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Mandey, S.H.L.

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A role for geranylgeranylation in interleukin-1β secretion

Saskia H.L. Mandey*, Loes M. Kuijk*, Joost Frenkel, Hans R. Waterham

Both authors contributed equally to this work

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A role for geranylgeranylation in interleukin-1β secretion

Saskia H.L. Mandey ¹, Loes M. Kuijk ², Joost Frenkel ², Hans R. Waterham¹

¹Laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma Children’s Hospital, Academic Medical Center, University of Amsterdam
²Departments of General Pediatrics and Pediatric Immunology, Division of pediatrics, University Medical Center, Utrecht

Abstract

Objective
Mevalonate kinase deficiency (MKD) is an autosomal recessive disorder characterized by recurring episodes of inflammation. The enzyme mevalonate kinase (MK) catalyzes the phosphorylation of mevalonic acid, which is an early step in isoprenoid biosynthesis. We noticed that peripheral blood mononuclear cells (PBMCs) from MKD patients secrete high levels of interleukin (IL)-1β when stimulated with lipopolysaccharide (LPS). Why a depressed activity of MK leads to inflammation is still unknown, but may be due to a temporary shortage of certain isoprenoid end products and/or the accumulation of mevalonic acid.

Methods
We studied the effect of addition of intermediate metabolites and inhibitors of the isoprenoid biosynthesis pathway on IL-1β secretion by PBMCs from MKD patients and from healthy controls.

Results
We show that inhibition of enzymes involved in geranylgeranyl pyrophosphate (GGPP) synthesis or geranylgeranylation of proteins leads to a marked increase of lipopolysaccharide (LPS)-stimulated IL-1β secretion in peripheral blood mononuclear cells (PBMCs) from control subjects. Furthermore, the increased IL-1β secretion by PBMCs from MKD patients is reversed by supplementation with GGPP as well as with mevalonic acid. IL-1β secretion was increased only when control PBMCs were incubated with excessive amounts of mevalonic acid. Finally, a reduction in IL-1β secretion by MKD PBMCs was also observed when sterol biosynthesis was inhibited favouring non sterol isoprenoid biosynthesis.

Conclusions
Our results indicate that a shortage of geranylgeranylated proteins rather than an excess of mevalonate is likely to cause increased IL-1β secretion by PBMCs of MKD patients.

Introduction

Mevalonate kinase deficiency (MKD) is an autosomal recessive auto-inflammatory disorder characterized by recurring episodes of high fever associated with headache, arthritis, nausea, abdominal pain, diarrhea, and skin rash (1:2). Originally, two distinct syndromes had been defined, classic mevalonic aciduria (MA) (3) and hyper-IgD and periodic fever syndrome (HIDS)(4), which, after the discovery that both
disorders are caused by a deficient activity of the enzyme mevalonate kinase (MK)(5,6), are now recognized as the severe and mild presentation of MKD. Patients with the HIDS presentation typically show the recurrent episodes of fever with associated inflammatory symptoms (1), whereas patients with the MA presentation in addition to these episodes show developmental delay, dysmorphic features, ataxia, cerebellar atrophy, and psychomotor retardation and may die in early childhood (2). Cells of patients with the HIDS presentation show a residual MK enzyme activity of 1-8% (6-8), but in cells of patients with the MA presentation the enzyme activity is below detection level (2,9,10). This difference in residual enzyme activity is also reflected in the occurrence of high levels of mevalonic acid in plasma and urine of patients with the MA presentation and low to moderate levels of mevalonic acid in patients with the HIDS presentation.

Blood analyses during the episodes of fever indicate an acute inflammatory state, with a marked rise in serum levels of pro-inflammatory cytokines, such as interleukin (IL)-6 and interferon gamma (IFN-γ) (11,12). Also, between attacks, isolated peripheral blood mononuclear cells (PBMCs) from MKD patients secrete increased amounts of pro-inflammatory cytokines, such as IL-1β (13,14). IL-1β is the prototypic proinflammatory cytokine and appears to play an important role in the pathogenesis of several autoinflammatory diseases including MKD (15). This is supported by the
MK catalyzes the ATP-dependent phosphorylation of mevalonate to produce 5-phosphomevalonate and is the first enzyme to follow the rate-limiting and highly regulated enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in the isoprenoid biosynthesis pathway (figure 1) (23). The isoprenoid biosynthesis pathway provides cells with several bioactive molecules, including isoprenyl groups, the polypropyl chain of heme A, dolichol and sterols. The isoprenyl groups come in two forms: the farnesyl groups (from farnesylpyrophosphate) and the geranylgeranyl groups (from geranylgeranylpyrophosphate). Both can be attached to proteins of the Ras superfamily. Although the precise molecular mechanism by which a depressed activity of MK leads to increased IL-1β secretion and fever episodes is still unknown, there are indications that it is due to a temporary shortage of certain isoprenylated proteins (24).

Recently, Simon et al. reported the outcome of simvastatin treatment of 6 patients with the HIDS phenotype, which led to shorter periods of fever in most patients (25). The rationale for testing simvastatin in these patients was based on the assumption that the elevated mevalonic acid levels in patients are causing the inflammation. Since statins, such as simvastatin, are competitive inhibitors of HMG-CoA reductase, this treatment will lead to a lowering of mevalonate levels and thus was predicted to reduce inflammation.

In contrast, we previously reported that not the elevated mevalonic acid levels but a shortage of isoprenoid end products contribute to the inflammation in MKD (14:24:26). In order to resolve this apparent discrepancy, we studied the inflammatory response of both MKD and control PBMCs by measuring IL-1β secretion upon stimulation with lipopolysaccharide (LPS) after exposing the cells to a concentration range of mevalonate. Furthermore, we studied the effect of various enzyme inhibitors and intermediate metabolites of the isoprenoid biosynthesis pathway on IL-1β secretion by PBMCs of MKD patients and controls. Our results indicate that increased IL-1β secretion is correlated with a shortage of certain non-sterol isoprenoids rather than elevated mevalonic acid levels.

**Materials and methods**

After approval by the ethical review board and written informed consent by their parents, blood was drawn from MKD patients by venipuncture in sterile pyrogen-free heparinized tubes (Vacuette, Greiner Bio-one). Healthy volunteers served as controls. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep™ according to the supplier’s protocol (Axis-Shield, Oslo, Norway). 1x10^5 cells per well were seeded in 96 well-flat-bottom microtiter plates in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 10 mM HEPES (Gibco) and 1% penicillin-streptomycin. Next, PBMCs were incubated with culture medium containing the indicated compound at 37°C in a humidified atmosphere containing 5% CO₂ in air. After 18 hours of incubation, *E. coli* 055:B5 LPS (Sigma, final concentration of 100 ng/ml) or medium was added to the cultures followed by 24 hours of additional incubation. After this, supernatants were collected and stored at –20 °C until analysis.
Mevalonate was prepared by hydrolyzation of mevalonic acid lactone with 0.1 M NaOH, followed by neutralization and stabilization at pH 7.4 with 1 M HEPES and 0.1 M HCl. Simvastatin and Zaragozic acid A (ZAA) were prepared as previously described (27). Farnesyl transferase inhibitor and geranylgeranyl transferase inhibitor (FTI-277 and GGTI-298, Calbiochem) were also dissolved in DMSO (20 mM). Pamidronate (a gift from Novartis) was dissolved in distilled water (10 mM).

**Cytokine Measurements**

IL-1β concentrations were measured in duplicate in thawed supernatant samples, using commercially available enzyme-linked immunosorbent assays (Pelikine-compact™ human IL-1β ELISA kit, Sanquin Amsterdam, the Netherlands) according to the manufacturer’s instructions.

**Statistic analysis**
P-values were calculated using a Friedman paired nonparametric ANOVA test followed by Dunn’s multiple comparisons test or a two tailed Wilcoxon matched pairs test. All results are expressed as mean ± standard error of the mean.

**Results**

**Effect of inhibition upstream of MK and of mevalonic acid supplementation**

The effect of inhibiting HMG-CoA reductase, which is the enzyme preceding MK, was studied by incubation of PBMCs from MKD patients and control subjects with or without 5 µM simvastatin, followed by stimulation with LPS. In the absence of simvastatin, the stimulation of IL-1β secretion by LPS was higher in MKD PBMCs than in control PBMCs, whereas LPS-stimulated IL-1β secretion was markedly increased after incubation with simvastatin both in control and MKD PBMCs (figure 2A). These results indicate that lowering of the levels of mevalonate and/or downstream isoprenoids, leads to an increase in IL-1β secretion.

To investigate whether elevated mevalonate levels also can be pro-inflammatory, we next determined LPS-induced IL-1β secretion of PBMCs from control subjects and MKD patients when these are exposed to increasing concentrations of mevalonate. Incubation of freshly isolated PBMCs from control subjects with mevalonate concentrations ranging from 0 to 10 mM only resulted in a noticeable increase of LPS-stimulated IL-1β secretion at 5 and 10 mM (figure 2B). When PBMCs of MKD patients were incubated with the same mevalonate concentration range, we observed a marked decrease in IL-1β secretion with increasing mevalonate concentrations (figure 2B).
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Figure 2: The effect of inhibition upstream of MK and mevalonic acid supplementation.
A: IL-1β secretion by PBMCs from 4 controls (shaded bars) and 4 MKD patients (closed bars). Cells were incubated in the absence or presence of simvastatin for 18 hours and stimulated for 24 hours with LPS. B: The effect of mevalonate on IL-1β secretion by PBMCs from controls (shaded bars) and MKD patients (closed bars). Presented are the mean IL-1β concentrations determined in independent incubations using PBMCs obtained from 7 different controls and 3 different MKD patients. Incubations with 10 mM mevalonate were performed in PBMCs from 3 controls and 2 MKD patients. Data are expressed as mean ± standard error of the mean.

Effect of inhibition of enzymes located downstream of MK

The reduced IL-1β secretion observed after mevalonate supplementation supports the hypothesis that the LPS-induced IL-1β secretion by PBMCs from MKD patients is due to a shortage of one of the isoprenoid end products and not to elevated mevalonate levels. To further substantiate this we studied the effect of inhibiting different enzymes located downstream of MK in the isoprenoid biosynthesis pathway. To this end, we incubated control PBMCs with increasing concentrations of pamidronate, which inhibits farnesyl pyrophosphate synthase, the enzyme that catalyzes the formation of geranyl pyrophosphate and farnesyl pyrophosphate (figure 1). This incubation resulted in a significant elevation of the LPS-stimulated IL-1β secretion (figure 3A), confirming that a shortage of isoprenoid end products plays a role in the elevated LPS-stimulated IL-1β secretion.

Both simvastatin and pamidronate inhibit the synthesis of sterol as well as non-sterol isoprenoid end products. Because our previous results indicated that the shortage underlying the symptoms in MK deficiency most probably concerns non-sterol isoprenoid end products (14;24;26), we next studied the effect of increasing concentrations of Zaragozic acid A (ZAA) on IL-1β secretion by control and MKD PBMCs. ZAA is a specific inhibitor of squalene synthase, the first enzyme committed exclusively to sterol isoprenoid biosynthesis. Thus, ZAA will inhibit the synthesis of sterol isoprenoids and, by doing so, promote the synthesis of non-sterol isoprenoids (figure 1). Moreover, the reduction in the synthesis of sterol end products will lead to increased transcription of early isoprenoid biosynthetic genes (23). Consequently, it can be expected that the incubation of MKD PBMCs with ZAA results in a reduced LPS-stimulated IL-1β secretion, which was indeed observed (figure 3B).
A role for geranylgeranylation in IL-1β secretion

The previous experiment pointed to a role for non-sterol isoprenoid end products in IL-1β secretion. The most prominent non-sterol isoprenoids with a known biological function are the farnesyl and geranylgeranyl moieties, derived from farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively. Both can be covalently attached to proteins in a process known as protein isoprenylation. To determine whether the increased IL-1β secretion is due to a shortage of farnesyl or geranylgeranyl moieties, we tested the effect of incubation of control PBMCs with farnesyl transferase inhibitor (FTI) or geranylgeranyl transferase inhibitor (GGTI) on LPS-stimulated IL-1β secretion. This showed that inhibition of geranylgeranyl transferase led to a marked increase in LPS-stimulated IL-1β secretion, whereas addition of FTI did not have a noticeable effect on the secretion of IL-1β (figure 4A). In agreement with these findings, we also found that the increased LPS-stimulated IL-1β secretion observed when control PBMCs are incubated with simvastatin could be completely reversed when the cells were incubated with GGPP (figure 4B). Also, the increased LPS-stimulated increase in IL-1β secretion in PBMCs of MKD patients could be reduced to control levels with GGPP (figure 4C & D).
Discussion

Patients with the HIDS and the MA phenotype experience similar inflammatory episodes despite marked differences in both residual MK activity and accumulating levels of mevalonic acid. This suggests that mevalonate itself has no major effect on the inflammatory response. This is confirmed in our study, in which only a small increase in LPS-stimulated IL-1β secretion is observed when control PBMCs are incubated with 5 mM and 10 mM mevalonate but not at lower concentrations. Because 5 mM mevalonate is 10 times higher than the mevalonate levels that can be found in blood of MA patients and even 1000 times higher than that found in blood of HIDS patients, it seems very unlikely that mevalonic acid is the main cause for the inflammatory response. In fact, the increase in LPS-stimulated IL-1β secretion by
MKD PBMCs is even reversed by the addition of mevalonate. These data are in agreement with previously reported results (26), which showed that MA and HIDS cells compensate for the reduced MK activity by elevating their intracellular mevalonate levels through increasing HMG-CoA reductase activity. This increased HMG-CoA reductase activity was also downregulated when MKD cells were incubated with the isoprenoid precursors farnesol (FOH), geranylgeraniol (GGOH) or mevalonate (26). Thus, in order to maintain the flux through the isoprenoid biosynthesis pathway it seems important to increase the intracellular mevalonic acid levels. Failure of this compensatory mechanism might even lead to increased IL-1β secretion and inflammation, as is suggested by the severe inflammatory crises provoked by lovastatin treatment of two MA patients in an attempt to lower the mevalonic acid levels in these patients (2).

Other studies showed that incubation of control PBMCs with lovastatin or fluvastatin increased IL-1β secretion (14;28;29) and that lovastatin was able to further increase the elevated IL-1β secretion in MKD PBMCs (14). This was also observed in the present study when simvastatin was added to PBMCs from MKD patients and controls. These observations confirm that mevalonate, by itself, is not the cause of the LPS-stimulated hypersecretion of IL-1β by PBMCs of MKD patients.

The results of our study strongly indicate that it most probably is a shortage of a non sterol isoprenoid end product, notably geranylgeranyl groups, that leads to the increased IL-1β secretion in MKD. This is concluded from the fact that inhibition of enzymes involved in GGPP synthesis or geranylgeranylation of proteins, i.e. HMG-CoA reductase, farnesyl pyrophosphate synthase and geranylgeranyl transferase, all led to a marked increase of LPS-stimulated IL-1β secretion by control PBMCs. Moreover, the increased IL-1β secretion in PBMCs from MKD patients could be reversed by supplementation with GGPP, whereas the inhibition of sterol synthesis with ZAA, which promotes the synthesis of non-sterol isoprenoids, also results in a reversal of the LPS-stimulated IL-1β secretion by MKD PBMCs. Together, these findings clearly indicate an important role for non sterol isoprenoids, notably geranylgeranyl groups, in the regulation of the inflammatory response.

There are currently no established therapies for MKD, although treatment with the recombinant form of IL-1 receptor antagonist anakinra was shown to be effective in relieving the symptoms (19). Our results here indicate that raising the availability of geranylgeranyl moieties in MKD, for example by increasing the pathway flux towards non sterol isoprenoid biosynthesis, may provide another option to treat MKD.

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