkin Elmer Life Sciences, Boston, MA), dark adapted overnight and counted by liquid scintillation counting.

Estimation of relative mRNA abundance by Real-Time Reverse Transcriptase PCR (RT-PCR): Total RNA was extracted from cells using TRIZOL reagent (Invitrogen, Carlsbad, CA). DNase-free RNA was reverse transcribed using SuperScript III (Invitrogen, Carlsbad, CA) and then subjected to real-time quantitative PCR using Sybr Green (Applied Biosystems, Foster City, CA) and the appropriate forward and reverse primers on an ABI Prism model 7000 sequence-detection system (ABI, Foster City, CA). Results are expressed relative to control after normalizing to 18S rRNA. Single products for each RT-PCR reaction were obtained. Primers used for the different genes examined are shown in Table 1.

Estimation of protein expression by Western Blotting: Following incubation with the various treatments, cells were rinsed and scraped in PBS and collected by low speed centrifugation. The cell pellet was lysed in 10mM HEPES, pH 7.4 containing 100mM sodium chloride, 5mM EDTA, 5mM EGTA, 1% Triton X100, 0.1% SDS, 0.5%
sodium deoxycholate, 1mM DTT and protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Cells were mechanically disrupted by repeated passage through 27-gauge syringe and needle and centrifuged for 5 minutes at 14,000rpm in a tabletop microfuge. Equivalent amounts of the supernatant containing the solubilized proteins were separated by SDS/PAGE on 15% gels (for the detection of caspase-3) or 7.5% gels (for all other proteins) and transferred to PVDF membranes. For detection of prelamin A proteins were transferred to nitrocellulose membranes. PVDF membranes were blocked for 1 hour in blocking buffer (TBS containing 5% non-fat milk and 0.05% Tween-20). Nitrocellulose membranes were blocked in TBS containing 10% non-fat dry milk and 0.05% Tween-20. Membranes were then incubated for an additional hour with anti-SREBP-1 (diluted 250-fold), anti-SREBP-2 (diluted 250-fold), anti-lamin A (diluted 200-fold) or anti-caspase-3 (diluted 100-fold) antibodies dissolved in blocking buffer containing 5% milk. For the detection of prelamin A nitrocellulose membranes were incubated with blocking buffer containing anti-prelamin A antibody diluted 500-fold. Membranes were washed six times for five minutes each with TBS containing 0.05% Tween-20. They were then incubated for 1 hour with their respective secondary antibodies diluted 500-fold in blocking buffer containing 5% milk. After washing six times with TBS containing 0.05% Tween-20, the membranes were exposed to HRP substrates for 1 hour. SuperSignal West Pico substrate detection kit was used for the detection of SREBP proteins and ECL detection kit was used for the other proteins. All incubations and washes were performed at 37°C. Exposures to Blue sensitive autoradiography X-ray films (Midwest Scientific, St. Louis, MO) were between 1-30 minutes. X-ray films were scanned and the intensity of bands was determined using the image processing and analysis software, Image J (1.34s, NIH, USA).

**Determination of FPP:** Cells were incubated for 18 hours with 25µM lovastatin. FPP, 10µM, was added per well for an additional hour in the continued presence of lovastatin. After the incubation cells were washed with PBS, trypsinized and collected by low speed centrifugation. Cells were then extracted with butanol/75mM ammonium hydroxide/ethanol, 1:1.25:2.75, v/v and processed for determination of unesterified FPP mass by HPLC as described previously (16).

**Cell Viability:** The number of viable and non-viable cells remaining after incubation with the treatments was counted by Trypan Blue exclusion. In addition, cell viability was also assessed by the conversion of the tetrazolium compound, MTT, to its colored formazan product by mitochondrial dehydrogenase as described previously (17). Relative to control cells, none of the treatments at the concentrations examined, significantly altered the number of viable cells, mg protein/well or mitochondrial dehydrogenase activity. To evaluate the effect of lovastatin on apoptosis, cells were incubated for 24 hours with either M199 or M199 containing 10µM lovastatin. Following incubation cellular proteins were extracted and separated by SDS/PAGE on 15% gels and immunoblotted for caspase-3.

**Statistical Analyses:** Differences between treatments were assessed for statistical significance using ANOVA followed by Tukey’s T test (18).

**RESULTS**

**Effect of mevalonate-depletion on fatty acid synthesis -** To address the effect of mevalonate and its derivatives on fatty acid synthesis, CaCo-2 cells were depleted of mevalonate by incubation for 24 hours with Lovastatin. Lovastatin is a potent inhibitor of HMG-CoA reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate in the rate-limiting step of the mevalonate pathway (Figure 1). During the last 2 hours of incubation with lovastatin, cells were pulsed with radiolabeled acetate, and the incorporation of the precursor into fatty acids was estimated. Results are shown in Figure 2. Lovastatin caused a dose-dependent increase in fatty acid synthesis (Figure 2A) and, as expected, decreased the synthesis of sterols (Figure 2B). Lovastatin did not alter cell viability, cell number or protein concentration (data not shown). Thus, results were similar whether expressed per mg protein or per well. Neither were these results due to changes in apoptosis. In cells incubated with either medium alone or medium supplemented with lovastatin there was little appreciable
cleavage of caspase-3, an early marker of apoptosis (Figure 2C). In contrast, etoposide, a known inducer of apoptosis, caused breakdown of the enzyme into its active products. Lovastatin was therefore used at 10µM in all subsequent experiments. The incorporation of labeled acetate into fatty acids was linear with increasing times of incubation with the precursor and was greater in cells incubated with lovastatin (Figure 2D). To examine the time-dependent effects of lovastatin on fatty acid synthesis, cells were incubated for increasing times with the inhibitor and the incorporation of labeled acetate into fatty acids was estimated. Results are shown in Figure 3 and demonstrate that fatty acid synthesis increased significantly in cells incubated with lovastatin only after 12 hours of incubation. To confirm that the effects of lovastatin were due to mevalonate depletion, fatty acid synthesis was estimated in cells incubated with lovastatin and increasing concentrations of mevalonate. In results shown in Figure 4, mevalonate prevented the effects of lovastatin on fatty acid synthesis in a dose-dependent manner. At the highest concentration examined, there was no difference in fatty acid synthesis between cells incubated with mevalonate alone and cells incubated with mevalonate and lovastatin. At this concentration, compared to control cells, mevalonate alone caused a modest increase in fatty acid synthesis.

Effect of isoprenoids on fatty acid synthesis - The above results suggest that mevalonate and/or its downstream products are involved in regulating fatty acid synthesis. Oxysterol end-products of the mevalonate pathway are ligands for LXR and can therefore stimulate the expression of LXR target genes such as SREBP-1c and genes involved in fatty acid synthesis (2,5-7). However, as lovastatin caused a marked inhibition in the synthesis of sterols it is unlikely that oxysterol-mediated activation of LXR was responsible for the increase in fatty acid synthesis observed in mevalonate-depleted cells. Instead, these results suggest that derivatives of mevalonate upstream of sterols are involved in fatty acid synthesis. To identify this derivative/s, cells were incubated for 24 hours with lovastatin alone or together with each of the isoprenoid intermediates of the mevalonate pathway. These intermediates were added to cells in concentrations that are equivalent in terms of their conversion to downstream products in the pathway. Incorporation of labeled acetate into fatty acids was then determined. Results shown in Figure 5A demonstrate that none of the immediate downstream derivatives of mevalonate, IPP, or its isomer DMAPP, or GPP, significantly altered lovastatin-induced increase in fatty acid synthesis. In contrast, the longer 15-carbon isoprenoid, FPP, prevented the effect of lovastatin on fatty acid synthesis. Fatty acid synthesis in cells incubated with lovastatin and FPP were not significantly different from cells incubated with vehicle alone. Compared to vehicle alone FPP alone caused a modest decrease in fatty acid synthesis. Similar effects were observed in cells incubated with farnesol (FOH), the alcohol derivative of FPP. As FPP is the precursor for the synthesis of sterols, GGPP, and ubiquinone, the effect of this sesquiterpene could have been due to conversion to any one or more of these products. However, this was found to be unlikely. Incubation with GGPP did not alter fatty acid synthesis in cells incubated with lovastatin. Addition of squalene, the sterol precursor, to mevalonate-depleted cells only modestly decreased fatty acid synthesis compared to cells incubated with lovastatin alone. Incubation with 100µM cholesterol, a concentration significantly higher than what would be produced from 10µM FPP, did not attenuate the effects of lovastatin. If anything, it increased fatty acid synthesis in the presence of lovastatin. Cholesterol, at a lower concentration of 5µM that would be produced from 10µM FPP, did not alter statin-induced increase in fatty acid synthesis (results not shown). Ubiquinone, similar to squalene, caused only a modest decrease in fatty acid synthesis in mevalonate-depleted cells. Thus the effects of FPP were either due to itself, a farnesylated protein or its degradation products.

The negative charge on pyrophosphate esters at physiological pH is thought to limit the entry of isoprenoid pyrophosphates into cells. Specificity of results obtained with incubating cells with these compounds has therefore been questioned. Recent reports, however, demonstrate the that they cross the cell membrane and are metabolically active (14,19). To confirm that isoprenoid pyrophosphates are taken up and incorporated into CaCo-2 cells, cells were pre-incubated for 18 hours with 25µM lovastatin and then incubated for
an additional hour with 10µM FPP. Intracellular accumulation of free, unesterified FPP was then estimated in these cells. Results are shown in Figures 5B. As expected, compared to control cells, lovastatin decreased FPP mass secondary to its inhibition of mevalonate synthesis. When FPP was added to cells incubated with lovastatin, intracellular levels of free FPP significantly increased by 2-fold compared to cells incubated with lovastatin alone. To evaluate whether the FPP that enters cells is incorporated into proteins, the ability of the isoprenoid to prevent the effects of lovastatin on the farnesylation of prelamin A was determined. Following farnesylation, prelamin A is cleaved at its carboxy terminus to yield mature lamin A (20). Inhibition of farnesylation, by either inhibiting the farnesylation reaction or by limiting FPP availability, would prevent this proteolytic cleavage and leave the carboxy terminus of prelamin A intact. Thus, an antibody directed against the carboxy terminus of prelamin A enables its detection under conditions that inhibit its farnesylation (21). Using this antibody, the expression of prelamin A was determined in cells incubated for 24 hours with 10µM lovastatin, conditions which inhibit the synthesis of FPP. Results are shown in Figure 5C. As expected, the expression of prelamin A was determined in cells incubated with lovastatin and FPP. When FPP was added to these cells, however, the amount of prelamin A was reduced to levels that were almost undetectable. In control cells or cells incubated with FPP alone prelamin A was undetectable. These results indicate that exogenous FPP is utilized for farnesylating prelamin A. None of the treatments altered the expression of lamin A. Taken together, the above results demonstrate that exogenous FPP is taken up, retained as such and is functionally active in CaCo-2 cells. These results therefore validate the specificity of effects observed with FPP in these cells.

Effect of farnesylation on fatty acid synthesis - FPP is a substrate for farnesyl transferase which catalyzes the post-translational modification of proteins containing the conserved CAAX consensus sequence by covalent thioester linkage of FPP to the cysteine residue (22,23). As several of these proteins function in various signaling pathways, we questioned whether the effects of FPP were due to farnesylated protein/s. To address this, cells were incubated for 24 hours with lovastatin alone or together with FPP and/or increasing concentrations of a potent peptidomimetic inhibitor of farnesylation, FTI-277. Fatty acid synthesis was then determined. Results are shown in Figure 6A. As observed in Figure 5, the increase in fatty acid synthesis in cells incubated with lovastatin was significantly impaired by addition of FPP. When FTI-277 was added to cells incubated with lovastatin and FPP, the inhibitory effects of FPP were not altered by FTI at any of the concentrations examined. FTI alone modestly decreased fatty acid synthesis. To verify that FTI-277 was effective in inhibiting farnesylation of proteins the expression of prelamin A was determined in cells incubated with the inhibitor. As with the previous experiment shown in Figure 5, the expression of prelamin A was determined using an antibody directed against its carboxy terminus. Results are shown in Figure 6B, and as expected, they demonstrate that the abundance of prelamin A increased with increasing concentrations of FTI-277 indicating that the inhibitor was indeed inhibiting farnesyl pyrophosphate transferase activity. Thus, inhibition of farnesylation in mevalonate-depleted cells was unable to prevent the effects of FPP on fatty acid synthesis. It is therefore unlikely that the effect of FPP could have been due to a farnesylated protein.

Effect of β-oxidation on fatty acid synthesis - FOH has been observed to increase the transcription of PPAR target genes involved in fatty acid oxidation (24). FPP and FOH are inter-convertible and FOH has been previously demonstrated to reverse the effects of mevalonate depletion on Ras isoprenylation (25,26). This is consistent with the observation in the present study that FOH, similar to FPP, inhibited fatty acid synthesis in mevalonate-depleted cells. As the synthesis and oxidative degradation of fatty acids are reciprocally related, it is possible that FPP decreased fatty acid synthesis in mevalonate-depleted cells by increasing its oxidation. To examine this, cells were incubated with lovastatin and/or FPP in the presence or absence of etomoxir, a known inhibitor of carnitine palmitoyl transferase-1 and of mitochondrial β-oxidation of fatty acids (27,28). Fatty acid synthesis was then estimated. Results are shown in Figure 7. As
observed previously the increase in fatty acid synthesis in mevalonate-depleted cells was inhibited by FPP (Figure 7A and B left panel). Addition of etomoxir to cells incubated with lovastatin and FPP did not prevent the inhibitory effects of FPP on fatty acid synthesis (Figure 7B). Etomoxir by itself or in the presence of lovastatin modestly decreased fatty acid synthesis by about 25% (Figure 7A). The efficacy of etomoxir in inhibiting β-oxidation of fatty acids was assessed by measuring the release of [14C]-CO2 from [1-14C]-palmitic acid. After one hour of incubation with etomoxir, the release of labeled carbon dioxide was inhibited by about 60% (3.5 ± 0.48 vs 1.34 ± 0.05 pmoles of [14C]-CO2 /million cells). These results suggest that the effects of FPP were not due to increased β-oxidation of fatty acids.

Effect of mevalonate-depletion on mRNA abundance of genes involved in fatty acid synthesis - Ligand-mediated activation of LXR would not be expected in statin-incubated cells deficient in cholesterol and its oxidized derivatives. Increased fatty acid synthesis in these cells is therefore unlikely to be due to increased expression of SREBP-1c and its lipogenic targets. To examine whether this was true, cells were incubated for 24 hours with lovastatin alone or together with mevalonate or FPP. Total RNA was extracted and the mRNA abundance of ACL, ACC, FAS and SREBP-1c was determined by quantitative real-time RT-PCR. Results shown in Figure 8 are normalized to 18S rRNA and are expressed relative to control values. Of all the genes that were examined, only FAS mRNA was significantly increased by mevalonate depletion (Figure 8A). Compared to control cells, mRNA levels of FAS increased 2-fold following mevalonate depletion. When mevalonate or FPP was added together with lovastatin, the abundance of FAS mRNA decreased to levels that were not different from control values (Figure 8B). Mevalonate or FPP alone did not significantly alter the expression of this gene. SREBP-1c mRNA levels were modestly decreased following mevalonate depletion and remained suppressed even after supplementation with exogenous mevalonate.

The selective increase in FAS but not in ACC and ACL gene expression and the lack of increase in SREBP-1c mRNA suggests that SREBP-1c was not activated in mevalonate-depleted cells. To verify whether this was associated with unaltered levels of the active form of the SREBP-1 protein, the expression of both the precursor 125kDa protein and of the mature cleaved product (65kDa) was estimated in cells incubated with lovastatin in the presence or absence of mevalonate or FPP. The results are shown in Figures 9A and C.

Compared to control cells, in cells incubated with lovastatin the amount of the precursor protein was significantly decreased (Figures 9A and 9C). Addition of mevalonate, but not FPP, together with lovastatin prevented the inhibitory effects of lovastatin. When added alone, mevalonate and FPP had very modest effects on the abundance of the precursor protein. In contrast to the precursor protein, abundance of the mature protein was significantly less (Figure 9A). It was either very modestly increased (Figure 9C bottom left panel) or remained unaltered (Figure 9C bottom right panel) by mevalonate depletion. Addition of either mevalonate or FPP to mevalonate-depleted cells did not alter the amount of mature protein. Similarly, neither mevalonate nor FPP alone affected the abundance of the mature protein.

These results demonstrate that the precursor SREBP-1 protein is more sensitive to mevalonate depletion than the mature protein. It is therefore unlikely that depletion of mevalonate significantly affects the amount of active SREBP-1 protein. This is consistent with the lack of increase in mRNA levels of the SREBP-1c itself and its gene targets, ACC and ACL. Thus, it is unlikely that the regulation of fatty acid synthesis in mevalonate-depleted cells is dependent on SREBP-1c activation and instead is likely due to a selective increase in the expression of the FAS gene.

Effect of mevalonate-depletion on SREBP-2 activation - Recently, the FAS gene was shown to be one of several targets of SREBP-2 (29). SREBP-2 would be expected to be activated under conditions of sterol deficiency. In animals fed a cholesterol-depletion diet, activation of SREBP-2 was associated with increased fatty acid synthesis (30). Thus, it is possible that mevalonate depletion increases fatty acid synthesis by activating SREBP-2 and addition of FPP, a precursor for the synthesis of sterols, restores fatty acid synthesis and FAS mRNA by decreasing SREBP-2 activation. To address this possibility SREBP-2
protein expression was estimated in cells incubated for 24 hours with lovastatin in the presence or absence of mevalonate or FPP. Cells were lysed and the precursor and mature forms of SREBP-2 protein determined by immunoblotting. Results are shown in Figures 9B and D. The abundance of the precursor protein was significantly less than that of the mature protein (Figure 9B). Mevalonate depletion decreased the amount of precursor protein in the presence of methanol, the vehicle in which FPP was delivered to cells (Figures 9B bottom panel and 9D top right panel). FPP did not prevent this decrease. In comparison to control cells, FPP alone did not alter the amount of precursor protein. The expression of precursor SREBP-2 did not appear to change with lovastatin, mevalonate or both in the absence of methanol (Figures 9B top panel and 9D top left panel). In contrast to the precursor protein, the amount of mature SREBP-2 was dramatically increased following mevalonate depletion (Figures 9B and 9D bottom panels). Addition of mevalonate, but not FPP, prevented this increase. The amount of mature SREBP-2 was not significantly different between cells incubated with FPP and lovastatin and cells incubated with lovastatin alone. Moreover, the amount of mature protein in cells incubated with lovastatin and FPP remained significantly greater than the amount of protein in control cells. Neither mevalonate nor FPP alone altered the abundance of the mature protein. Thus, FPP does not prevent the increase in amount of mature active SREBP-2 in mevalonate-depleted cells.

These results suggest that FPP does not interfere with the increased transcription of SREBP-2 target genes in mevalonate-depleted cells. To verify whether this was true the mRNA levels of four SREBP-2 target genes, HMG CoA synthase, HMG CoA reductase and squalene synthase were examined in cells incubated with lovastatin alone or in the presence of mevalonate or FPP. The results are shown in Figure 10 (A-D). As expected, mRNA levels of all four gene targets of SREBP-2 were increased in cells incubated with lovastatin consistent with increase in SREBP-2 activation in sterol-depleted cells. Addition of mevalonate, the precursor for sterols, prevented this increase (Figure 10 A-D top panels). Addition of FPP, however, did not alter the effects of lovastatin on HMG CoA synthase, HMG CoA reductase and squalene synthase and only modestly attenuated the effects of lovastatin on FPP synthase mRNA levels (Figures 10 A-D bottom panels). In other experiments FPP synthase mRNA levels, despite being attenuated by about 20% in mevalonate-depleted cells by the addition of FPP, remained elevated at values at least 2-fold greater than control (data not shown). These results are consistent with the effects of FPP on SREBP-2 protein expression in mevalonate-depleted cells. Thus, SREBP-2 activation is probably not involved in FPP-mediated inhibition of fatty acid synthesis and FAS mRNA in mevalonate-depleted cells.

Effect of inhibition of transcription on fatty acid synthesis – In addition to transcriptional regulation, the activity and steady-state mRNA levels of enzymes involved in fatty acid synthesis may be regulated post-transcriptionally (31,32). To address whether the increase in fatty acid synthesis and FAS mRNA in mevalonate-depleted cells could have occurred by post-transcriptional mechanisms, cells were incubated with lovastatin in the presence or absence of the general inhibitor of transcription, actinomycin D. Fatty acid synthesis and FAS mRNA abundance were then estimated. Inhibition of transcription prevented the increase in fatty acid synthesis (Figure 11A) and FAS mRNA (Figure 11B) observed in mevalonate-depleted cells without altering cell viability. Thus, mevalonate-depletion likely enhances fatty acid synthesis and FAS mRNA by transcriptional mechanisms.

DISCUSSION

In cells depleted of mevalonate and all of its derivatives, including oxysterols, fatty acid synthesis was enhanced. This occurred by a mechanism that was likely independent of SREBP-1c activation as neither gene expression of SREBP-1c itself nor of its targets, ACC and ACL was increased in mevalonate-depleted cells. In fact, compared to control cells, in cells incubated with lovastatin there was a modest decrease in SREBP-1c mRNA. This was accompanied by decreased levels of SREBP-1 precursor protein levels. The amount of the mature protein, however, remained unaltered suggesting a lack of increase in SREBP-1 activation. The difference in
response of the mature and precursor protein to mevalonate depletion is likely due to the differential regulatory mechanisms of SREBP-1 activation. Both transcriptional and post-transcriptional regulation of SREBP proteins has been described (1). Lack of cholesterol availability in mevalonate-depleted cells would inhibit oxysterol-mediated activation of LXR and LXR-dependent gene transcription of SREBP-1c. This would lead to decreased synthesis of SREBP-1c protein. This is consistent with a lower amount of precursor SREBP-1 protein in mevalonate-depleted cells. As considerably more precursor than mature protein was detected, mevalonate depletion decreased total SREBP-1 protein levels as well (data not shown). In contrast, post-transcriptional mechanisms increase the amount of mature active SREBP-1 protein under conditions of sterol deficiency. Depletion of mevalonate and sterols would promote sterol sensitive SREBP cleavage-activating protein (SCAP)-mediated escort of SREBP-1 from the endoplasmic reticulum to the Golgi where cleavage of the precursor SREBP-1 protein to its mature isoform occurs. While the amount of mature protein did not increase in cells incubated with lovastatin, the relative amount of precursor protein that was cleaved to its mature isoform was significantly greater in these cells as compared to control cells. Thus, post-transcriptional regulation was likely responsible for maintaining the amount of mature protein despite decreased SREBP-1 precursor and total protein levels in cells depleted of mevalonate. The lack of increase in mature SREBP-1 protein in mevalonate-depleted cells compared to control cells was consistent with the inability of mevalonate depletion to cause a consistent increase in gene expression of all the lipogenic targets of SREBP-1c examined. Only FAS mRNA levels were increased in cells incubated with lovastatin whereas mRNA levels of other enzymes, ACC and ACL, remained unaltered. In a previous study incubation with a 5-fold greater concentration of a statin than was used in the present study significantly decreased SREBP-1c mRNA levels as well as SREBP-1 precursor and mature protein levels (33). Our results, although following the same trend as this previous study, were less dramatic and this is probably due to the differences in concentrations of statins used. It is possible that at these high concentrations, statins would suppress SREBP-1c gene transcription and protein synthesis enough to decrease the amount of mature protein as well. Even if high doses of statin were to similarly affect CaCo-2 cells and decrease SREBP-1c activation, it is unlikely that they would decrease fatty acid synthesis as well. In results not shown, fatty acid synthesis compared to control cells was increased in cells incubated with lovastatin at concentrations 10-fold greater that what we report in the present study.

Unlike other enzymes in the fatty acid biosynthetic pathway, such as ACC, which are regulated both transcriptionally and post-transcriptionally, FAS is primarily regulated at the level of gene transcription (31,32). The induction in FAS mRNA in refed animals was inhibited by an inhibitor of gene transcription and was shown to be due to increased promoter activity of the FAS gene (32). Consistent with this observation, we observed that the increase in FAS mRNA and fatty acid synthesis following mevalonate depletion was prevented in the presence of an inhibitor of gene transcription. The FAS gene is under tight transcriptional control by several hormonal and nutritional factors many of which recruit SREBP-1c for mediating their effects (31,32). However, regulation of fatty acid synthesis and of FAS gene transcription also occurs independently of SREBP-1 (15,30,34,35). Fatty acid synthesis was still maintained, albeit at levels lower than control, in cells defective in SREBP processing suggesting regulation by factors other than SREBP-1 (34). Similarly, involvement of an SREBP-1-independent mechanism was also demonstrated in the nutritional regulation of the FAS gene. SREBP-1 could only partially account for the increase in FAS gene transcription in fasted rats fed a high glucose diet (35). In the intestinal epithelial cells, an influx of saturated, monounsaturated and polyunsaturated fatty acids all suppressed de novo fatty acid synthesis but only the polyunsaturated fatty acids decreased SREBP-1 protein and SREBP-1c mRNA levels (15). Similarly, dramatic changes in cholesterol flux in the intestine altered fatty acid synthesis by a mechanism that was independent of SREBP-1c (30). It was suggested that regulation of fatty acid synthesis following changes in cholesterol flux involved SREBP-2. This notion is supported by the observation that the FAS gene is a target for
SREBP-2 (29). As SREBP-2 is activated under conditions of sterol deficiency, we examined whether the selective increase in FAS mRNA in cells depleted of mevalonate could have been due to increased activation of SREBP-2. This possibility, however, was ruled out because FPP, which prevented the increase in fatty acid synthesis and FAS mRNA in statin-treated cells, did not alter the increase in gene expression of SREBP-2 targets, HMG CoA synthase, HMG CoA reductase, and squalene synthase. The increase in FPP synthase mRNA in mevalonate-depleted cells was only modestly attenuated by the addition of FPP. Moreover, FPP was unable to prevent the increase in mature SREBP-2 observed in cells treated with lovastatin. This is surprising given that FPP is a precursor for the synthesis of sterols and should therefore have caused a sterol-dependent decrease in the activation of SREBP-2 and the expression of its target genes. However, it is possible that at the concentration used, 10µM, FPP was not sufficiently converted to sterols. This is consistent with the inability of cholesterol to mimic the effects of FPP on fatty acid synthesis in mevalonate-depleted cells. These results strongly suggest an SREBP- and sterol-independent mechanism of regulation by FPP of fatty acid synthesis in mevalonate-depleted cells.

Besides SRE binding sites, other regions in the FAS promoter confer transcriptional regulation. LXR has been shown to bind directly to its response elements located in the promoter region of the FAS gene and activate transcription of the gene even in the absence of SREBP-1c (36). If LXR were mediating the effects of mevalonate depletion on fatty acid synthesis in mevalonate-depleted cells. These results strongly suggest an SREBP- and sterol-independent mechanism of regulation by FPP of fatty acid synthesis in mevalonate-depleted cells.

A unique site in the FAS promoter has been identified to mediate the stimulatory effects of glucose independently of insulin (44). This site, termed the carbohydrate response element, is located upstream of the insulin response sequence and includes a CCAAT box that binds CCAAT enhancer binding protein (C/EBP). Interestingly, statins have been shown to augment the transcription of CCAAT containing genes, such as, TNF-α, IL-12p40 and COX-2, in a (C/EBP)-dependent manner (45,46). Furthermore, similar to the results obtained in the present study, the effects of statins on IL-12p40 could be reversed by mevalonate and isoprenoids but not by sterols (45). As C/EBP is expressed in the intestine (47) and has been shown to promote lipogenesis by increasing the transcription of FAS (48), it is possible that the stimulation of fatty acid synthesis in cells incubated with lovastatin was due to enhanced C/EBP-mediated induction in FAS gene transcription. The mechanism/s by which FPP and/or its metabolites regulate the expression of this transcription factor, its binding to promoter elements in the FAS gene or its interaction with repressors or activators of transcription need to be elucidated.

FOH and to a lesser extent geranylgeraniol, has been shown to induce the activity of PPAR and the transcription of PPAR-responsive genes involved in fatty acid β-oxidation (24). As fatty acid oxidation and synthesis are coordinately regulated in a reciprocal manner, FPP could have prevented
the increase in fatty acid synthesis in statin-treated cells by increasing its oxidation. This possibility was ruled out, as inhibition of mitochondrial β-oxidation of fatty acids did not alter the effects of FPP in mevalonate-depleted cells. This observation is not entirely unexpected. We have previously observed that fatty acids are not significantly oxidized in the gut (49). Unlike the liver, β-oxidation of fatty acids in the intestine is not a significant metabolic pathway for fatty acids and therefore does not likely account for changes in fatty acid synthesis in this organ.

Results from the present study demonstrate that although FPP is a sterol precursor, its effects were not mediated by sterols. Neither were its effects due to a farnesylated protein or its downstream derivative GGPP. Instead, these results suggest that the effects of FPP could have been due to the isoprenoid itself or its degradation products. A previous study demonstrated that FOH suppresses triglyceride synthesis in the liver (50). It was suggested that the effects of the isoprenoid were due to its dicarboxylic acid degradation products (50,51). Whether FPP is degraded in non-hepatic tissues is unknown. Future studies are underway to examine the intracellular metabolism of FPP and to further explore possible mechanisms by which the isoprenoid and/or its metabolites regulate fatty acid synthesis.

Results from the present study demonstrate that besides sterols, the mevalonate pathway also provides another product, the upstream non-sterol isoprenoid, FPP, that has regulatory effects on fatty acid synthesis. To the best of our knowledge, the effects of this isoprenoid on fatty acid synthesis has not been reported so far. We demonstrate that, in contrast to sterols, FPP regulates fatty acid synthesis by a mechanism independent of SREBP but one likely involves transcription of the FAS gene. A systematic study of the FAS promoter will enable the identification of region/s that are regulated by FPP and possibly of transcription factors that mediate the effects of the isoprenoid. We believe that results presented in the present study form the basis for such future studies. Our results suggest that fatty acid synthesis may be differentially regulated by sterol and non-sterol products of this mevalonate pathway and that flux through the pathway could be an important factor in the regulation of lipogenesis.

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FOOTNOTES

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Abbreviations:
ACC, acetyl-Coenzyme A carboxylase; ACL, ATP-citrate lyase; DMAPP, dimethylallyl pyrophosphate; FAS, fatty acid synthase; FOH, farnesol; FPP, farnesyl pyrophosphate; FTI, farnesyl transferase inhibitor; FXR, farnesoid X receptor; GPP, geranyl pyrophosphate; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; HMG-CoA reductase, 3-hydroxy 3-methylglutaryl coenzyme A reductase; IPP, isopentenyl pyrophosphate; LXR, liver X receptor; RXR, retinoid X receptor; SREBP, sterol regulatory element binding protein; TLC, thin layer chromatography.

FIGURE LEGENDS

Figure 1. The Mevalonate Pathway. The synthesis of isoprenoids from acetyl CoA is shown. Enzymes with their respective inhibitors used in the study are shown in italicized and bold characters, respectively. FPTase, farnesyl pyrophosphate transferase and GGPTase, geranylgeranyl pyrophosphate transferase.

Figure 2. Effect of mevalonate-depletion on fatty acid synthesis. Cells were incubated for 24 hours with increasing concentrations of lovastatin. For the last 2 hours of incubation, 1µCi of $[^{14}C]$-acetate was added per well and the incorporation of radiolabeled acetate into fatty acids (A) and total sterols (B) determined as described in Methods. Results are shown as mean±SEM of pmoles of acetate incorporated per well. N=3/treatment. *p<0.05 versus no lovastatin. C. Cells were incubated for 24 hours with M199 alone or medium containing 10µM lovastatin. As a positive control cells were incubated with 50µM etoposide (ETP). Following incubation, cells were lysed and caspase-3 cleavage detected by Western Blotting. Results from one out of two representative experiments are shown. N=3/control or lovastatin. D. Cells were incubated for 24 hours with M199 alone (●) or with 10µM lovastatin (◊). For the last 15, 30, 60 and 120 minutes of the incubation cells were incubated with 1µCi/well of $[^{14}C]$-acetate and the incorporation of labeled acetate into fatty acids was determined. Results from one out of two representative experiments shown as mean±SEM of pmoles of acetate incorporated per well. N=3/treatment, *p<0.05 versus control.

Figure 3. Time-dependent effects of mevalonate-depletion on fatty acid and sterol synthesis. Cells were incubated for increasing times with M199 (●) alone or with 10µM lovastatin (◊) and pulsed with radiolabeled acetate for the last 2 hours of every incubation. The incorporation of acetate into fatty acids was estimated. Results from one out of two experiments are shown as mean±SEM of percent acetate incorporation relative to control at each time point. N=3/treatment, *p<0.05 versus control.

Figure 4. Effect of mevalonate on fatty acid synthesis. Cells were incubated for 24 hours with M199 alone or with 10µM lovastatin and/or increasing concentrations of mevalonate. During the last 2 hours of the incubation cells were pulsed with labeled acetate and the incorporation of acetate into fatty acids determined. Results from one out of two representative experiments are shown and demonstrate mean±SEM of percent incorporation of acetate per well relative to control. Symbols: open bar, -Lovastatin and cross-hatched bar, +lovastatin. N=3/treatment, a: p<0.05 versus –lovastatin for each treatment group, b: p<0.05 vs –lovastatin-mevalonate, c: p<0.05 vs +lovastatin-mevalonate.

Figure 5. Effect of intermediates of the mevalonate pathway on fatty acid synthesis. A: Cells were incubated for 24 hours with 0.1% ethanol and 0.3% methanol in the presence or absence of 10µM lovastatin and/or 30µM IPP, 30µM DMAPP, 10µM GPP, 10µM FPP, 10µM GGPP, 5µM squalene, 100µM cholesterol or 50µM ubiquinone. Radiolabeled acetate was added to cells for the last 2 hours of the incubation and its incorporation into fatty acids determined. Results from one out of at least two experiments are shown as mean±SEM of percent incorporation of acetate
per well relative to control. Symbols: open bar, -lovastatin and cross-hatched bar, +lovastatin. N=3/treatment, a: p<0.05 versus -lovastatin control for each isoprenoid, b: p<0.05 vs vehicle-lovastatin, c: p<0.05 vs vehicle+lovastatin. B: Cells were pre-incubated for 18 hours with M199 alone or with 25μM lovastatin (Lov) before being exposed to 10μM FPP for 1 hour. Following incubation, cells were washed and lipids extracted and processed for estimating FPP mass as described in Methods. Results from one out of three representative experiments are shown as mean ±SEM of pmoles of FPP/well. N=3/treatment, *: p<0.05 vs. Control, **: p<0.05 vs Lov. C: Cells were incubated for 24 hours with 0.3% methanol in the presence or absence or 10μM lovastatin (Lov), 10μM FPP or both lovastatin and FPP together. Following incubation, cellular proteins were extracted, separated by SDS/PAGE and transferred to PVDF or nitrocellulose membranes for the detection, by Western blotting, of lamin A and prelamin A, respectively. Results from one out of two representative experiments are shown. N=3/treatment.

Figure 6. Effects of inhibition of farnesylation on fatty acid synthesis. A. Cells were incubated for 24 hours with 10μM lovastatin alone or together with 10μM FPP and/or increasing concentrations of FTI-277, an inhibitor of farnesyl transferase. Control cells received 0.02% DMSO and 0.3% methanol. Radiolabeled acetate was added to cells for the last 2 hours of the incubation and fatty acid synthesis was estimated as described. Results from one out of three representative experiments are shown as means±SEM of percent incorporation of acetate per well relative to control. N=2/treatment, *p<0.05 versus Control. B: Cells were incubated for 24 hours with FTI-277 at the same concentrations as indicated in A. Control cells received 0.02% DMSO alone. As a positive control cells were incubated for 24 hours with 25μM lovastatin (Lov). Following incubation, cellular proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes and the abundance of prelamin A determined by Western blotting as described in Methods. Results from one out of three representative experiments are shown. N=2/treatment.

Figure 7. Effect of inhibition of beta-oxidation on fatty acid synthesis. Cells were incubated for 24 hours with 10μM lovastatin alone and/or 10μM FPP in the presence or absence of 10μM etomoxir (ETO), an inhibitor of mitochondrial β-oxidation. Control cells were incubated with 0.1% ethanol and 0.3% methanol. During the last 2 hours of incubation, cells were incubated with radiolabeled acetate and its incorporation into fatty acids was estimated. Results from one of two representative experiments are shown as means±SEM of pmoles of acetate incorporated/well (A) and as percent incorporation of acetate per well relative to control (B, left panel) or ETO (B, right panel). Open bar, -lovastatin and cross-hatched bar, +lovastatin. N=3/treatment, a: p<0.05 versus –lovastatin for each treatment, b: p<0.05 versus vehicle–lovastatin, c: p<0.05 versus vehicle+lovastatin.

Figure 8. Effect of mevalonate-depletion and FPP on mRNA levels of SREBP-1c and enzymes involved in fatty acid synthesis. Cells were incubated for 24 hours with 10μM lovastatin (Lov) alone or together with 5mM mevalonate (Mev) (A). Another set of cells was incubated with vehicle alone (0.3% methanol) or with 10μM Lov and/or 10μM FPP (B). Total RNA was extracted and mRNA levels of SREBP-1c, ACC, ACL, and FAS estimated by quantitative real-time RT-PCR. Results from one of three representative experiments were normalized to 18S rRNA levels and expressed as means±SEM of arbitrary units relative to control. N=3/treatment. *p<0.05 versus control, **p<0.05 versus lovastatin alone.

Figure 9. Effect of mevalonate-depletion and FPP on SREBP activation. Cells were incubated for 24 hours with 10μM lovastatin (Lov) alone or together with 5mM mevalonate (Mev) (top panels). Another set of cells was incubated with vehicle alone (0.3% methanol) or with 10μM Lov and/or 10μM FPP (bottom panels). Cells were lysed and precursor and mature SREBP-1 (A) and SREBP-2 (B) proteins were separated by SDS/PAGE and estimated by Western blotting. Representative autoradiograms from one out of two experiments are shown. Intensity of bands corresponding to
the precursor and mature SREBP-1 (C) and SREBP-2 (D) proteins are shown as mean±SEM densities relative to control. * p<0.05 versus control, ** p<0.05 versus lovastatin alone. N=3/treatment.

Figure 10. Effect of mevalonate-depletion and FPP on mRNA levels of HMG CoA synthase, HMG CoA reductase, FPP synthase and squalene synthase. Cells were incubated for 24 hours with 10µM lovastatin (Lov) alone or together with 5mM mevalonate (Mev). Another set of cells was incubated with 0.3% methanol alone or with 10µM lovastatin and/or 10µM FPP. Total RNA was extracted and mRNA levels of HMG CoA synthase (A), HMG CoA reductase (B), FPP synthase (C) and squalene synthase (D) were estimated by quantitative, real-time RT-PCR. Results from one of three representative experiments were normalized to 18S rRNA levels and expressed as mean±SEM of arbitrary units relative to control. N=3. *p<0.05 versus control, **p<0.05 versus lovastatin alone.

Figure 11. Effect of actinomycin D on fatty acid synthesis and FAS mRNA. Cells were incubated for 24 hours with 10µM lovastatin alone or together with 4µM actinomycin D (Act D) in 0.05% ethanol. Control cells received vehicle alone. At the end of the incubation, fatty acid synthesis (A) and FAS mRNA (B) were estimated as described in Figures 2 and 8, respectively. Results from one out of two representative experiments are shown as mean±SEM. Symbols: open bar: -lovastatin; cross-hatched bar: +lovastatin. N=3/treatment. *p<0.05 versus control.

TABLES

| Gene                  | Primers                              | Size (bp) | Accession Number/Reference |
|-----------------------|--------------------------------------|-----------|-----------------------------|
| SREBP-1c              | Forward 5'-GGA GGG GTA GGG CCA ACG GCC T-3' | 80        | S66167 (52)                 |
|                       | Reverse 5'-CAT GTC TTC GAA AGT GCA ATC C-3' |           |                             |
| ACL                   | Forward 5'-CCC AGA CAT GCG AGT GCA G-3' | 81        | NM_001096                   |
|                       | Reverse 5'-TCC AGT GCA TAA TCG AGC AGA-3' |           |                             |
| ACC                   | Forward 5'-GAA GGG CTT ATA TTG CCT ATG AAC TTA AC-3' | 92        | U19822                      |
|                       | Reverse 5'-GGG CAG CAT GAA CTG GAA TT-3'' |           |                             |
| FAS                   | Forward 5'-ACA GGG ACA ACC TGG AGT TCT-3' | 151       | NM_004104                   |
|                       | Reverse 5'-CTG TGG TCC CAC TTG ATG AGT-3' |           |                             |
| HMG-CoA Synthase      | Forward 5'-TTT CCT CTC GTG CCG CTC-3' | 67        | X66435                      |
|                       | Reverse 5'-GCA GTC TCC AGG TCT GTC ACT G-3' |           |                             |
| Gene                        | Forward  | Reverse     | Accession |
|-----------------------------|----------|-------------|-----------|
| HMG-CoA Reductase           | 5’-CAG CTT GTG TGT CCT TGG TAT TAG A-3’ | 5’-GCT GAG CTG CCA AAT TGG A-3’ | NM_000859 |
| FPP Synthase                | 5’-TCT CCC AGA TCG TTA GGG TG-3’ | 5’-TCC CGG AAT GCT ACT ACC AC-3’ | NM_002004 |
| Squalene Synthase           | 5’-GAA ATG CCT TGG CCA CCC-3” | 5’-ACC TTC CGC TTG CCC C-3’ | X69141    |
| 18S rRNA                    | 5’-TAA GTC CCT GCC CTT TGT ACA CA-3’ | 5’-GAT CCG AGG GCC TCA CTA AAC-3’ | K03432    |
Figure 2

A) Fatty Acids

Incorporation of $[^{14}\text{C}]$-acetate x 10^{-3} (pmol/well)

Lovastatin, µM

B) Sterols

Incorporation of $[^{14}\text{C}]$-acetate x 10^{-3} (pmol/well)

Lovastatin, µM
Figure 2

C)

| Size (kDa) | Con | Lov | Etp |
|-----------|-----|-----|-----|
| 37        |     |     |     |
| 25        |     |     |     |
| 20        |     |     |     |
| 15        |     |     |     |
| 10        |     |     |     |
Figure 2

D)

Incorporation of \(^{14}\text{C}\)-acetate \(\times 10^{-3}\) (pmol/well) vs. Minutes

Control
Lovastatin

* denotes significance.
Figure 3

Incorporation of $^{14}$C-acetate (% of Control)

- Control
- Lovastatin
Mevalonate, mM

Incorporation of [14C]-acetate (% of Control)

Figure 4

- Lovastatin
+ Lovastatin
Figure 5

A) Incorporation of $[14C]$-acetate (% of Control)

- Lovastatin
- +Lovastatin

Vehicle
IPP
DMAPP
IPP/DMAPP
GPP
FPP
FOH
GGPP
Squalene
Cholesterol
Ubiquinone
Figure 5

B)
Figure 5 C)

75 kDa

---

Prelamin A

75 kDa

---

Lamin A

Control  Lov  FPP  Lov & FPP
Figure 6

A)
Figure 6

B)

| FTI-277, µM | 0  | 0.01 | 0.1 | 1   | 10  | Lov (25µM) |
|-------------|----|------|-----|-----|-----|------------|
Figure 7

A) Incorporation of \([^{14}\text{C}]-\text{acetate}\) (pmol/well) in Control, FPP, ETO, and FPP & ETO groups with and without Lovastatin treatment.

B) Incorporation of \([^{14}\text{C}]-\text{acetate}\) (% of Control) in Control, FPP, ETO, and FPP & ETO groups with and without Lovastatin treatment.
Figure 9

|       | SREBP-1 |       | SREBP-2 |       |
|-------|---------|-------|---------|-------|
|       | Control | Lov   | Mev     | Lov/Mev |
|       |         |       |         |         |
| Vehicle | Lov | FPP   | Lov/FPP |         |
|       |       |       |         |         |
|       |         |       |         |         |

**A) SREBP-1**

**B) SREBP-2**

- **Control**
- **Lov**
- **Mev**
- **Lov/Mev**

- **Vehicle**
- **Lov**
- **FPP**
- **Lov/FPP**

**Precursor**

**Mature**
Figure 9

C) SREBP-1

![Bar charts showing mean density relative to control for precursor and mature forms of SREBP-1 under different conditions.](http://www.jbc.org/Downloaded_from)
A) HMG CoA Synthase

Figure 10
B) HMG CoA Reductase

Arbitrary Units Relative to Control

Control
Lov
Mev
Lov & Mev

Vehicle
Lov
FPP
Lov & FPP
C) FPP Synthase

Figure 10
D) Squalene Synthase

![Graph showing arbitrary units relative to control for Squalene Synthase. The graph compares control, Lov, Mev, Lov & Mev, Vehicle, Lov, FPP, Lova & FPP conditions. Significant differences are indicated by asterisks (* and **).]
Regulation of fatty acid synthesis by farnesyl pyrophosphate
Shubha Murthy, Huaxiang Tong and Raymond J. Hohl

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