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Statin synergizes with LPS to induce IL-1β release by THP-1 cells through activation of caspase-1

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Abstract

Mevalonate kinase deficiency (MKD) is a hereditary syndrome characterized by recurring episodes of fever and inflammation. Peripheral blood mononuclear cells from MKD patients secrete high levels of interleukin (IL)-1β when stimulated with lipopolysaccharide (LPS), which is thought to be a primary cause of the inflammation. However, the link between a deficient mevalonate kinase and excessive IL-1β release remains unclear. To investigate this we made use of a model in which monocytic cells (THP-1) were treated with simvastatin. Statins are compounds that inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase and thereby artificially impair the isoprenoid biosynthesis pathway, mimicking mevalonate kinase deficiency.

Our study revealed that LPS-stimulated THP-1 cells treated with simvastatin had an increased caspase-1 mediated processing of proIL-1β. This increased processing was caused by enhanced autoprocessing of caspase-1, rather than enhanced transcription or translation of caspase-1 or proIL-1β. Simvastatin-induced activation of caspase-1 was caused by an impairment of non-sterol isoprenoid biosynthesis, as the isoprenyl intermediate GGPP could block activation of caspase-1 and mIL-1β release. In addition, inhibition of both farnesyl pyrophosphate synthase and geranylgeranyltransferase I also induce mIL-1β release.

Taken together, these results demonstrate that simvastatin augments LPS-induced IL-1β release post-translationally, by inducing caspase-1 activity. These findings suggest that MKD patients may have overactive caspase-1, causing enhanced IL-1β processing and subsequent inflammation in response to bacterial components.

Keywords: Autoinflammatory; Inflammation; Simvastatin; Mevalonate kinase; Interleukin-1β

1. Introduction

The hyperimmunoglobulinemia D and periodic fever syndrome (HIDS; MIM#260920), is an autosomal recessive disorder, characterized by recurrent fever attacks and an elevated level of serum IgD (>100 IU/ml) (Drenth et al., 1994). The febrile attacks are accompanied by painful cervical lymphadenopathy and often by abdominal pain, vomiting and diarrhea. A variety of other symptoms including headache, skin rashes, mucosal ulcers, myalgia and arthralgia may also occur (Frenkel et al., 2001, 2000; Drenth et al., 1994). During the fever episodes an acute phase response is observed, with leukocytosis and elevated acute-phase reactants. Serum levels of proinflammatory cytokines, such as interleukin-6 (IL-6) and interferon-γ (IFN-γ), rise during fever attacks (Drenth et al., 1995a,b). Also, between attacks, isolated peripheral blood mononuclear cells (PBMC) from HIDS patients secrete large amounts of IL-1β (Drenth et al., 1996), which further increases during fever (Drenth et al., 1995b).

In 1999, the genetic defect of HIDS was identified: patients were shown to have mutations in the gene *MVK*, which codes for the enzyme mevalonate kinase (Houten et al., 1999; Drenth et al., 1999). Since this discovery, HIDS and its more severe

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allelic phenotype, mevalonic aciduria, are jointly referred to as mevalonate kinase deficiency (MKD). Mevalonate kinase is an important enzyme in the isoprenoid biosynthesis pathway (Houten et al., 2003). This pathway produces cholesterol and a number of non-sterol isoprenoids. The latter play a vital role in the prenylation of a variety of proteins, mostly of the Ras GTPase superfamily. Recently, it has become apparent that impairment of the isoprenoid pathway has widespread effects on immune function, both anti-inflammatory and pro-inflammatory (Kwak et al., 2000; Ikeda et al., 2000; Martinez-Gonzalez et al., 2001; Sadeghi et al., 2000; Greenwood et al., 2006; Montero et al., 2000). Several studies have shown that the secretion of IL-1β by activated PBMC was greatly augmented by the inhibition of isoprenoid biosynthesis using hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors, also known as statins. This increased cytokine release appeared to be specifically due to a lack of isoprenoids, since the addition of mevalonic acid, the product of HMG-CoA reductase, reduced cytokine production to control levels (Montero et al., 2004, 2000; Frenkel et al., 2002). In PBMC from patients suffering from mevalonate kinase deficiency it is the lack of isoprenoid products, specifically of geranylgeranylated proteins, that raises IL-1β production (Mandey et al., 2006). This IL-1β production may be largely responsible for the inflammation and fever observed in MKD patients. However, it is not known how impaired isoprenoid biosynthesis leads to increased IL-1β release.

Unlike most cytokines, IL-1β is synthesized as an inactive precursor (proIL-1β), lacking a conventional leader sequence. Instead of passing through the endoplasmic reticulum and the Golgi complex, proIL-1β is translated in the cytosol. There, the inactive proform requires processing by caspase-1, which cleaves proIL-1β directly after the aspartic acid residue at position 116 (Thornberry et al., 1992; Cerretti et al., 1992). In addition to IL-1β, caspase-1 can also cleave interleukin-18 (Ghayur et al., 1997; Gu et al., 1997) and recently, interleukin-33 was also identified as a caspase-1 substrate (Schmitz et al., 2005). Caspase-1 itself is synthesized as an inactive zymogen of ~45 kDa that, via induced proximity to another caspase-1 zymogen, can undergo autocleavage, creating 10 kDa and 20 kDa subunits. Two p10 and two p20 subunits form the fully functional heterodimeric enzyme. In analogy to the apoptotic caspase-9 (Acehan et al., 2002), caspase-1 auto-activates itself in a complex of proteins termed the inflammasome (Martinon et al., 2002). Caspase-1 contains an N-terminal caspase recruitment domain (CARD), which forms a homotypic interaction (CARD-CARD interaction) with apoptosis-associated speck-like protein containing a CARD (ASC). This adaptor protein then binds to other members of the inflammasome (Srinivasula et al., 2002) via similar homotypic interactions, enabling oligomerization and autocleavage. Active caspase-1 can then process proIL-1β into mature IL-1β (mIL-1β), which is subsequently secreted. The exact export mechanism of mIL-1β remains unclear.

To investigate the regulation of increased mIL-1β production in mevalonate kinase deficiency, we studied the monocytic cell line THP-1 in which the isoprenoid biosynthesis pathway was artificially impaired using simvastatin. We examined the effect of this impairment on transcription and translation of (pro)caspase-1 and (pro)IL-1β and on caspase-1 enzyme activity. Our study revealed that simvastatin treatment induced an increase in caspase-1 mediated processing of proIL-1β by LPS-stimulated THP-1 cells. This increased processing was caused by enhanced autoprocessing of caspase-1, rather than enhanced transcription or translation of either caspase-1 or proIL-1β. The simvastatin-induced activation of caspase-1 was caused by an impairment of non-sterol isoprenoid biosynthesis, as the isoprenyl intermediate GGPP could completely block activation of caspase-1 and mIL-1β release and the inhibition of geranylgeranyltransferase I enhanced IL-1β release, similar to simvastatin.

2. Materials and methods

2.1. Reagents

Simvastatin, lipopolysaccharide (LPS; E. coli 0127:B8), geranylgeranylpyrophosphate (GGPP) and Actinomycin-D were purchased from Sigma–Aldrich. Simvastatin was prepared by dissolving the prodrug in ethanol, followed by hydrolysis of the lactone by adding NaOH. After neutralization with 1 M HEPES pH 7.4 and HCl the solution was sterilized by filtration through a 0.2 μm filter and stored as aliquots at −20 °C. GGTI-298 was obtained from Calbiochem, benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (z-VAD-FMK) from R&D Systems. Pralnacasan (a specific caspase-1 inhibitor) was a kind gift from Vertex Pharmaceuticals (Boston, U.S.A.). Pamidronate (a specific farnesyl PP synthase inhibitor) was a kind gift from Novartis (Basel, Switzerland).

2.2. Cell culture

THP-1 cells were routinely grown in RPMI 1640 (Life Technologies) containing 2 mM glutamine, 100 U/ml penicillin-streptomycin (RPMI^{++}) and 10% Fetal Calf Serum (FCS). Cells (at a density of 1×10^6/ml) were cultured in 12-well microtiter plates in RPMI^{++}/5% FCS. Incubations were performed at 37 °C in a humidified atmosphere containing 5% CO_2 in air, in the presence or absence of 5 μM simvastatin. After 24 h of incubation, LPS at a final concentration of 200 ng/ml was added to the cells without any other change in the culture medium. The incubations were prolonged for an additional 4 h after which supernatants were removed and either frozen at −20 °C or assayed immediately. Cell pellets were stored at −20 °C or snap frozen in liquid nitrogen and stored at −80 °C for RT-PCR analysis. For the proIL-1β ELISA assay, cell pellets were thawed, taken up in lysis buffer (150 mM NaCl, 20 mM HEPES, 10 mM EDTA, 1% Triton X-100) containing protease inhibitors (Roche) and incubated on ice for 30 min. Cell lysates were centrifuged at 17,000 × *g* for 15 min and the supernatants were used for determination of proIL-β content.

2.3. Cytokine measurements

Cytokine detection was carried out using commercially available ELISA kits: Pelikine-compact^{TM} human IL-1β and IL-6

ELISA kits (Sanquin, Amsterdam), human proIL-1β/IL-1F2 Quantikine ELISA Kit (R&D Systems), human IL-18 module set (Bender MedSystems). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate.

2.4. LDH assay

THP-1 cells were cultured for 24 h in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 h with 200 ng/ml LPS. Simvastatin-mediated effects were blocked by addition of 10 μM geranylgeranylpyrophosphate (GGPP) to the culture medium. After treatment the cells were thoroughly resuspended and 200 μl cell suspension was used for the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate.

2.5. Quantitative real-time RT-PCR analysis

The relative expression levels of IL-1β and caspase-1 to β-actin RNA were measured with the LightCycler® system (Roche, Mannheim, Germany). To this end, total RNA was isolated from THP-1 cells with trizol extraction. First strand cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR (Roche) according to the manufacturer's instructions. The IL-1β fragment was amplified using primers: IL-1β Fw 5′-AGA AGA ACC TAT CTT CTT CGA C-3′ and IL-1β Rev 5′-ACT CTC CAG CTG TAG AGT GG-3′. Caspase-1 primers were: Fw 5′-CTT CCT TTC CAG CTC CTC AG-3′ and Rev 5′-CCT GTG ATG TCA ACC TCA GC-3′. The β-actin fragment was amplified using the following primer set: Fw 5′-GGC ACC AGG GCG TGA TGG-3′ and Rev 5′-GTC TCA AAC ATG ATC TGG GTC-3′. Data were analyzed using LightCycler Software, version 3.5 (Roche) and the program LinRegPCR, version 7.5 (Ramakers et al., 2003) for analysis of real-time PCR data. To adjust for variations in the amount of input RNA, the IL-1β and caspase-1 mRNA levels were normalized against the mRNA levels of the housekeeping gene β-actin.

2.6. Measurement of caspase-1 cleavage

Caspase-1 p20 detection was carried out using a commercially available ELISA kit: Quantikine human caspase-1 immunoassay (R&D Systems) The assay was performed according to the manufacturer's instructions and all samples were tested in duplicate.

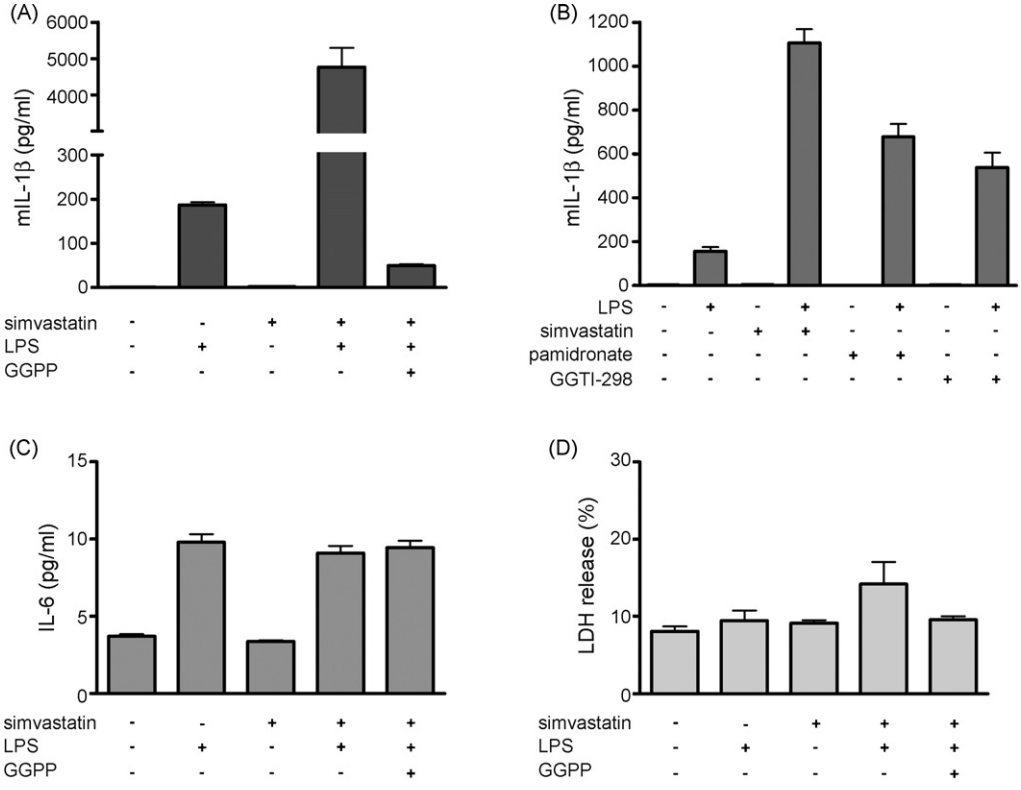

Fig. 1. Simvastatin augments LPS-induced mIL-1β release in THP-1 cells. THP-1 cells were cultured for 24 h in the presence or absence of simvastatin (5 μM), pamidronate (100 μM) or GGTI-298 (50 μM) and stimulated for an additional 4 h with 200 ng/ml LPS. Simvastatin-mediated effects were blocked by addition of 10 μM geranylgeranylpyrophosphate (GGPP) to the culture medium (A). Release of the pro-inflammatory cytokines IL-1β (A and B) and IL-6 (C) were determined by ELISA. Data are represented as means ± S.E.M. ($n = 4$, $n = 3$ and $n = 3$, respectively). Cell viability was determined by an LDH assay (D). Data are represented as means ± S.E.M. of two independent experiments performed in duplicate.

3. Results

3.1. Simvastatin augments LPS-induced mIL-1β release in THP-1 cells

THP-1 cells were cultured for 24 h in the presence or absence of simvastatin and stimulated for an additional 4 h with LPS, after which supernatant was assayed for the presence of mIL-1β. THP-1 cells treated with a combination of simvastatin and LPS displayed dramatically increased mIL-1β release as compared to LPS stimulation alone (Fig. 1A). Incubation with simvastatin alone did not induce detectable mIL-1β release. The addition of GGPP completely inhibited the simvastatin/LPS-induced mIL-1β release. In Fig. 1B inhibitors for two other enzymes of the isoprenoid biosynthesis pathway were included: geranylgeranyltransferase I (GGT I) is the enzyme that attaches GGPP to the target protein and farnesyl pyrophosphate synthase, which is the enzyme just prior to GGT I in the pathway (Houten et al., 2003). When THP-1 cells were incubated with these inhibitors IL-1β release was enhanced, similar to simvastatin, indicating that increased IL-1β release was specifically caused by a shortage of geranylgeranylpyrophosphate. Simvastatin treatment affected only IL-1β release, since there was no effect on release of the proinflammatory cytokine IL-6 (Fig. 1C). In addition, the enhanced IL-β release in the presence of simvastatin/LPS was not due to passive 'leakage' of IL-1β, since there were no major changes in lactate dehydrogenase release, indicating that the cell membrane remained intact during this treatment (Fig. 1D).

3.2. Simvastatin-induced mIL-1β release is caspase-1 dependent

Since IL-1β is known to be a substrate of caspase-1 (Cerretti et al., 1992; Thornberry et al., 1992; Gu et al., 1997; Ghayur et al., 1997) it is likely that caspase-1 is actively involved in simvastatin-induced mIL-1β release. To test this hypothesis, THP-1 cells were cultured as before in the presence or absence of simvastatin. Prior to LPS stimulation either a general caspase inhibitor (Z-VAD-FMK) or the specific caspase-1 inhibitor pralnacasan was added to the culture medium at the indicated concentrations. After 4 h mIL-1β levels were determined. Both the general caspase inhibitor and the specific caspase-1 inhibitor inhibited mIL-1β release in a dose-dependent manner (Fig. 2). Addition of 10 μM inhibitor reduced mIL-1β release to levels comparable to LPS stimulation alone. These data indicate that simvastatin-induced mIL-1β release requires caspase-1 activity.

3.3. Simvastatin synergizes with LPS to increase IL-1β mRNA levels

The increased caspase-1-mediated mIL-1β release could be due to increased availability of either proIL-1β or of caspase-1 or both. We therefore investigated whether simvastatin-induced mIL-1β release was due to an increase in transcription of either procaspase-1 or of proIL-1β or both. THP-1 cells were cultured as before with simvastatin and/or LPS and after stimulation mRNA levels for caspase-1 and proIL-1β were determined using quantitative real-time RT-PCR analysis. Statin/LPS treatment had no major effect on procaspase-1 mRNA levels (Fig. 3A). For IL-1β mRNA levels, LPS alone induced a 120-fold increase compared to the untreated control cells (Fig. 3B) (Fenton et al., 1987). The combination of LPS and simvastatin had a modest synergistic effect (150-fold compared to untreated control cells) that was reversed by the isoprenylpyrophosphate GGPP. Thus, increased levels of IL-1β mRNA could, at least in part, be responsible for the increased mIL-1β release observed after statin/LPS treatment. However, the finding that LPS treatment alone does not lead to major IL-1β release despite inducing a dramatic increase in IL-1β mRNA levels, argues against transcriptional regulation as the underlying mechanism for our observations.

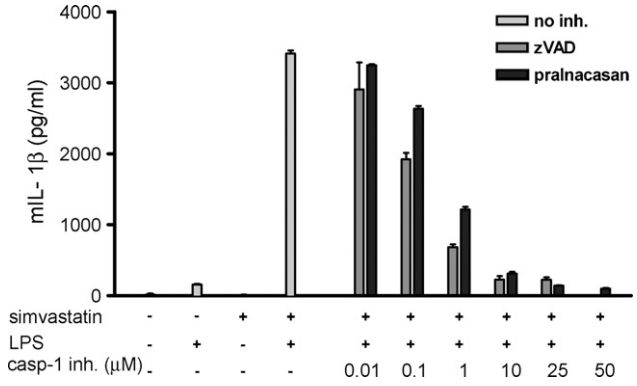

Fig. 2. Simvastatin-induced mIL-1β release is caspase-1 dependent. THP-1 cells were cultured for 24 h in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 h with 200 ng/ml LPS. Prior to LPS stimulation either a general caspase inhibitor (Z-VAD-FMK) or the specific caspase-1 inhibitor pralnacasan was added to the culture medium at concentrations ranging from 0.01 to 50 μM. Data are represented as means ± S.E.M. ($n = 2$).

3.4. Simvastatin treatment does not increase intracellular proIL-1β protein levels

We next wished to determine if the observed synergistic effect of simvastatin and LPS on proIL-1β transcription also leads to enhanced production of proIL-1β protein. THP-1 cells were cultured as before in presence or absence of LPS and/or simvastatin and after 4 h of stimulation cells were harvested and cell extracts were prepared as previously described. ProIL-1β protein levels were determined by a specific proIL-1β ELISA, which does not recognize the mature processed form. In accordance with the dramatic rise in mRNA levels, LPS induced a large increase in proIL-1β protein levels (Fig. 4A). This increase was a direct consequence of induced transcription as incubation with the transcription inhibitor Actinomycin-D (Act-D) reduced intracellular proIL-1β protein to an undetectable level. Furthermore, Act-D treatment reduced mIL-1β release to control levels in a dose-dependent manner (data not shown). The modest synergistic induction of transcription by additional

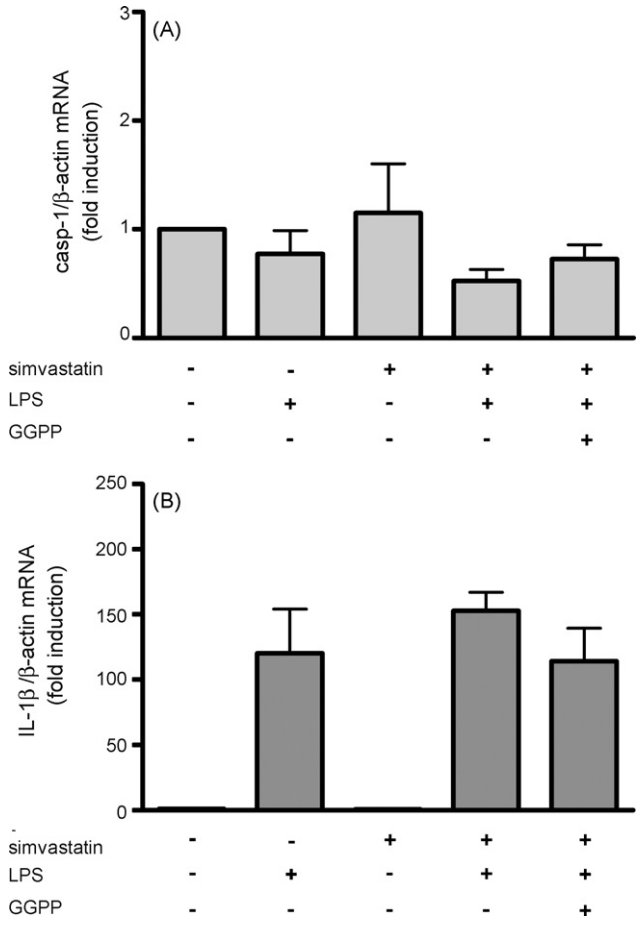

Fig. 3. Simvastatin has no effect on caspase-1 transcription (A), but synergizes with LPS to increase IL-1β mRNA levels (B). THP-1 cells were cultured for 24 h in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 h with 200 ng/ml LPS. Simvastatin-mediated effects were blocked by addition of 10 μM geranylgeranylpyrophosphate (GGPP) to the culture medium. Data are represented as means ± S.E.M. ($n = 4$).

simvastatin treatment (Fig. 3B) did not result in increased levels of proIL-1β protein. The intracellular pool of proIL-1β actually decreased in the presence of both simvastatin and LPS as compared to LPS alone (Fig. 4B), possibly due to increased processing and release of mIL-1β as demonstrated in Fig. 1.

To determine whether this decrease was indeed due to a higher turnover of proIL-1β into the mature, secreted form, we incubated the cells, in addition to LPS and simvastatin, with the specific caspase-1 inhibitor pralnacasan at concentrations known to inhibit mIL-1β release efficiently (see Fig. 2). Incubation with this inhibitor led to a restoration of intracellular proIL-1β protein levels in a dose-dependent manner (Fig. 4B), suggesting that the observed decrease was indeed due to caspase-1 mediated proteolysis. However, maximum inhibition of caspase-1 activity did not result in an accumulation of proIL-1β protein, indicating that the synergistic induction of transcription by statin/LPS does not correlate with enhanced translation into proIL-1β protein. Taken together, these results further argue against an important role for enhanced transcription in increasing mIL-1β release.

3.5. Simvastatin augments LPS-induced mIL-1β release via activation of caspase-1

These data suggest that simvastatin-mediated mIL-1β release is regulated at a post-translational level. Therefore, we investigated whether simvastatin had an effect on activation of caspase-1 or on the export of IL-1β protein or both. If simvastatin specifically, and exclusively, induces export of IL-1β, without affecting caspase-1 activation, then inhibition of caspase-1 in the presence of simvastatin and LPS should lead to increased export of inactive proIL-1β. Studies by Thornberry et al. and our own unpublished observations support the concept that proIL-1β can be released independently of processing by caspase-1 (Thornberry et al., 1992). THP-1 cells cultured in the presence of the inhibitors Z-VAD-FMK and pralnacasan did not show an increase in proIL-1β release (data not shown), suggesting that statin treatment does not exclusively target export of IL-1β. However, caspase-1 activation and subsequent export of the mIL-1β have been described to be very closely linked processes (Dinarello, 2005).

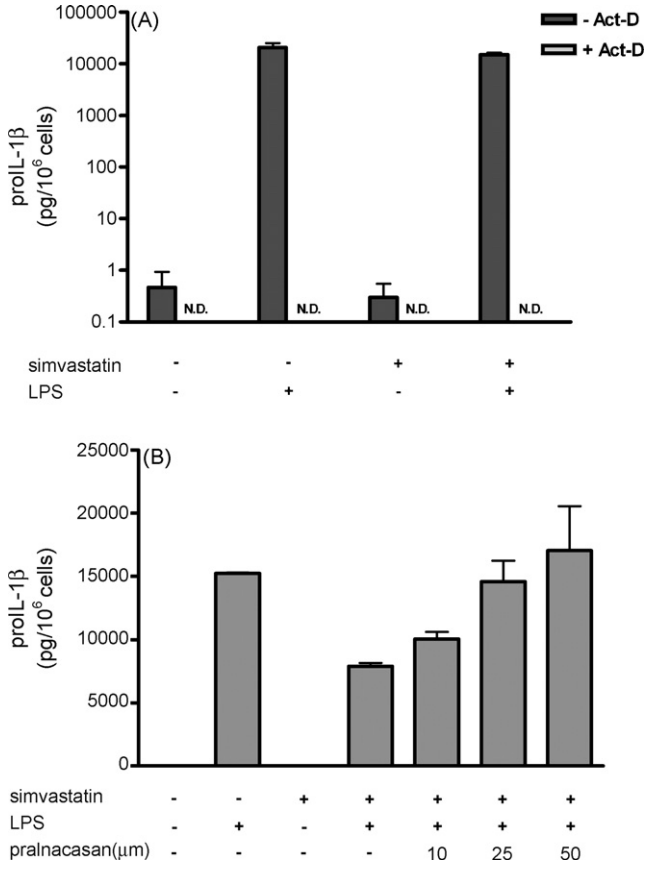

Fig. 4. Simvastatin treatment does not increase intracellular proIL-1β protein levels. THP-1 cells were cultured for 24 h in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 h with 200 ng/ml LPS. After stimulation proIL-1β protein levels were determined in cell extracts. (A) 10 min prior to LPS stimulation the transcription inhibitor Actinomycin-D was added to the culture medium at a concentration of 2 μg/ml. N.D. = non-detectable. Data are represented as means ± S.E.M. ($n = 3$). (B) Prior to LPS stimulation the specific caspase-1 inhibitor pralnacasan was added to the culture medium at the indicated concentrations. Data are represented as means ± S.E.M. ($n = 2$).

To further test the hypothesis that simvastatin induces mIL-1β release through activation of caspase-1 we examined autoprocessing of caspase-1. Since caspase-1 subunits have been shown to be readily secreted upon activation (Andrei et al., 2004), autocleavage of caspase-1 was determined by measuring caspase-1 p20 in culture supernatant. Treatment with simvastatin resulted in a time-dependent (Fig. 5A) and dose-dependent increase of caspase-1 p20 subunits in culture supernatants (Fig. 5B). Furthermore, LPS induced some caspase-1 p20 release on its own, but clearly synergized with simvastatin in the release of p20. GGPP completely blocked the statin-induced, but not the LPS-induced release of caspase-1 p20. Taken together, these data suggest that simvastatin alone or in combination with LPS can induce processing and activation of caspase-1.

3.6. Simvastatin induces IL-18 release

In addition to IL-1β, caspase-1 is also known to process IL-18. However, processing of IL-18 differs from IL-1β in that proIL-18 protein is already expressed without the need for LPS-induced transcription (Mehta et al., 2001; Puren et al., 1999). To test the hypothesis that simvastatin primarily affects activation of caspase-1, we treated THP-1 cells as before in presence or absence of simvastatin for 24 h and stimulated for an additional 4 h with LPS, after which supernatant was assayed for the presence of IL-18. Similar to mIL-1β, LPS stimulation alone induced a moderate increase and coincubation of LPS and simvastatin a very strong increase in IL-18 levels in culture supernatant (Fig. 6). However, in contrast to mIL-1β, treatment with simvastatin alone enhanced IL-18 release to the same extent as when

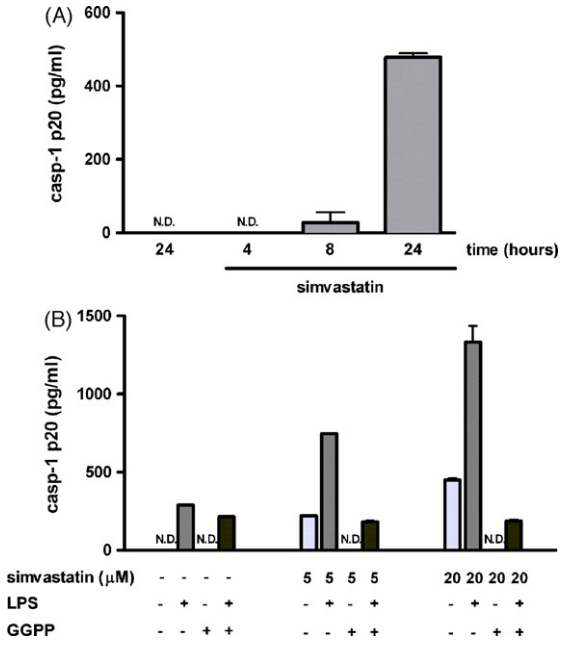

Fig. 5. Simvastatin induces release of caspase-1 p20. (A) THP-1 cells were cultured in the presence or absence of simvastatin (20 μM) for the indicated time periods. (B) THP-1 cells were cultured in the presence or absence of GGPP (10 μM) and/or simvastatin (5 or 20 μM). After 24 h the cells were stimulated with 200 ng/ml LPS for 4 hours. N.D. = non-detectable. Data are represented as means ± S.E.M. ($n = 2$).

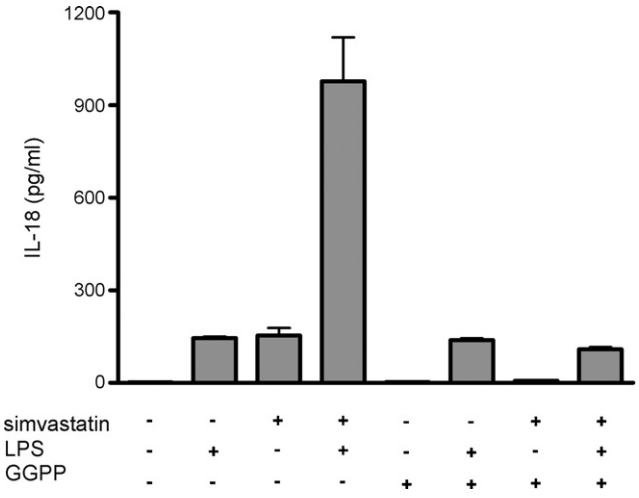

Fig. 6. Simvastatin induces IL-18 release. THP-1 cells were cultured for 24 h in the presence or absence of simvastatin (5 μM) and/or GGPP (10 μM). Cells were subsequently stimulated for an additional 4 h with 200 ng/ml LPS. Data are represented as means ± S.E.M. ($n = 4$).

cells were stimulated with LPS. Simvastatin-induced, but not LPS-induced IL-18 release could be completely reversed by coincubation with GGPP. These data support the conclusion that simvastatin acts by stimulating caspase-1 activity.

4. Discussion

Mevalonate kinase deficiency is a metabolic disease, caused by a genetic defect in isoprenoid biosynthesis (Houten et al., 1999; Drenth et al., 1999). However, clinically it is characterized by periodic fever accompanied by inflammation of joints, skin and serosa, suggesting an inflammatory disorder. Our previous studies have shown that inhibition of non-sterol isoprenoid biosynthesis can induce mIL-1β release by activated PBMC (Mandey et al., 2006). This effect has also been reported by several other groups (Montero et al., 2000; Kiener et al., 2001; Coward et al., 2006). In the current study we show that monocytic THP-1 cells pre-treated with simvastatin and subsequently stimulated with LPS also show augmented mIL-1β release (Fig. 1). These results indicate that IL-1β release in response to impairment of non-sterol isoprenoid biosynthesis is independent of T lymphocyte activation. The enhanced mIL-1β release could be abrogated by addition of geranylgeranylpyrophosphate (GGPP), a non-sterol intermediate of the pathway, which specifically restores one branch of non-sterol isoprenoid biosynthesis. In addition, specific inhibition of this branch by addition of GGTI-298 enhanced IL-1β release (Fig. 1B). These data demonstrate that simvastatin-mediated IL-1β release is specifically due to a lack of geranylgeranylpyrophosphate. As expected, the simvastatin-enhanced mIL-1β release was mediated by caspase-1 (Fig. 2).

Simvastatin-induced mIL-1β release could potentially be regulated at various levels: enhanced transcription or translation of either caspase-1 or of proIL-1β, increased proteolytic cleavage of proIL-1β or increased export of mIL-1β. We observed that simvastatin slightly enhanced LPS-induced transcription

of IL-1β (Fig. 3B). However, since LPS treatment alone led to a dramatic increase in mRNA levels without major effects on mIL-1β release, we thought it unlikely that the observed synergistic effect on transcription by simvastatin/LPS could account for the dramatic increase in mIL-1 release. On a translational level, we observed that after simvastatin/LPS treatment intracellular proIL-1β protein levels were not increased, but actually somewhat reduced compared to LPS treatment alone (Fig. 4A). A similar decrease in cell-associated proIL-1β levels was observed by Sutterwala et al. after stimulation of LPS-primed macrophages (Sutterwala et al., 2006). Blocking of caspase-1 activity resulted in a restoration of intracellular proIL-1β levels, but not to further accumulation (Fig. 4A). All these data imply that the observed simvastatin-enhanced proIL-1β transcription does not result in increased levels of the proIL-1β protein and is therefore not responsible for increased release of mIL-1β. Therefore, simvastatin-induced mIL-1β release is most likely regulated at a post-translational level, either by regulating caspase-1 activity or by regulation of the export mechanism of mIL-1β. Since we did not find an increased release of proIL-1β in the presence of caspase-1 inhibitors, it is unlikely that simvastatin exclusively targets the export mechanism of mIL-1β. Consequently, we continued by looking at the effect of simvastatin alone or in combination with LPS on autoprocessing of procaspase-1. Simvastatin treatment induced an increase in extracellular caspase-1 p20 in a time- and dose-dependent manner (Fig. 5), suggesting that simvastatin can activate caspase-1. LPS again worked synergistically with simvastatin in inducing release of p20.

Taken together, our data suggest a "two-step" model where LPS stimulation is needed for efficient transcription of the IL-1β gene resulting in high levels of intracellular proIL-1β protein. In addition, inhibition of the isoprenoid biosynthesis pathway via simvastatin induces proteolytic activity of caspase-1. Active caspase-1 can then subsequently process proIL-1β protein into mIL-1β, which is secreted together with the caspase-1 subunits.

This model could also account for the finding that there is an increase in IL-18 release after simvastatin treatment. THP-1 cells are likely to express low levels of proIL-18 without the need for LPS priming, similar to monocytes and PBMC (Mehta et al., 2001; Puren et al., 1999). Thus, statin-induced activation of caspase-1 would then indeed be sufficient for IL-18 processing and release.

Although little is known about the newly discovered caspase-1 substrate IL-33, it would be very interesting to investigate the effect of simvastatin on secretion of this new cytokine, which is thought to induce a T helper type 2 associated cytokine profile (Schmitz et al., 2005). An increased proteolytic activation of IL-33 could possibly help to explain symptoms like the skin rashes that are often observed in MKD patients.

Unfortunately, the exact mechanism behind simvastatin-induced caspase-1 activation remains unclear. However, the observations that addition of GGPP can completely counteract the effects induced by simvastatin and that both farnesyl pyrophosphate synthase inhibitor and geranylgeranyltransferase I inhibitor enhance IL-1β release clearly indicate that a shortage of one or more geranylgeranylated proteins is causing enhanced activation of caspase-1. This group of proteins includes the Rho subfamily of small GTPases and the gamma subunits of all heterotrimeric G proteins. Lack of geranylgeranylation of these proteins may cause mislocalization of the protein, since they lack the proper membrane localization anchor. In addition, it may cause an overactivity of the protein, since without the geranylgeranyl fatty acid tail, guanine nucleotide dissociation inhibitors (GDIs) can no longer bind and the protein can more easily change to its active GTP-bound state (Cordle et al., 2005). Exactly which GTPases are involved and how they are connected to caspase-1 activation and inflammasome assembly remains to be investigated. Very interesting in this context are the findings by Basak et al. (Basak et al., 2005) who describe a role for Rac1/PAK1 signaling in activation of caspase-1. *Helicobacter pylori* LPS induced direct interaction between PAK1 and caspase-1, which was inhibited in cells transfected with dominant-negative Rac1. This would imply that in an environment where Rac1 is overactive, for example in statin-treated cells, there could be increased activation of caspase-1. Whether Rac1 and PAK1 are involved in statin-induced caspase-1 activation is currently being investigated.

Taken together, the current study provides evidence that simvastatin can activate caspase-1. These findings suggest that patients suffering from mevalonate kinase deficiency may have overactive caspase-1, at least during fever episodes, causing cells of their immune system to more readily secrete mIL-1β in response to bacterial components like LPS. Although further investigations are necessary, our data suggest that therapies aimed at blocking the activity of caspase-1 or of its product mIL-1β may prove beneficial for MKD patients. Indeed, inhibition of IL-1 receptor function has been reported to be effective in several patients with MKD (Rigante et al., 2006; Nevyjel et al., 2007).

Conflict of interest

None.

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