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Mevalonate kinase deficiency affects isoprenylation and activation of RhoA and Rac1

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Summary

Mevalonate kinase (MK) deficiency is caused by mutations in the MVK gene, resulting in depressed activity of the encoded enzyme MK. MK is an enzyme in the isoprenoid biosynthesis pathway and, although the reduced MK activity in MKD in principle affects the biosynthesis of all isoprenoids, it seems to have in particular an effect on non-sterol isoprenoid biosynthesis. Because small GTPases highly depend on isoprenylation (i.e. geranylgeranylation) for their proper signalling function, we studied the effect of MK deficiency on the isoprenylation and activation of the two small GTPases RhoA and Rac1. Incubation with simvastatin and GGTI-298 inhibited the geranylgeranylation of RhoA and Rac1 in MKD cells due to a shortage of geranylgeranyl pyrophosphate (GGPP). Moreover, the shortage of GGPP resulted in increased activation of RhoA and Rac1. Due to the temperature-sensitivity of MK mutations, isoprenoid biosynthesis in MKD cells became more impaired after incubation of the cells at high temperatures resulting in a reduction of the level of membrane-bound RhoA. Remarkably, incubation of MKD cells for 24 hours at 40°C led to a marked increase in the activation of cytosolic RhoA, while the level of RhoA protein in the cytosol remained more or less similar, suggesting deregulation of RhoA activation in response to high temperatures. The effect of this disturbed (over)activation of RhoA, and possibly other GTPases, on their downstream targets may provide important insights into the pathophysiology underlying the inflammatory episodes in patients with MKD.

Introduction

The isoprenoid biosynthesis pathway provides cells with a variety of sterol and non-sterol isoprenoids that are vital for diverse cellular processes. Important products composed of isoprenoids are heme-A, dolichol, ubiquinone-10, sterols, and isoprenylated proteins. Protein isoprenylation is the posttranslational covalent addition of the isoprenoids farnesyl pyrophosphate or geranylgeranyl pyrophosphate to cysteine residues at the carboxy terminus of proteins (1). Isoprenylation is important for the function of many regulatory proteins such as small G proteins, because it enables their...
membrane localization, which allows the interaction with downstream signalling effectors. The small G proteins are monomeric GTPases of 20-40 kDa (2). The best-known family of these small GTPases is the Ras superfamily, which comprises of five major subfamilies; Ras, Rho/Rac, Rab, Sar1/ARF, and Ran (3). Small GTPases participate in the regulation of a wide variety of cellular functions, including cell cycle progression, morphology and migration, cytoskeletal function, vesicle trafficking, and gene transcription (1-3).

In addition to regulation by isoprenylation, small GTPases also act as molecular switches. They cycle between an active GTP-bound state and an inactive GDP-bound state (4). Activation of small GTPases is controlled by so-called guanine nucleotide exchange factors (GEFs) that catalyse the exchange of GDP for GTP, whereas GTPase-activating proteins (GAPs) release the interaction with downstream effectors by accelerating the hydrolysis of GTP by the small GTPases (5,6).

Among the different enzyme defects of isoprenoid biosynthesis currently known (7), only mevalonate kinase deficiency (MKD; MIM# 251170, 260920) affects the synthesis of all isoprenoids. Mutations in the MVK gene cause a deficiency of mevalonate kinase (MK), the first enzyme following the highly regulated HMG-CoA reductase. Mevalonic aciduria (MA; MIM# 251170) and hyper-IgD and periodic fever syndrome (HIDS; MIM# 260920) represent the severe and mild clinical and biochemical ends of the MKD spectrum (8-12). Patients with MKD typically have recurrent episodes of high fever associated with headache, skin rash, abdominal pain, arthritis, nausea and diarrhea (13,14). MA patients show, in addition to these episodes, developmental delay, dysmorphic features, ataxia, cerebellar atrophy, and psychomotor retardation; and may die during early childhood (13).

Although the MK enzyme activity is hardly detectable in fibroblasts from MA patients, the de novo biosynthesis of cholesterol and protein isoprenylation can be rather normal when these fibroblasts are cultured under normal conditions (15-18). This is due to an increased activity of HMG-CoA reductase, which leads to elevated levels of mevalonate and a rather normal flux through the isoprenoid biosynthesis pathway (15,17,18). However, this flux can easily be disturbed, which follows from the observation that MA fibroblasts are more sensitive to inhibition of HMG-CoA reductase by simvastatin than control fibroblasts (18).

Although the deficient MK enzyme activity in principle affects the biosynthesis of all isoprenoids, there are indications that in particular a temporary shortage (or dysfunction) of one or more geranylgeranylated proteins allows the onset of the fever episodes these patients suffer from (18-21). Indeed, under normal conditions, the flux through the pathway in MKD patients appears sufficiently high for most cellular processes requiring isoprenoids (18,19). However, as we postulated previously, this flux may easily be disrupted by fairly harmless events such as physical stress or vaccinations that lead to a small increase in body temperature (19). Because most mutations in the MVK gene give rise to a temperature-sensitive effect on MK protein
maturation and stability (19,21), this small increase will result in a rapid decrease in MK enzyme activity and, consequently, a block in the isoprenoid biosynthesis pathway leading to a shortage of end products.

Because for their proper signalling function, small GTPases highly depend on isoprenylation and in MKD cells the MK deficiency in particular seems to affect non-sterol isoprenoid biosynthesis (18-21), we studied the effect of MK deficiency on the isoprenylation (i.e. geranylgeranylation) and activation of two small GTPases, RhoA and Rac1. For these studies, we used cultured primary skin fibroblasts from a healthy control and an MA patient, in which the isoprenoid biosynthesis pathway can be readily manipulated by varying the culturing conditions. We studied the effects when these cells were cultured in the presence of simvastatin or GGTI, an inhibitor of geranylgeranyl transferase, or at a higher temperature of 40°C.

Materials and Methods

Cell culture
Primary skin fibroblast cell lines obtained from an MK-deficient patient with the MA presentation and a healthy control individual with a similar passage number were cultured in standard medium, which is nutrient mixture Ham’s F-10 with L-glutamine and 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; Invitrogen, Breda, The Netherlands), supplemented with 10% fetal calf serum (FCS; Invitrogen) and grown until confluency. Subsequently, the medium was replaced with fresh culture medium (2 sets of flasks) supplemented with either 20 µM geranylgeranyltransferase inhibitor (GGTI-298; Calbiochem, La Jolle, CA), 200 ng/ml lipopolysaccharide (LPS; Escherichia coli O55: B5 LPS, Sigma, St. Louis, MO), or different concentrations of simvastatin (a gift from Merck, Sharpe and Dohme BV, Haarlem, The Netherlands) as indicated. The cells were then cultured for two days at 37°C. Cells that were used for the temperature experiment, received medium supplemented with LPS 24 hours prior to the switch to 40°C and were then cultured for the indicated time periods. After incubation, cells of one flask were harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and once with 0.9% NaCl, snap-frozen as pellets in liquid nitrogen, and stored at -80°C to be used later for membrane and cytosol separation (see below). The other flask was used directly for the RhoA and Rac1 activation assays (see below).

Simvastatin was prepared as a 10 mM stock solution as described previously (18). GGTI was prepared as a 20 mM stock solution by dissolving the drug in DMSO (Merck, Darmstadt, Germany) and LPS was prepared as a 10 mg/ml stock solution dissolved in PBS.
Isoprenylation and activation in MK deficiency

RhoA and Rac1 activation assay
RhoA and Rac1 activation assays were performed as described previously (22,23). Briefly, fibroblasts were washed three times with ice-cold PBS, lysed in the flask using lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 10% glycerol, 1% NP-40, 2 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptine, 10 µg/ml aprotinin, 1 mM benzamidin, 1 mM DTT, 1 mM vanadate), and scraped. The lysed cell homogenates were then centrifuged (10 min, 12,000 x g) and the supernatants transferred to 1.5 ml tubes. Equal amounts of protein (400-500 µg in 500 ml) were incubated for 60 min at 4° C with bacterially produced GST-RBD [Rhotekin] (24) (for RhoA pull-downs) or GST-PAK (23) (for Rac1 pull-downs) bound to glutathione-agarose beads (Sigma, ST. Louis, MO). The beads were washed three times with lysis buffer followed by centrifugation (10 sec, 12,000 x g). The bound proteins were eluted by boiling in SDS-sample buffer and analyzed by sodium dodecyl (lauryl) sulfate-polyacrylamine gel electrophoresis (SDS-PAGE).

Membrane and cytosol separation
Membrane and cytosol separation was performed as described previously (18). The amount of protein was measured in the sonicated lysates and adjusted with hypotonic buffer. Equal amounts of protein (1-1.5 mg/ml) were used for ultracentrifugation. The cytosolic and membrane fractions were analyzed by SDS-PAGE.

Membrane and cytosol separation and RhoA activation combination assay
For the combination experiments, fibroblasts were incubated with 0, 0.2, or 0.02 µM simvastatin for 48 hours or at 37° C or 40° C for 24 hours. The fibroblasts were washed three times with ice-cold PBS, lysed in the flask using lysis buffer without 1% NP-40, and scraped. The amount of protein was measured in the sonicated lysates and adjusted with lysis buffer without 1% NP-40. Equal amounts of protein (800-900 µg in 900 µL) were used for ultracentrifugation. The supernatant was transferred to another tube and NP-40 was added (end concentration 1% NP-40). The pellet was dissolved in lysis buffer (with 1% NP-40) and sonicated. Prior to incubation of the supernatant (cytosolic) and pellet (membrane) fractions with GST-RBD [Rhotekin] bound to glutathione-agarose beads for 60 min at 4° C, a sample was taken to determine the distribution of RhoA. The beads were washed three times with lysis buffer followed by centrifugation (10 sec, 12,000 x g). The bound proteins were eluted by boiling in SDS-sample buffer and analyzed by sodium dodecyl (lauryl) sulfate-polyacrylamine gel electrophoresis (SDS-PAGE).

Immunoblot analysis
Proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose by semidry blotting. To verify equal transfer of proteins, each blot was reversibly stained with Ponceau S. prior to incubation with antibodies. Membranes were then incubated with RhoA monoclonal antibody (sc-418) diluted 1:1,000 (Santa Cruz Biotechnology,
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Santa Cruz, CA) or Rac1 antibody (dilution 1:10,000) (Upstate Biotechnology, Lake Placid, NY). Antigen-antibody complexes were visualized with Rabbit anti-mouse horseradish peroxidase conjugate (DAKO, Glostrup, Denmark), using the enhanced chemiluminescence system (Amersham biosciences, Little Chalfont, UK). Densitometric analysis of immunoblots was performed using Advanced Image Data Analyzer (AIDA) software (Raytest, Strauenhardt, Germany).

Statistical analysis
Statistical analysis was performed using one-way analysis of variance [ANOVA] followed by Dunnett’s 2-sided \( t \)-test or a 2-tailed paired \( t \)-test where appropriate. \( P \) values less than 0.05 were considered significant.

Results
Effect of simvastatin on RhoA and Rac1 isoprenylation and activation
Previously, we showed that despite the MK deficiency, fibroblasts from MA patients have normal levels of geranylgeranylated RhoA in their membranes under standard culturing conditions, but that interference at the level of HMG-CoA reductase results in a reduction of membrane-bound RhoA (18). Using the same approach, i.e. blocking the pathway by incubating cells from a healthy control individual and an MA patient with simvastatin, an inhibitor of HMG-CoA reductase, we now studied both the effect on isoprenylation and activation of RhoA and Rac1. Because MKD is an autoinflammatory disorder and previous work indicated that geranylgeranyl groups have an important role in the regulation of the inflammatory response (21), we also incubated the cells with LPS.

![Graphs A and D showing the effect of simvastatin on RhoA and Rac1 isoprenylation and activation](image)
Figure 1. Effect of simvastatin and LPS on localization and activation of RhoA and Rac1 protein. A control and MA cell line were incubated with 0 (white bar), 0.2 (grey bar), or 0.02 µM (black bar) simvastatin for two days in the presence or absence of 200 ng/ml LPS. Relative expression of RhoA (a) and Rac1 (d) protein associated with the membranes. Relative expression of RhoA (b) and Rac1 (e) protein in the cytosol. Relative expression of active RhoA (c) and Rac1 (f). Bars show the mean and SEM of 3 independent experiments. Immunoblots show the results of 1 representative experiment. Statistical analysis was performed using one-way analysis of variance followed by Dunnett’s t-test. * = P < 0.05.

When the control and MA cell lines were cultured in the absence of simvastatin, similar levels of RhoA and Rac1 in the membranes were observed (fig.1a and 1d), confirming our previous observations that in MA fibroblasts, proteins are isoprenylated normally (18). Incubation with simvastatin decreased the amount of RhoA associated with the membrane fraction in control and MA fibroblasts in a concentration-dependent manner. As reported previously (18), MA fibroblasts display a higher sensitivity to
treatment with simvastatin, with decreased levels of membrane-bound RhoA observed after incubation with low concentrations of simvastatin, whereas, the amount of RhoA protein in the cytosolic fraction increased in both control and MA cell lines (fig.1b). The relative increase in the level of cytosolic RhoA is similar for control and MA fibroblasts, although the amount of RhoA present in the cytosol is higher in MA cells as shown by the immunoblot. The presence of LPS did not significantly affect the localization of RhoA.

The levels of membrane-bound Rac1 also decreased upon incubation with increasing simvastatin concentrations, whereas the amount of Rac1 in the cytosolic fraction increased (fig.1d and 1e). However, with respect to Rac1 localization the MA cells do not appear to be more sensitive towards inhibition with simvastatin than control cells (fig.1d and 1e). Remarkably, in contrast to what is observed in the MA cells, no simvastatin-dependent decrease of membrane-bound Rac1 was observed when control cells were incubated with simvastatin in the presence of LPS (fig.1d). Overall, the effects of simvastatin treatment were more pronounced for RhoA than for Rac1.
Incubation with simvastatin leads to an increase in the amount of active RhoA and Rac1 in both normal and MA cells. The relative increase in active RhoA is larger than the relative increase in active Rac1 (fig.1c and 1f). There was no effect of LPS on the activation of RhoA and Rac1.

The effect of GGTI on RhoA and Rac1 isoprenylation and activation
Earlier studies indicated that the effect of simvastatin on the localization of RhoA is due to a shortage of GGPP and can be rescued by the addition of GGOH (18). To confirm that also the observed effect of simvastatin on the activation of RhoA and Rac1 is due to a shortage of GGPP, we studied the effect of GGTI, an inhibitor of geranylgeranyl transferase, on the isoprenylation and activation of RhoA and Rac1.

Our results showed that GGTI had a similar effect as simvastatin on the localization and activation of RhoA and Rac1. Incubation with GGTI led to a reduction in membrane-bound RhoA and Rac1 that was similar for both control and MA fibroblasts (fig.2a and 2d), and increased the level of cytosolic RhoA and Rac1 (fig.2b and 2e). As observed with simvastatin, inhibition by GGTI also led to the activation of RhoA and Rac1 in both the control and MA cells (fig.2c and 2f).
Figure 3. Effect of temperature and LPS on localization and activation of RhoA and Rac1 protein. A control and MA cell line were incubated at 40°C for 0 (white bar), 4 (light grey bar), 8 (dark grey bar), or 24 hours (black bar). Cells received 200 ng/ml LPS 24 hours before the switch to 40°C. Relative expression of RhoA (a) and Rac1 (d) protein associated with the
membranes. Relative expression of RhoA (b) and Rac1 (e) protein in the cytosol. Relative expression of active RhoA (c) and Rac1 (f). Bars show the mean and SEM of 3 independent experiments. Immunoblots show the results of 1 representative experiment. Statistical analysis was performed using one-way analysis of variance followed by Dunnett’s t-test. * = P < 0.05.
**Effect of temperature on RhoA and Rac1 isoprenylation and activation**

Because most mutations in MKD have a temperature-sensitive effect on MK enzyme activity, we previously postulated that fever episodes in MKD will result in a rather instant inhibition of the isoprenoid biosynthesis pathway, leading to a shortage of geranylgeranyl groups, compromising the function of geranylgeranylated proteins (18-20). To study the effect of elevated temperature on protein isoprenylation and activation, we simulated a fever episode by incubation of the cells at 40°C with or without LPS. This incubation had no marked effect in control cells, but clearly decreased the level of membrane-bound RhoA in time in MA cells, especially in the presence of LPS (fig.3a). Furthermore, incubating at 40°C increased cytosolic RhoA in a time-dependent manner (fig.3b).

We observed a small increase in the level of membrane-bound Rac1 in control cells (fig.3d), while cytosolic Rac1 remained similar in time (fig.3e). In MA cells, Rac1 localization was not affected by elevated temperature or LPS.

Incubation for 24 hours at 40°C resulted in a marked increase in active RhoA in MA cells (fig.3c). After 4 hours of incubation at 40°C, a small decrease in active Rac1 was observed in all cells at all conditions (fig.3f).

**Effect of simvastatin and temperature on activation of cytosolic and membrane-bound RhoA**

To determine if the observed induction of active RhoA and Rac1 due to the different culturing conditions relates to activation of cytosolic or membrane-bound RhoA and Rac1 proteins, we studied the effect of simvastatin or high temperatures on the activation of cytosolic and membrane-bound RhoA separately.

When the control and MA cell lines were cultured in the absence of simvastatin, the amount of active RhoA protein in the cytosolic fraction was already much higher in MA cells (fig.4a), while the level of active membrane-bound RhoA was similar in both control and MA cells (fig.4b). Incubation with simvastatin increased active cytosolic RhoA and decreased active membrane-bound RhoA in a concentration-dependent manner in both control and MA fibroblasts. Again, MA cells displayed a higher sensitivity to treatment with simvastatin, with increased levels of active cytosolic RhoA and decreased levels of active membrane-bound RhoA observed after incubation with low concentrations of simvastatin (fig.4a and 4b).

When we studied the effect of incubating the cells at 40°C for 24 hours on the activation of cytosolic and membrane-bound RhoA, we observed no visible effect in control cells. In the MA cells, however, we observed a marked increase in active RhoA protein in the cytosolic fraction (fig.4c). As for simvastatin, incubating at 40°C led to a decrease in the amount of active membrane-bound RhoA in both control and MA cells (fig.4d).
Figure 4. Effect of simvastatin and temperature on activation of cytosolic and membrane-bound RhoA. Immunoblot analysis of active RhoA in cytosol (a) and membrane fractions (b) of a control and MA cell line, incubated with 0, 0.02, or 0.2 µM simvastatin for 2 days. Immunoblot analysis of active RhoA in cytosol (c) and membrane fractions (d) of a control and MA cell line, incubated at 40°C for 24 hours. Each experiment was repeated four times, and showed the same tendency. Shown are data from two representative experiments.

Discussion
Although the reduced MK activity in MKD in principle affects the biosynthesis of all isoprenoids, it seems to have in particular an effect on non-sterol isoprenoid biosynthesis. Yet, even though the MK enzyme activity is hardly detectable in all cells with the MA presentation, protein isoprenylation can be rather normal when these cells
are cultured under normal conditions (18). This is due to increased activity of HMG-CoA reductase, which leads to elevated levels of mevalonate and a rather normal flux through the isoprenoid biosynthesis pathway (15,17,18). Because MA fibroblasts depend on elevated levels of mevalonate to maintain the flux through the pathway, they are more sensitive to simvastatin, an inhibitor of HMG-CoA reductase, the enzyme producing mevalonate (18). Because most isoprenylated proteins function in the membrane and require farnesyI or geranyIgeranyl moieties to become associated with the membrane, inhibition of HMG-CoA reductase by simvastatin also leads to an increase in non-isoprenylated proteins in the cytosolic fractions and a decrease of isoprenylated proteins in the membrane fraction (25,26). In MA fibroblasts, low concentrations of simvastatin already inhibit the pathway, as demonstrated by the substantial reduction in membrane-bound RhoA. Furthermore, although the relative decrease of membrane-bound Rac1 is similar for control and MA fibroblasts, the immunoblot showed less Rac1 in the membrane in MA cells after incubation with simvastatin, confirming the higher sensitivity of MA cells to simvastatin (18). However, the effect of inhibiting the isoprenoid biosynthesis pathway on isoprenylation of Rac1 is less pronounced than for RhoA, which may be due to different turnover rates of these two proteins. Although there are a few studies that show that non-isoprenylated proteins may also have functional effects (27,28), most small GTPases need the isoprenyl moiety to translocate to the membrane to allow binding to their effectors. Therefore, inhibition of the isoprenoid biosynthesis pathway is expected to disturb proper function of the small GTPases.

The observed effect of simvastatin on protein isoprenylation is due to a shortage of geranylgeranyl pyrophosphate and it was previously shown that this shortage can be replenished by the addition of mevalonate or GGOH (18). The experiments with GGTI confirm that also the increased activation of RhoA and Rac1 by simvastatin is the result of a shortage of geranylgeranyl moieties.

The increase in active RhoA due to simvastatin is primarily if not solely caused by activation of cytosolic RhoA and not membrane-bound RhoA. A possible explanation for this could be the difference in the ability of isoprenylated and non-isoprenylated proteins to interact with regulatory proteins. Because the isoprenyl moiety plays a critical role in the interaction with Rho-GDI (29-31), non-isoprenylated GTPases will no longer be inhibited by Rho-GDI and consequently intrinsic nucleotide exchange, i.e. release of GDP and binding of GTP, may occur. Moreover, it has been shown that non-isoprenylated Rac1 and RhoA have no or a much weaker interaction with GEFs (30,32) and GAPs (33) than the isoprenylated forms, implying that also the activation and inactivation of non-isoprenylated GTPases is compromised. Because intracellular GTP levels are higher than GDP levels, binding to GTP will be preferred over GDP (34). Therefore, non-isoprenylated GTPases are likely to accumulate in the GTP-bound form, provided that the rate of nucleotide exchange exceeds the rate of hydrolysis.
The documented effect of inhibitors of protein isoprenylation varies widely in different studies. Our results are in line with studies that showed that mevastatin, GGTL-298, and N-bisphosphonates, inhibitors of FPP synthase, activate Rac in J774 macrophages after 30 hours of incubation (35). Moreover, culturing THP-1 cells for 18 hours with simvastatin or lovastatin activated Rac and Rho proteins in a concentration-dependent manner, whereas incubation with GGTL-298 also led to the activation of Rac (36). Finally, atorvastatin and cerivastatin were shown to increase GTP-bound Rac in HUVEC cells (37). Conversely, other studies have shown that inhibitors of protein isoprenylation negatively regulate the GTP loading of small GTPases. For example, the activity of RhoA is inhibited by statins in monocytes (38), GGTL-298 blocked FCS-stimulated RhoA activation in human mesangial cells (39), and mevastatin significantly blocked LPS-induced activation of Rac in THP-1 cells (40). Reduction in active RhoA was also shown after 6 hours of incubation with fluvastatin in vascular smooth muscle cells (41).

In our study, LPS did not activate RhoA and Rac1, in contrast to other studies where LPS was able to activate Rac1 and RhoA (40,42). The reason for this may be the use of different cell types although in one study activation of Rac1 after LPS incubation was shown in rat-2 fibroblasts (43). In the other studies, LPS-induced activation of Rac1 and RhoA was observed already after an incubation of several minutes, so maybe our incubation of two days is not suitable to see an effect on activation of RhoA and Rac1. Remarkably, however, LPS seemed to have an effect on Rac1 localization in control cells, because the simvastatin-dependent decrease of membrane-bound Rac1 was not seen after co-incubation with LPS. This could indicate that Rac1 has a function in LPS signalling and is specifically recruited to the membrane. There are studies that support this hypothesis. For example, stimulation of Toll-like receptor 2, involved in the recognition and response to Gram-negative and Gram-positive bacterial cell wall components activates and recruits Rac1 to the membrane-bound receptor, leading to the activation of NF-κB (44). In addition, it has been reported that Rac1 plays a role in LPS-induced stimulation of ERK and p38 MAP kinase in fibroblasts (43). Moreover, Rac1 is a member of the NADPH oxidase complex, which is activated after LPS stimulation and recruited to the membrane (45). In MA cells the effect of LPS on localization was not seen, which may be caused by the fact that MA cells do not have enough geranylgeranyl moieties available. It is possible that this decreased ability of Rac1 to localize to membranes contributes to the pathophysiology in MKD.

Previously, we postulated that in MA cells a small raise in temperature will lead to a decrease in MK enzyme activity, due to the temperature-sensitive effect of most mutations on MK protein maturation and stability. When MK has become rate-limiting in these cells, this will lead to an instant disturbance of the pathway flux and consequently affect the isoprenylation of proteins. Here, we confirmed this postulation because elevated temperatures resulted in a reduction of the level of membrane-bound RhoA in MA cells, indicating that isoprenoid biosynthesis is inhibited at high temperatures. The
level of active RhoA bound to the membrane is also decreased, which will most likely disturb proper functioning of RhoA, because most small GTPases need to bind to their effectors and perform their functions in the membrane. Incubating MA cells for 24 hours at 40°C led to a marked increase in the activation of cytosolic RhoA, while the level of RhoA protein in the cytosol remained more or less similar, suggesting deregulation of RhoA activation in response to high temperatures. Hypothetically, this may be due to the fact that the cell tries to compensate for not having sufficient active membrane-bound RhoA to perform its essential function, because of the lack of geranylgeranyl moieties. As it has been reported that non-isoprenylated RhoA also has some functional activity, it is possible that this abnormal (over)activation of RhoA leads to an inflammatory response or prevent down-regulation of the inflammatory response.

In summary, we showed that simvastatin, GGTI-298, and high temperatures inhibit protein isoprenylation in MA cells. Moreover, inhibition of isoprenoid biosynthesis by simvastatin or GGTI-298 results in activation of RhoA and Rac1, whereas incubation with simvastatin or at high temperatures both induced the activity of cytosolic RhoA and decreased the level of active membrane-bound RhoA. In future studies the effect of this disturbed, ectopic activation of these GTPases on their down-stream targets will be studied, which may provide important insights into the pathophysiology underlying the inflammatory episodes in patients with MKD.

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