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Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients

Marit Schneiders, Sander M. Houten, Ronald J.A. Wanders and Hans R. Waterham

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Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients

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Summary

Mevalonic aciduria (MA) and hyper-IgD and periodic fever syndrome (HIDS) are both caused by depressed mevalonate kinase (MK) activity. MK is the first enzyme to follow the highly regulated 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (HMGR), which catalyzes the rate-limiting step in the isoprenoid/cholesterol biosynthesis pathway. In fibroblasts of MA patients, but not of HIDS patients, HMGR activity is elevated under normal growth conditions. This activity is down-regulated when the cells are supplemented with the isoprenoid precursors geraniol, farnesol, or geranylgeraniol, or a mixture of 25-hydroxycholesterol and cholesterol. This indicates that the regulation of the pathway in these cells is not disturbed. The elevated HMGR activity is probably due to a shortage of non-sterol isoprenoid end products, as indicated by normal HMGR mRNA levels in MA fibroblasts. Furthermore, the HMGR activity in MA cells was more sensitive to geranylgeraniol suppression and less sensitive to sterol suppression than the HMGR activity in low density lipoprotein receptor-deficient cells. HMGR activity in MA cells was down-regulated also by addition of its product mevalonate to the culture medium. Indicating that the elevation of mevalonate levels, which are high in MA patients and moderate in HIDS patients, allows the cells to compensate for the depressed MK activity. Indeed, the isoprenylation of Ras and RhoA protein appeared normal in HIDS and MA fibroblasts under normal conditions but showed increased sensitivity toward inhibition of HMGR by simvastatin. Our combined results indicate that MK-deficient cells maintain the flux through the isoprenoid/cholesterol biosynthesis pathway by elevating intracellular mevalonate levels.

Introduction

Mevalonic aciduria (MA, MIM 251170) and hyper-IgD and periodic fever syndrome (HIDS, MIM 260920) are two autosomal recessive disorders both caused by a deficient activity of the enzyme mevalonate kinase (MK, ATP:mevalonate-5-phosphotransferase, EC 2.7.1.36) due to functional significant mutations in the encoding gene (MVK) (1-5). MA is a severe and often fatal multisystemic disease, characterized by psychomotor
retardation, failure to thrive, hepatosplenomegaly, anemia, and recurrent febrile episodes. HIDS is a more benign condition, in which patients suffer, as in MA, from recurrent fever episodes associated with lymphadenopathy, arthralgia, gastrointestinal problems, and skin rash.

MK enzyme activity in MA is usually below detection levels when measured in cultured skin fibroblasts of MA patients (3). In HIDS, however, a residual MK activity ranging between 1 and 7% of the control value can be measured both in fibroblasts and leukocytes from patients (4,6). As a result of the MK deficiency, excretion of mevalonic acid in urine occurs, which correlates with disease activity in both syndromes. The basal level of excreted mevalonic acid is 100- to 1000-fold higher in MA when compared with HIDS and may reach concentrations of over 500 µM in plasma (3,7).

MK is the first enzyme to follow the highly regulated 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGR, EC 1.1.1.34) in the isoprenoid/cholesterol biosynthesis pathway and converts mevalonate into 5-phosphomevalonate. This pathway provides cells with isoprenoids that are vital for diverse cellular processes. The main end products include isoprenylated proteins, heme A, dolichol, ubiquinone-10, isopentenyl tRNAs, and sterols.

Feedback regulation of isoprenoid biosynthesis by cholesterol is achieved predominantly through activation of transcription of genes that govern the synthesis of cholesterol (HMG-CoA synthase and HMGR) and its receptor-mediated uptake from plasma lipoproteins (LDL receptor) (8). This regulation is performed by a class of transcription factors called sterol regulatory element-binding proteins (SREBPs) (9). The SREBPs are conditional positive transcription factors that enhance transcription when sterols are absent but are not required for basal transcription when sterols are present (8). SREBP cleavage-activating protein (SCAP) binds to the regulatory domain of SREBP and is both an escort protein and a sensor of sterols. At high sterol concentrations, SCAP strongly interacts with one of the two Insig proteins, causing the SREBP-SCAP complex to retain in the ER. At low sterol concentrations, this interaction is weakened allowing SCAP to transport SREBP from the ER and activate transcription (10-12). HMGR, which performs the rate-limiting enzyme step in isoprenoid biosynthesis, is also subject to several post-transcriptional regulation mechanisms. These include translational efficiency of the HMGR mRNA and turnover of the HMGR protein (8). The rate of translation of HMGR mRNA is dictated by the cell's demand for non-sterol isoprenoids, whereas the degradation rate of the HMGR protein is regulated by the cell's demand for both sterol and non-sterol isoprenoids (8). The sterols together with one of the Insig proteins bind to the membrane-spanning domain of HMGR, which is a so-called "cholesterol sensing domain", leading to ubiquitination and proteasomal degradation (13). Geranylgeraniol can enhance this sterol-accelerated degradation of HMG-CoA reductase via Insig (14). Farnesol (FOH) also has been implicated to be a non-sterol regulator of HMGR degradation (15-21), however, this is still a matter of debate (14,22). Combined, these different regulatory mechanisms can induce a 200-
fold increase in HMGR protein in response to statins (8), potent competitive inhibitors of HMGR that are used widely to treat atherosclerosis and familial hypercholesterolemia. These drugs block the synthesis of mevalonate and, as a consequence, lower the endogenous synthesis of isoprenoids, including cholesterol. Although the MK enzyme activity in fibroblasts from MA patients is hardly detectable with the standard enzyme assays, the biosynthesis of cholesterol from radiolabeled precursors can be (near) normal in these cells (23-25). Thus, it appears that MA fibroblasts are able to compensate for their defect in MK and that the flux through the cholesterol biosynthesis pathway may be rather normal. This is due to increased activity of HMG-CoA reductase and the LDL receptor pathway in such cells (23,25). It has been reported that the increased activity of HMG-CoA reductase is insuppressible by exogenous LDL cholesterol and can be up-regulated further under cholesterol-free culture conditions (23). We have extended these studies by measuring the effect of non-sterol and sterol end products as well as different inhibitors on HMGR activity and determining the effect of HMGR inhibition on protein isoprenylation in fibroblasts from both MA and HIDS patients. Our data indicate that MK-deficient cells maintain the flux through the isoprenoid biosynthesis pathway by elevating the intracellular mevalonate levels.

Materials and Methods

Materials
HMG-CoA, geraniol (GOH), FOH, geranylgeraniol (GGOH), cholesterol, and 25-hydroxycholesterol were obtained from Sigma. Mevalonolactone, 3-methyl-3-buten-1-ol, and 3-methyl-2-buten-1-ol were obtained from Fluka. Farnesyltransferase inhibitor (FTI-277) and geranylgeranyltransferase inhibitor (GGTI-298) were obtained from Calbiochem. Simvastatin was a gift from Merck Sharp and Dohme, Zaragozic Acid A (ZAA) was a gift from Merck, and Pamidronate was a gift from Novartis. Radiolabeled [14C]HMG-CoA was obtained from Amersham Biosciences. When necessary, the obtained batch was purified further by ethyl acetate extraction. The antibody against Ras was obtained from Transduction Laboratories; the antibody against RhoA was obtained from Santa Cruz Biotechnology.

Cell Culture
Fibroblast cell lines obtained from confirmed MK-deficient patients (HIDS and MA) and a homozygous familial hypercholesterolemia (FHC) patient were cultured in nutrient mixture Ham's F-10 with L-glutamine and 25 mM HEPES (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) or 10% delipidated FCS (Roche Molecular Biochemicals) as indicated. For each experiment, cells were seeded in a T-75 culture flask (Costar) and grown until confluency. Two days prior to a particular treatment, the cells received fresh culture medium. To treat cells, culture medium with the indicated
compounds or, as a control, solvent was added to the cells and the incubation was continued for another 2 days. For enzyme and immunoblot analysis, cells were harvested after trypsinization and washed twice with PBS, and either used directly or snap-frozen in liquid nitrogen and stored at -80°C until use. Mevalonolactone, GOH, FOH, and GGOH were dissolved in ethanol as 250× stock solutions. A 250 mM solution of mevalonic acid was prepared by dissolving the mevalonolactone in a 0.1 N NaOH solution. Pamidronate was dissolved in water as a 10 mM stock solution and both FTI and GGTI were dissolved in DMSO as a 20 mM stock solution. ZAA was prepared as a 50 mM stock solution by dissolving the drug in DMSO. 10 mM simvastatin stock solution was prepared by dissolving the prodrug in pure ethanol, followed by hydrolysis of the lactone by adding 0.1 N NaOH. After neutralization with 50 mM HEPES, pH 7.4, and 0.1 N HCl, the solution was sterilized by filtration through a 0.2-µm filter and stored in aliquots at -20°C (end concentration ethanol 25% (v/v)).

**Quantitative PCR**

The relative expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and HMGR mRNAs were determined using the Lightcycler system (Roche Molecular Biochemicals). For the experiment where the expression levels of HMGR mRNA were related to the expression levels of GAPDH mRNA, total RNA (free of genomic DNA) was isolated with the SV total RNA isolation system (Promega) after which first-strand cDNA was prepared as described by IJlst et al. (26). The relative mRNA expression levels of HMGR and GAPDH were determined using a plasmid containing the corresponding gene as standard. The GAPDH fragment was amplified using the following primer set: GAPDH Fw, 5'-ACC ACC ATG GAG AAG GCT GG-3', and GAPDH Rev, 5'-CTC AGT GTA GCC CAG GAT GC-3'. The HMGR fragment was amplified using primers: HRED Fw2, 5'-TCA AAG GGT ACA GAG AAA CCA C-3', and HRED Rev2, 5'-TAT GCT CCC AGC CAT GGC AG-3'. In every sample the expression of GAPDH and HMGR was determined in duplicate.

For the experiment where the expression levels of HMGR mRNA were related to the expression levels of β-actin mRNA, total RNA was isolated from primary skin fibroblasts using TRIzol (Invitrogen, Carlsbad, CA) extraction, after which complementary DNA (cDNA) was prepared using a first-strand cDNA synthesis kit for RT-PCR (Roche). The β-actin fragment was amplified using primers: β-actin fw, 5'-GGC ACC AGG GCG TGA TGG-3', and β-actin rev, 5'-GTC TCA AAC ATG ATC TGG GTC-3'. In every sample the expression of β-actin and HMGR was determined in duplicate. Data were analyzed using LightCycler software, version 3.5 (Roche) and the program LinRegPCR, version 7.5 (27) for analysis of real-time PCR data.

**HMGR Enzyme Assay**

HMGR was measured essentially as described by Brown et al. (28) with some modifications. The fibroblast pellets were dissolved in HMGR assay buffer containing
100 mM KPi, 200 mM KCl, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, and 10 μg/ml leupeptin (pH 7.1). The cells were disrupted by sonication (twice at 8-watt output, 40 J, at room temperature). One-hundred microliters of the resulting homogenate was preincubated for 10 min at 37 °C with 60 μl of cofactor-mix containing 66.7 mM glucose 6-phosphate, 10 mM NADPH, 16.7 mM EDTA, and 25 units/ml glucose-6-phosphate dehydrogenase. The enzyme reactions were started with the addition of 1.7 nmol of [14C]HMG-CoA and 5.6 nmol of HMG-CoA in 40 µl of H2O. After a 30-min incubation period at 37 °C, reactions were terminated by adding 50 µl of 1.2 N HCl. After 30 min, the product was extracted three times with 2 ml of ethyl acetate. The extracts were evaporated to dryness and analyzed by silica thin layer chromatography using a solvent system toluene:acetone (1:1) dried with Na2SO4. The formed product was quantified by phosphorimaging (Fuji FLA-3000) with the aid of the Aida software package using samples with known amounts of [14C]mevalonate. In every sample, the activity of HMGR was determined in duplicate.

<table>
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<th>Table 1. Relative mRNA expression levels of HMGR and HMGR enzyme activity in control, HIDS, and MA cultured skin fibroblasts</th>
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<td>Relative HMGR expression, 500 x HMGR/GAPDH</td>
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Membrane and Cytosol Separation

Cell pellets were dissolved in hypotonic buffer containing 5 mM Tris-Cl, pH 7.0, 5 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 2 mM EGTA, 2 mM DTT, and Complete protease inhibitor mixture (Roche Molecular Biochemicals). The cells were lysed by sonication (twice at 8-watt output, 40 J, with cooling between the pulse periods). The membranes were separated from the cytosolic fraction by a 30-min ultracentrifugation step in an Airfuge (Beckman, 15 p.s.i., 100,000xg). The supernatant was designated as the cytosolic fraction. The membrane pellet was dissolved in radioimmune precipitation assay (RIPA++) buffer containing 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 mM NaH2PO4, 5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1% sodium deoxycholic acid, 0.1% SDS, 1 mM DTT, and Complete protease inhibitor mixture and homogenized by sonication (once 7-watt output, 40 J). Both fractions were boiled in Laemmli sample buffer and stored at -20 °C.

Immunoblot Analysis

Equal amounts of protein (measured in the sonicated lysates prior to ultracentrifugation) were resolved on a 15% SDS-polyacrylamide gel and transferred
onto nitrocellulose by semi-dry immunoblotting. To verify equal transfer of protein, the blots were stained reversibly with Ponceau S. Membranes were blocked using blocking buffer containing 5% nonfat dry milk and 1% bovine serum albumin in PBS with 0.1% Tween. Membranes were probed with either the Ras antibody (1:1000) or the RhoA antibody (1:1000) in blocking buffer, which was diluted 10 times in PBS with 0.1% Tween. Detection of the antigen-antibody complexes was performed with horseradish peroxidase-conjugated secondary antibody (DAKO) and using the enhanced chemiluminescence kit (ECL, Amersham Biosciences).

![Figure 1. Suppression of HMGR activity by sterols.](image)

**Figure 1.** Suppression of HMGR activity by sterols.
HMGR specific activities in control (C) and two different MA (M1 and M2) fibroblast cell lines were determined after 48 h of culturing in the presence or absence of various mixtures of 25-hydroxycholesterol (25-OH chol) and cholesterol (chol). The error bars indicate 1 S.D.

**Results**

**Elevated HMGR Activity Is Suppressed by Isoprenoid Precursors and Sterols**
In accordance with previous observations by Gibson et al. (23), we observed an elevation of HMGR activity in cultured skin fibroblasts of MA patients. In HIDS fibroblasts, however, HMGR activity was within the normal range (Table I). Gibson et al. (23) also reported that the elevated HMGR enzyme activity in MA fibroblasts could not
be suppressed by exogenous LDL cholesterol and was up-regulated further when the cells were cultured with LDL-cholesterol (lipid) depleted FCS (23). We also observed this up-regulation in MA cells when cultured under lipid-depleted conditions (unpublished data). In addition and similar to data previously shown in normal cells (8,29) and our control cells, we observed efficient down-regulation of HMG activity when MA fibroblasts were grown in the presence of various mixtures of 25-hydroxycholesterol and cholesterol (Fig. 1). This down-regulation was dose-dependent and occurred in fibroblasts of MA patients with different genotypes. These include cells from mildly affected MA patients with mutations resulting in a stable MK with an elevated Km for mevalonate (A334T; M1) and from severe MA patients with mutations mainly affecting the stability of the MK protein (I268T; M2) (30-32).

Next, we tested whether the elevated HMG activity in MA fibroblasts could be suppressed by non-sterol isoprenoids. To this end, MA, HIDS, and control fibroblasts were cultured for 2 days with 60 µM GOG, FOH, or GGOH (Fig. 2A). The treatment with GGOH caused a decrease in HMG enzyme activity in all cell lines, whereas the treatment with FOH caused only a decrease in the MA cell line. GOG was ineffective in all three tested cell lines at this concentration. To determine the effective concentration
Figure 2. Suppression of HMGR activity by isoprenoid precursors.
A, HMGR specific activities in control (C), HIDS (H), and MA (M1) fibroblast cell lines were determined after 48 h of culturing in the presence or absence of 60 µM GOH, FOH, and GGOH. B, HMGR specific activities in control (C) and two different MA (M1 and M2) fibroblasts cell lines were determined after 48 h of culturing in the presence or absence of GOH, FOH, GGOH, and IPOH at different concentrations. The error bars indicate ±1 S.D.

range of GOH, FOH, and GGOH, we cultured MA fibroblasts in the presence of different concentrations of these compounds (Fig. 2B). This showed a dose-dependent decrease of the HMGR enzyme activity for all three compounds. 

GGOH was the most effective in suppressing HMGR enzyme activity and consequently showed significant cytotoxicity at 75 µM leading to death of the cells.
within 2 days. FOH was more effective than GOH. The suppression of HMGR enzyme activity by these non-sterol isoprenoids was fast with an almost maximal effect reached already after 2 h (unpublished data). Similar as the effect of sterols, the effect was observed in cells with different genotypes (A334T; M1, I268T; M2). The suppressive effect of these compounds appeared specific, because the alcohol of the closely related isoprenoid isopentenyl pyrophosphate (IPOH, 3-methyl-3-buten-1-ol) did not have any effect on HMGR activity even up to a concentration of 1 mM (Fig. 2B). These findings indicate that the regulatory mechanisms of the isoprenoid/cholesterol biosynthesis pathway are not disturbed in MA patients.

To determine whether the elevated HMGR activity was due to an increase in HMGR gene transcription, we analyzed HMGR mRNA by quantitative PCR and determined the activity of HMGR in the same sample. This showed that the elevated activity in the MA fibroblasts was not due to a higher transcription rate of the HMGR gene, because HMGR mRNA levels were similar in all cell lines, whereas HMGR activity was elevated only in the MA cell line (Table 1). When, as a control, the same cell lines were cultured under lipid-depleted conditions, however, we observed the expected increase in HMGR mRNA levels indicating that HMGR gene transcription per se is not disturbed in these cells (unpublished data).

Differential Suppression of HMGR Activity in MA and FHC Cells by GGOH and Sterols
The finding of similar HMGR mRNA levels in the MA cells suggest that the elevated HMGR activity in the cells is not due to the sterol-dependent activation of the SREBP pathway but to one of the post-transcriptional non-sterol-dependent regulatory mechanisms. To obtain additional support for this we studied the suppressive effect of various mixtures of 25-hydroxycholesterol and cholesterol and of GGOH on the HMGR activity in MA fibroblasts and in fibroblasts of a homozygous familial hypercholesterolemia (FHC) patient. The latter cell line also exhibits elevated HMGR activity, which is not due to MK deficiency but to a complete deficiency of the LDL receptor, responsible for LDL-cholesterol import. We observed that the suppression of HMGR activity in the MA cell line was more sensitive to supplementation with GGOH (which can only be used as precursor for non-sterol isoprenoids) than in the FHC cell line (Fig. 3A). The reverse was true for supplementation with the sterol mixtures, which showed that the suppression of HMGR activity in the FHC cell line was more sensitive than in the MA cell line (Fig. 3B).

Elevated HMGR activity in MA cells is caused by shortage of non-sterol isoprenoids
We next studied the effect of different enzyme inhibitors of the isoprenoid biosynthesis pathway on the HMGR activity in MA and control fibroblasts. Inhibition of the synthesis of both sterol and non-sterol isoprenoids by pamidronate, an inhibitor of farnesyl pyrophosphate synthase, resulted in an increase in HMGR enzyme activity in the
Figure 3. Differential suppression of HMGR activity in MA and FHC cells by GGOH and sterols. A, suppression of the elevated HMGR activity in MA fibroblasts and in fibroblasts of a homozygous familial hypercholesterolemia (FHC) patient by various concentrations of the non-sterol isoprenoid precursor GGOH. B, suppression of the elevated HMGR activity in MA fibroblasts and in fibroblasts of a homozygous familial hypercholesterolemia (FHC) patient by various mixtures of 25-hydroxycholesterol (25-OH) and cholesterol (Chol). The error bars indicate ±1 S.D.
control cells as well as a further increase in the MA cells (fig 4A). This increase appears to be due to a shortage of non-sterol isoprenoids, because the incubation with ZAA (inhibitor of squalene synthase), which inhibits solely the synthesis of sterol isoprenoids and simultaneously promotes the synthesis of non-sterol isoprenoids, did not have an effect on the HMGR enzyme activity in control cells. Moreover, ZAA incubation resulted in a decrease of the HMGR activity in MA cells. To confirm that elevated levels of non-sterol isoprenoids reduce the increased HMGR enzyme activity in MA cells, these cells were incubated with FTI and GGTI. Because FTI and GGTI are inhibitors of farnesylation and geranylgeranylation of proteins respectively, this incubation will lead to higher levels of FPP and/or GGPP in the cell. Indeed, both inhibitors reduced the HMGR activity in MA cells as efficiently as ZAA. The highest reduction was observed with a combination of the inhibitors (Fig. 4a). FTI had no effect in the control cells, but GGTI caused a small increase in HMGR activity in the control cells.

To determine whether the effects of the different inhibitors on HMGR activity were due to an altered HMGR gene transcription, we analyzed HMGR mRNA levels in the same samples. The marked increase in HMGR enzyme activity due to incubation with pamidronate in both control and MA cells was not due to an increased transcription rate of the HMGR gene, as the HMGR mRNA levels were similar to those of the untreated samples (Fig. 4B). This suggests that the effect of pamidronate is regulated by a post-transcriptional mechanism, which would be consistent with regulation by non-sterol isoprenoids. ZAA incubation resulted in somewhat increased HMGR gene transcription, which could be caused by a small activation of SREBPs due to decreased sterol
Regulation of isoprenoid biosynthesis in MK deficiency

Elevated HMGR activity in MA cells is caused by shortage of non-sterol isoprenoids.

Enzyme activity (A) and relative mRNA expression levels of HMGR (B) in two control (C1 and C2) and two MA (M3 and M4) fibroblast cell lines after 48 h of culturing in the presence 100 µM pamidronate, 50 µM ZAA, 20 µM FTI, 20 µM GGTI, or a combination of 10 µM FTI and 10 µM GGTI. The error bars indicate ±1 S.D.

Biosynthesis. Remarkably, although the HMGR enzyme activity was reduced in MA cells, incubation with GGTI and to a lesser extent the combination of FTI and GGTI led to a marked increase in the transcription rate of the HMGR gene in all cell lines.

Exogenous Mevalonate Suppresses HMGR Activity in MA Fibroblasts

The fact that fibroblasts from MA patients are still capable of synthesizing cholesterol and several other isoprenoids from radiolabeled precursors, like acetate, octanoate, and mevalonate (23-25 unpublished data), implies that MK activity is not entirely deficient in these cells, although it is below detection level in our MK enzyme assay. To test whether exogenous mevalonate is capable of normalizing HMGR enzyme activity in MA fibroblasts, these cells were cultured for 48 h in the presence of different concentrations of mevalonolactone or sodium mevalonate (Fig. 5). This treatment caused a dose-dependent decrease in HMGR enzyme activity. This down-regulation occurred not only in fibroblasts of an MA patient with a mutation affecting the Km of MK for mevalonate (A334T; M1) but also in an MA fibroblast cell line in which the stability of the MK protein is decreased (l268T; M2). Sodium mevalonate was more effective than mevalonolactone in suppressing HMGR enzyme activity. In controls, treatment with mevalonolactone reduced the HMGR activity to undetectable levels.
HIDS and MA Skin Fibroblasts Have Increased Sensitivity for Simvastatin

From our results we hypothesized that MA and HIDS fibroblasts compensate for their depressed MK activity by raising intracellular mevalonate levels. To test this hypothesis we determined the sensitivity of a control, an HIDS, and an MA fibroblast cell line to inhibition of HMGR activity by simvastatin. This was done by culturing these cell lines in the presence of different concentrations simvastatin for 48 h. After incubation, the cells were fractionated into a membrane and a cytosolic fraction, which were subjected to immunoblotting with antibodies against two isoprenylated proteins (Fig. 6). Most isoprenylated proteins function in the membrane and need farnesyI or geranylgeranyl moieties to become associated with the membrane. As a consequence of normal protein turnover, inhibition of HMGR by simvastatin will lead to an increase in non-isoprenylated (and non-functional) proteins in the cytosolic fraction and a decrease in isoprenylated proteins in the membrane fraction (33,34). We used antibodies to Ras, which is a farnesylated protein, and RhoA, which is a geranylgeranylated protein. All fibroblast cell lines cultured in the absence of simvastatin had similar levels of Ras and
Regulation of isoprenoid biosynthesis in MK deficiency

RhoA protein in the membrane fraction, indicating that MA and HIDS fibroblasts are capable of synthesizing isoprenylated proteins as efficiently as control fibroblasts (Fig. 6). Similar results were obtained in cultured lymphoblasts of MA and HIDS patients (unpublished data). It appears that in confluent grown fibroblasts the majority of the RhoA protein is localized in the cytosol fraction, whereas the majority of Ras is localized in the membrane fraction.

Depletion of cellular mevalonate levels induced by simvastatin treatment of the cells showed a marked difference. First, in all cell lines, total RhoA protein levels are increased in response to simvastatin treatment, a previously reported compensatory response to the lowered levels of functional RhoA in the membrane (33-36). In addition, the MA cell line had more RhoA in the cytosolic fraction than the control cell line. Moreover, in MA and HIDS fibroblasts, the increase of Ras (Fig. 6A) and RhoA (Fig. 6B) in the cytosolic fraction occurs at lower concentrations of simvastatin than in controls. The concomitant decrease of RhoA in the membrane fraction is much stronger than for Ras, suggesting a higher turnover rate for RhoA. In fibroblasts from an MA patient an effect was already visible at a simvastatin concentration as low as 8 nM. Also fibroblasts from an HIDS patient were more sensitive to simvastatin than a control cell line. Although both the control and the HIDS cell line start to accumulate RhoA in the cytosol at 40 nM simvastatin, this process is faster in the HIDS cell line. In addition, quantification of the immunoblots revealed that this process was already maximal at 200 nM in the HIDS cell line, whereas in the control cell line it was only half-maximal at this concentration. Thus, the extent of the MK deficiency reflects the sensitivity to simvastatin, with the cells displaying the lowest MK activity (and highest HMGR activity) being the most sensitive.

To study the effect of an increased pathway flux on the sensitivity to simvastatin, MA, HIDS, and control fibroblasts were cultured in lipid-free FCS. The sensitivity to inhibition of HMGR by simvastatin decreased in all cell lines, which most probably reflects the up-regulation of HMGR enzyme activity (Fig. 6B). In MA and HIDS fibroblasts, however, RhoA appears in the cytosolic fraction at lower concentrations of simvastatin than controls. Without addition of simvastatin, lipid-free FCS did not induce an apparent difference in isoprenylation between MA, HIDS and control fibroblast cell lines.

FOH and GGOH Can Rescue Deficient Isoprenylation in MA

FOH and GGOH can be utilized by cells for isoprenoid biosynthesis when added to the culture medium (37-39). We tested whether fibroblasts from an MA patient were also able to use FOH and GGOH for the rescue of protein isoprenylation when HMGR was inhibited by simvastatin. FOH rescued farnesylation of Ras (Fig. 7A) as judged from the decreased level of this protein in the cytosol, whereas GGOH rescued geranylgeranylation of RhoA (Fig. 7B) as judged from the increased level of this protein in the membrane fraction. A combination of both compounds rescued isoprenylation of both proteins. Also the addition of 1 mM mevalonate to the medium rescued
Figure 6. Effect of simvastatin on protein isoprenylation in HIDS and MA skin fibroblasts.

A, immunoblot analysis of Ras in cytosol and membrane fractions of fibroblast lysates (15 µg of protein/lane). The control, HIDS, and MA cell lines were cultured in the presence of different concentrations of simvastatin. The concentrations are indicated in the figure. B, immunoblot analysis of RhoA in cytosol and membrane fractions of fibroblast lysates (12.5 µg of protein/lane and 25 µg of protein/lane, respectively). The control, HIDS, and MA cell lines were cultured in the presence of different concentrations of simvastatin in medium containing FCS and medium containing lipid-free FCS. The concentrations are indicated in the figure. Shown are the results from one representative experiment from three independently performed experiments.
isoprenylation of RhoA, again indicating that MA fibroblasts can use mevalonate when concentrations are sufficiently high (Fig. 7b). In accordance with a rescue of protein isoprenylation, we observed that in response to simvastatin treatment the total RhoA protein levels decreased after treatment with FOH, GGOH, and mevalonate (Fig. 7B).

To verify if the rescue of protein isoprenylation by FOH and GGOH was specific, we tested whether the alcohols of the isoprenoids isopentenyl pyrophosphate and dimethylallyl pyrophosphate (3-methyl-2-buten-1-ol) could also rescue the deficient isoprenylation in the MA cells when HMGR was inhibited by simvastatin. No rescue was observed with any of the tested concentrations or with combinations of these compounds (unpublished data).

**Discussion**

MA and HIDS are both caused by a deficiency of MK enzyme activity but to variable degrees. Despite this deficiency, cholesterol biosynthesis in fibroblasts derived from MA patients can be near normal depending on the culture condition ((23-25), unpublished data). Thus, it appears that MA fibroblasts are able to compensate for their defect in MK. Previously, it has been postulated that this is due to an increased activity of HMGR and the LDL receptor pathway (23-25). HMGR that catalyzes the conversion of HMG-CoA into mevalonate, which is the enzyme step preceding the one catalyzed by MK, is believed to perform the main rate-limiting step in isoprenoid biosynthesis and is among the most highly regulated enzymes in nature (8). The increased activity of HMGR in MA fibroblasts had been reported as insuppressible by exogenous LDL cholesterol and was up-regulated further under cholesterol-free culture conditions (23). This suggests that the LDL receptor pathway is saturated and that the high basal HMGR activity in MA is not due to a shortage of sterol end products. Apparently, the lipoproteins normally present in the FCS provide the cells with sufficient cholesterol, thus preventing SREBP activation. In accordance with this, we found that HMGR mRNA levels in MA fibroblasts are similar to the levels in control cells, indicating that under standard growth conditions the sterol-dependent SREBP pathway, involved in transcriptional regulation, is not activated. We also found that the increased HMGR activity was down-regulated when the medium of MA cells was supplied with FOH, GGOH, sterols, or extra mevalonate. Moreover, we noted that the HMGR activity in an MA cell line was more sensitive to GGOH suppression than the HMGR activity in an FHC cell line, whereas the HMGR activity in the FHC cell line was more sensitive to sterol suppression than the HMGR activity in the MA cell line. Furthermore, we showed that promoting the synthesis of non-sterol isoprenoids by inhibiting enzymes of the isoprenoid biosynthesis pathway with ZAA, FTI and GGTI reduced the elevated levels of HMGR enzyme activity in MA cells. Together, these findings indicate that the regulation mechanisms of the isoprenoid/cholesterol biosynthesis pathway are still functional in MA fibroblasts and that under normal growth conditions one of the non-
sterol-dependent regulatory mechanisms causes the increase in HMGR activity. These mechanisms are post-transcriptional and involve higher mRNA translation efficiency and protein degradation. The role for farnesol and/or FPP as a non-sterol regulator of HMGR protein degradation is still a matter of debate, because several reports show that farnesol can induce degradation of HMGR, while others show the opposite (14-22). GGOH has been implicated to be a non-sterol regulator of HMGR activity, as Sever et al. (14) showed that GGOH can enhance the sterol accelerated degradation of HMGR via Insig. In our experiments, GGOH was more effective than FOH in down-regulating HMGR enzyme activity. Correll et al. (17) reported that FOH was more effective than GGOH in promoting HMGR protein degradation, so the effect by GGOH may be related to mRNA translation efficiency.

**Figure 7. Rescue of deficient protein isoprenylation in HIDS and MA skin fibroblasts by FOH and GGOH.** A, immunoblot analysis of Ras in cytosol and membrane fractions of fibroblast lysates of an MA patient (10 µg of protein/lane and 20 µg of protein/lane, respectively) showing the effect of 20 µM FOH and 20 µM GGOH alone or in combination on the treatment with simvastatin. B, immunoblot analysis of RhoA in cytosol and membrane fractions of fibroblast lysates of an MA patient (10 µg of protein/lane and 20 µg of protein/lane, respectively) showing the effect of 20 µM FOH and 20 µM GGOH alone or in combination on the treatment with 40 nM simvastatin (lanes 2-5) and the effect of 1 mM mevalonate on the treatment with 100 nM simvastatin (lanes 6 and 7). Shown are the results from one representative experiment from three independently performed experiments.
Non-sterol isoprenoid regulation usually occurs at the posttranscriptional level. However, in our experiments, incubation with GGTI reduced the HMGR activity in MA cells, but led to a marked increase in the level of HMGR mRNA in both control and MA cells. A study by Ong et al. (40) also showed an induction in the mRNA of HMGR due to non-sterol isoprenoids in hepatocytes from rats that were treated with FOH for eight weeks. The increase could be due to a compensatory up-regulation of HMGR gene expression following degradation of the enzyme by non-sterol isoprenoids. Another explanation could be that shortage of a certain geranylgeranylated protein leads to an increase in transcription of the HMGR gene via a mechanism that is independent of SREBPs.

The difference in efficacy of mevalonate and mevalonolactone is probably a reflection of the activity of specific hydrolases (esterases) to activate the mevalonolactone in fibroblasts and the different diffusion coefficients of both molecules for crossing the cellular membrane.

As reported for cholesterol biosynthesis from radiolabeled precursors (23-25) unpublished data), we now report that under normal conditions also protein isoprenylation in HIDS and MA fibroblasts is normal. In cells from both disorders, the farnesylated protein Ras and the geranylgeranylated protein RhoA were present in the membrane fraction as shown by cellular subfractionation followed by immunoblotting. From these observations it can be concluded that MA and HIDS cells are able to compensate for reduced MK activity by elevating their intracellular mevalonate levels. This was illustrated also by the fact that addition of extra mevalonate to the medium down-regulated the HMGR activity in MA fibroblasts. This implies that the elevated HMGR activity observed in MA fibroblasts mainly serves to compensate for the leakage of mevalonate (or mevalonolactone) out of the cell. This is inevitable because a higher mevalonate concentration in the cell will lead to an increased leakage. Indeed, MA fibroblasts are more sensitive to simvastatin than HIDS fibroblasts, whereas HIDS fibroblasts are more sensitive to HMGR inhibition than control fibroblasts as demonstrated by the variable accumulation of non-isoprenylated proteins in the cytosol after treatment of cells with different concentrations of statins. Furthermore, this is reflected by the 100- to 1000-fold higher mevalonic acid excretion in MA patients when compared with HIDS patients (3,7).

The elevation of intracellular mevalonate concentrations is expected to promote a normal flux through the isoprenoid/cholesterol biosynthesis pathway in the MK-deficient cells when the following three criteria are met: 1) MK is not saturated with substrate. (When MK is saturated, any elevation in mevalonate levels would have no effect.); 2) HMGR is able to generate mevalonate levels that are high enough for MK to function at a normal rate. (HMGR has to compensate for the leakage of mevalonate out of the cell.); 3) HMGR is not subjected to non-competitive product inhibition. (This is not the case because HMGR is insensitive to any form of product inhibition ((41), unpublished data).) When MA and HIDS fibroblasts are able to compensate largely for their MK
deficiency by elevation of intracellular mevalonate levels, one could imagine a pathogenetic mechanism in which toxic levels of mevalonate are the cause of the observed symptoms in HIDS and MA. Hoffmann et al. (3), however, reported that a trial with lovastatin in two MA patients, used in an attempt to lower their presumed toxic mevalonate levels, resulted in severe clinical crises. This indicates that an excess of mevalonate itself may not be the major pathogenic factor in MA but instead a shortage of one of the isoprenoid end products. In fact, the outcome of this trial illustrates the importance of maintaining elevated mevalonate levels. However, at this moment, it cannot be excluded that few of the symptoms, exclusively observed in the far more severe MA, are induced by toxic mevalonate levels.

In conclusion, our results suggest that supplementation of isoprenoid precursors, such as mevalonate, FOH, and GGOH, may be beneficial in the abortion and prevention of fever episodes in HIDS and MA. Because it is known that, in vitro, FOH and GGOH have substantial cytotoxicity and are able to down-regulate HMGR enzyme activity, studies to the in vivo effects of isoprenoid precursor supplementation will be needed.

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References


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