RESEARCH ARTICLE

Pharmacological inhibitors of the mevalonate pathway activate pro-IL-1 processing and IL-1 release by human monocytes

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ABSTRACT. Objective. The effects of statins (3-hydroxy-3-methylglutaryl coenzyme A reductase-HMG-coenzyme A reductase inhibitors) on the inflammatory response remain unclear. HMGR is implicated in the mevalonate pathway, directly upstream of cholesterol biosynthesis. We studied the impairment by this pathway of cytokine production by peripheral blood mononuclear cells (PBMCs) and THP-1 cells. The aim was to identify a specific cytokine “signature” of cells under simvastatin treatment in order to link pharmacological inhibition of the mevalonate pathway and inflammation. Methods. Normal human PBMCs and THP-1 cells were cultured with inhibitors of HMGR (simvastatin), geranylgeranyltransferase (GGTI-298), farnesyltransferase (FTI-277), and/or caspase-1 (Z-VAD(Ome)-FMK). Following culture, cytokine production, caspase-1 activity, IL-1β mRNA and Rac-1 activity were determined. Results. Pharmacological inhibition of the mevalonate pathway specifically enhanced the release of IL-1α, IL-1β and IL-18 and inhibited IL-1ra production by LPS-activated PBMCs and THP-1 cells. Simvastatin did not modify pro-IL-1β expression, but enhanced caspase-1 activity, the enzyme responsible for IL-1β and IL-18 maturation. GGTI-298 also enhanced IL-1-family cytokine production, showing that geranylgeranylation is involved in caspase-1 activation. Additionally, simvastatin enhanced Rac-1 activity. Conclusion. Pharmacological inhibition of the mevalonate pathway by statins highlighted the specific induction of proinflammatory cytokines of the IL-1 family whose maturation is either directly (i.e. IL-1β and IL-18), or indirectly (i.e. IL-1α) dependant on caspase-1.

Keywords: caspase-1, simvastatin, IL-1 family cytokines, THP-1, peripheral blood mononuclear cells

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and are extensively prescribed to reduce atherosclerotic, cardiovascular events by lowering low-density lipoprotein cholesterol [1-4]. Statins impair cholesterol synthesis by inhibiting the rate-limiting step in the mevalonate pathway by preventing the reduction of HMG-CoA to mevalonate. Inhibition of the mevalonate pathway is also accompanied by an increase in low-density lipoprotein receptors in the liver, leading to increased uptake and clearance of cholesterol from the plasma [3, 4]. Downstream of mevalonate and upstream of cholesterol, the mevalonate pathway also produces a number of non-sterol isoprenoids, especially geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP), which are essential for intracellular signaling. Indeed, FPP and GGPP are precursors for the isoprenylation of small G proteins [5]. This enzymatic process is necessary for small G proteins to localize at the plasma membrane, allowing their signal transduction activity. FPP allows farnesylation of Ras-family proteins, while most Rho-family proteins are geranylgeranylated [5]. In addition to a direct effect of statins on the reduction of cardiovascular events through the inhibition of cholesterol synthesis, indirect effects have been attributed to the ability of statins to modulate inflammation. Interestingly, both anti-inflammatory and proinflammatory properties have been attributed to statins [6-11]. Statins inhibit in vitro expression of IL-1β and TNF-α in monocytes from patients with hypercholesterolemia [12], and reduce IL-6 expression and secretion by...
human adipocytes [13]. An ex vivo study of proinflammatory cytokine production by LPS-activated whole peripheral blood from pravastatin-treated patients with hypercholesterolemia showed a decrease in TNF-α and IL-6 production after treatment, whereas IL-1β, IL-8 and IL-1 receptor antagonist (IL-1ra) were not affected [14]. Other studies reported the induction of secretion of IL-1β, TNF-α and IL-6 by LPS or anti-CD2/CD28-stimulated peripheral blood mononuclear cells (PBMCs) in the presence of statins [15, 16], or by monocytes from statintreated mice [17]. It has been suggested that such increases in cytokine release were due specifically to a lack of isoprenoids [15, 17]. Interestingly, this pharmacological model has physiopathological counterparts, as two genetics diseases characterised by the inhibition of the mevalonate pathway and recurrent inflammation. Indeed, the hereditary periodic fevers hyperimmunoglobulinemia D, periodic fever syndrome (HIDS; OMIM #260920) and the more severe mevalonic aciduria (MA; OMIM #310377), are rare, autoinflammatory diseases characterized as mevalonate kinase deficiencies (MKD) and are caused by recessive mutations in the MVK gene (locus 12q24) [18, 19]. These mutations lead to inactivation of mevalonate kinase (MK), the enzyme directly downstream of HMGR in the mevalonate pathway. The non-sterol isoprenoids GGPP [20] and FPP [18, 21] metabolites synthesized downstream of MK have been thought to play a crucial role in the inflammatory manifestations of the MKD patients. In HIDS patients, plasma levels of IL-6 and TNF-α are increased during attacks, whereas IL-1α, IL-1β and IL-10 are unchanged [22]. Another study has shown that ex vivo, unstimulated or LPS-activated PBMCs from patients release more IL-1β, IL-6 and TNF-α than PBMCs from control subjects [23].

The aim of the present study was to analyze in vitro the influence of the inhibition of the mevalonate pathway by simvastatin on the proinflammatory cytokine profile secreted from control donors’ PBMCs or the LPS-treated THP-1 monocytic cell line. We further studied whether farnesylation, geranylgeranylation and Rac-1 (a protein of the Rho-family), as well as post-translational mechanisms of cytokine maturation and release, are involved in these processes.

METHODS AND MATERIALS

Cell culture and reagents

Normal human PBMCs were isolated by Ficoll-Hypaque (Biochrom AG, Berlin, Germany) centrifugation. For all experiments, PBMCs and THP-1 cells were grown in culture medium made up of RPMI 1640 (Invitrogen Life Technologies, Cergy Pontoise, France), supplemented with Glutamax-I, 10% heat-inactivated fetal calf serum (Sigma Aldrich, Saint-Quentin Fallavier, France), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen Life Technologies). THP-1 cells were seeded in this medium at a concentration of 0.3 × 10^6/mL, 72 h prior to experimentation. One million PBMCs or THP-1 cells were cultured in 1 mL of medium in the presence or absence of 1 μg/mL E. coli LPS (SigmaAldrich), 10 μg/mL anti-CD3 mAb (OKT3, ATCC, Rockville, MA, USA) and 1 ng/mL phosphor-12-myristate 13-acetate (PMA, Sigma Idrich), 10 μM simvastatin (Merck Pharmaceuticals, West Drayton, Middlesex, UK), 0.5 μM FTI-277 (Calbiochem, Darmstadt, Germany), 15 μM GGTI-298 (Calbiochem), 100 μM mevalonate (Mevanolactone, Sigma Aldrich) and/or 10 μM Caspase-1 inhibitor (Z-VAD(ome)-FMK, Alexis Biochemicals, Switzerland).

The use of PBMCs for research studies was approved by the Ethics Committee of the Poitiers Hospital.

ELISA for cytokine measurements

Cytokines were measured by ELISA in 48-hour culture supernatants. Commercial kits were used to measure IL-1β, IL-18, IL-1RA (matched antibody pairs for ELISA, R&D Systems), IFN-γ, IL-1α, IL-2, IL-4, IL-6, TNF-α (Eli-pair, Diaclone, Besançon, France), and IL-4 (OptEIA, Pharmingen, San Diego, CA). ELISAs were performed according to manufacturers’ instructions. All assays were performed in duplicate, in 96-well, flat-bottomed Maxisorp microtiter plates (Nunc, Rochester, NY, USA).

Caspase-1 activity measurement

Caspase-1 activity was measured using a Caspase-1 Fluorometric Assay Kit according to the manufacturer’s instructions (BioVision, Mountain View, CA, USA) in 48 hour, THP-1 culture supernatants concentrated 50-fold with a 10 kDa cut-off centrifugal concentrator (Vivaspin 2, Sartorius, Palaiseau, France). Relative caspase-1 activity was expressed as a ratio between the fluorescence intensities determined from treated cells/ control cells.

Real-time RT-PCR analysis

Total cellular RNA from THP-1 cells was extracted using TRIzol reagent (Invitrogen Life Technologies) and treated with DNase I (0.05 U/μL; Roche, Basel, Switzerland). cDNA was synthesized from 4 μg of total RNA using random hexamer primers (GE Healthcare, Stockholm, Sweden). Quantitative RT-PCR was conducted using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche) and the following primers: pro-IL-1β (forward, 5'-CTGTCCTGCGTGTTGAAAGA-3', reverse,
were activated with anti-CD3 mAb and PMA, and cultured for 48 hours in the presence or absence of simvastatin, and/or mevalonate. Whereas mevalonate had no effect on the secretion of the set of cytokines tested, simvastatin decreased the secretion of IFN-γ and IL-4 but did not significantly alter the secretion of IL-6, IL-2, IL-10, TNF-α, IL-1β, IL-1α and IL-18 (figure 1). This effect was specific for the inhibition of HMGR, since the addition of mevalonate restored secretion to control levels. GGTI-298, an inhibitor of type-I geranylgeranyl transferase, decreased the secretion of IFN-γ by activated PBMCs, whereas FTI-277, an inhibitor of the farnesyl transferase, had no significant effect.

Regarding the production of monocyte-derived cytokines by LPS-activated PBMCs, simvastatin and GGTI, in contrast, strongly and specifically enhanced the production of cytokines of the IL-1 family: IL-1α, IL-1β and IL-18, whereas FTI-277 had no effect (figure 2). Mevalonate abrogated all the effects of simvastatin. Simvastatin, FTI-277 and GGTI-298 did not significantly alter the production of TNF-α, IL-6 and IL-10, and mevalonate alone had no significant effect on cytokine secretion by LPS-activated PBMCs.

We further analyzed the effects of simvastatin on IL-1α, IL-1β, IL-1ra, IL-18 and IL-6 production by the THP-1 monocytic cell line (figure 3). The secretion of IL-1β and IL-1α by unstimulated cells was very low and was not further enhanced by simvastatin (figure 3A, B). In the presence of LPS, we observed a release of IL-1β within the first hours of culture (figure 3A), whereas IL-1α release was not modified (figure 3B). Addition of simvastatin to LPS-treated THP-1 cells dramatically enhanced both IL-1β and IL-1α production after 24 hours of culture (figure 3A, B). IL-1β production by LPS-activated THP-1 cells increased in a dose-dependent manner in response to simvastatin, ranging from 2.5 to 20 μM, and decreased at higher concentrations (figure 3C). As observed with simvastatin-treated PBMCs, GGTI-298 enhanced IL-1β and IL-18 secretion by LPS-activated THP-1, while FTI-277 had no effect (figure 3D, E). Otherwise, simvastatin significantly inhibited IL-1ra release, but did not affect IL-6 production (figure 3F, G).

Simvastatin does not affect pro-IL-1β expression, but induces IL-1β maturation through caspase-1 activation

Our data showed a specific enhancement of the secretion of IL-1 family cytokines by monocytes in the presence of simvastatin. Interestingly, two members of this family, IL-1β and IL-18, have the peculiarity of being produced as inactive pro-cytokines, matured into active form by caspase-1. Therefore, we studied the expression levels of pro-IL-1β transcript and protein, and the maturation of pro-IL-1β to the active form under simvastatin treatment in THP-1 cells. Real-time quantitative RT-PCR (figure 4A) and western blot (figure 4B) experiments showed that although pro-IL-1β mRNA and intracellular protein levels were increased by LPS, they were not significantly modified by simvastatin. Whereas LPS induced the secretion of the 34kDa pro-IL-1β by THP-1 cells, the 17kDa mature IL-1β form was not detectable in the culture supernatant (figure 4C). Interestingly, simvastatin

| Subcellular fractionation pull-down assays

The cells were lysed on ice by successive passages through a 25 G needle in a fractionation buffer (20 mM Tris pH 8, 150 mM NaCl and 5 mM EDTA, 200 mM saccharose, 1 mM Na3VO4, and 1% protease inhibitor cocktail (Sigma-Aldrich). Cells were cleared by centrifugation (750 g for 10 min); the supernatant was ultra-centrifuged (100 000 g for 1 h) to separate cytoplasmic and membranous fractions. The resulting supernatant was removed and saved as the cytoplasmic fraction. The lipopillic membrane-containing pellet and the cytoplasmic fraction were then resuspended in Laemmli sample buffer for western blotting analysis. The pull-down assays for detection of activated Rac-1 were performed with GST-PAK-CRIB-domain bound to glutathione-agarose as previously described [25].

Western blot analysis

Proteins were separated by 12% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (GE Healthcare). Membranes were blocked for two hours in Tris-buffered saline (150 mM NaCl and 20 mM Tris, pH 7.5) containing 3% non-fat dry milk and 0.1% Tween 20, and incubated with a mouse anti-human Rac-1 mAb (murine IgG2b, 23A8 clone, Upstate, Lake Placid, NY, USA), with a rabbit anti-human IL-1β polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with a rabbit anti-human caspase-1 p20 subunit Ab (Sigma-Aldrich), with a rabbit anti-human phospho-p38 Ab or with a rabbit anti-human total p38 Ab (GE Healthcare) overnight at 4°C. Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Sigma Aldrich), and immunoreactive proteins were visualized using the enhanced chemiluminescence assay (ECL, GE Healthcare). Ponceau red staining was used to control loading homogeneity. Images of western blot were scanned (Perfection 1670, Epson, Tokyo, Japan) and analyzed using NIH ImageJ software for densitometric representation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA, USA). Significance was determined using one-way ANOVA, followed by the Newman-Keuls test.

RESULTS

Blocking of the mevalonate pathway by simvastatin enhances the release of IL-1 family cytokines by LPS-activated PBMCs

To study the effects of mevalonate pathway inhibition on cytokine production by T cells, normal human PBMCs were activated with anti-CD3 mAb and PMA, and cultured for 48 hours in the presence or absence of simvastatin, and/or mevalonate. Whereas mevalonate had no effect on the secretion of the set of cytokines tested, simvastatin decreased the secretion of IFN-γ and IL-4 but did not significantly alter the secretion of IL-6, IL-2, IL-10, TNF-α, IL-1β, IL-1α and IL-18 (figure 1). This effect was specific for the inhibition of HMGR, since the addition of mevalonate restored secretion to control levels. GGTI-298, an inhibitor of type-I geranylgeranyl transferase, decreased the secretion of IFN-γ by activated PBMCs, whereas FTI-277, an inhibitor of the farnesyl transferase, had no significant effect.

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5'-CTGGGCAGACTCAATTCCA-3'; designed with Primer 3 Output software) and hydroxymethylbilane synthase (HMBS) [24] as a housekeeping gene.
strongly enhanced the maturation of pro-IL-1β to active IL-1β (figure 4C). This effect was totally or partially abolished by the addition of mevalonate or by a caspase-1 inhibitor respectively (figure 4C). Independently, we showed that the activity of caspase-1 was enhanced in culture supernatants from simvastatin-treated THP-1 cells (figure 4D). Moreover, we showed that the active caspase-1 p20 subunit is enhanced by simvastatin in culture supernatants from THP-1 cells (figure 4E).
We also showed that the caspase-1 inhibitor decreased the simvastatin-induced secretion of IL-1β and IL-1α by both LPS-activated THP-1 cells (figure 4F) and normal PBMCs (figure 4G). In contrast, simvastatin decreased the secretion of IL-1ra by both LPS-activated normal PBMCs and THP-1 cells. As expected, this decrease...
was abolished by the addition of mevalonate, but not by addition of a caspase-1 inhibitor (figure 4F, G). Finally, neither simvastatin nor caspase-1 inhibitor significantly modulated the production of IL-6 by both LPS-activated THP-1 cells (figure 4F) and PBMCs (figure 4G).

Simvastatin increases GTP-loading activity of Rac-1, with a discrete effect on Rac-1 localization

Our data suggested that the modulation of protein geranylgeranylation is responsible for the simvastatin-induced
activation of caspase-1 and subsequent maturation of IL-1 family cytokines. We studied the effects of simvastatin on the GTP-loading activity and localization of Rac-1, a geranylgeranylated small G-protein of the Rho family that requires geranylgeranylation to be localized to the plasma membrane. Interestingly, Rac-1 is directly implicated in the activation of caspase-1 [26, 27]. Using GST-PAK-CRIB-domain pull down, we observed that the active form of Rac-1 (the GTP-loaded Rac-1) was clearly enhanced in the presence of simvastatin, both in resting and LPS-activated THP-1 cells (figure 5A). Moreover, we showed by western blot analysis that the level of Rac-1 was slightly increased in the cytoplasm, and decreased at the plasma membrane under simvastatin treatment (figure 5B). We also showed that p38 phosphorylation, an indicator of Rac-1 activation [28], was increased by simvastatin (figure 5C).

DISCUSSION

Simvastatin treatment of LPS-activated PBMCs and THP-1 cells strongly and specifically enhanced the secretion of IL-1α, IL-1β and IL-18, proinflammatory cytokines belonging to the IL-1 family. IL-1β and IL-18 have the common feature of being synthesized as inactive pro-cytokines that require enzymatic processing by caspase-1 in order to matured into the active form [29-31]. The involvement of post-translational events in IL-1β release induced by simvastatin was shown by the enhanced secretion of mature IL-1β by activated monocytes, without any effect on either pro-IL-1β mRNA or protein levels. Both caspase-1 activity and active caspase-1 p20 subunit level were increased in culture supernatants of simvastatin-treated THP-1 cells. In addition, inhibition of caspase-1 decreased simvastatin-induced maturation and release of IL-1β, whereas the production of the caspase-1-independent cytokines IL-6 and IL-1ra was not modified. These data are in agreement with previous studies showing that statins enhance caspase-1 activity and IL-1β release by Mycobacterium tuberculosis-primed PBMCs or by activated THP-1 cells [10, 17, 32]. Unlike IL-1β and IL-18, IL-1α is known to be synthesized as the biologically active cytoplasmic or membrane-bound pro-IL-1α, and is mainly secreted through a proteolytic cleavage by calpain [33]. Surprisingly, we showed herein IL-1α release under simvastatin treatment that is inhibited in the presence of a caspase-1

Figure 5
Effects of simvastatin on the GTP-loading activity and localization of Rac1, and p38 phosphorylation. THP-1 cells were cultured with or without 1 μg/mL LPS and/or 10 μM simvastatin for 24 hours. (A) Cells were lysed and GTP-loaded Rac1 was purified by GST-PAK-CRIB-domain pull-down. The presence of Rac-1 was revealed by western blot analysis (representative of four independent experiments). (B) Cytoplasm and membrane fractions were separated by 100 000 g, 1 h ultracentrifugation. Supernatants containing cytoplasm were collected and membrane pellets were solubilized in Laemmli sample buffer. Rac1 level was assessed by western blot analysis (representative of three independent experiments). (C) Total and phosphorylated p38 intracellular levels were assessed by western blot. Results are expressed as the relative phospho-p38/total p38 ratio detected in supernatants of stimulated cells as compared with control cells (representative of two independent experiments).
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inhibitor. IL-1β-induced IL-1α expression could account for this effect. As IL-6 and TNF-α are not over-expressed in our culture conditions, we suggest that it is not the primary mechanism. Since a caspase-1-dependent activation of calpain has been reported [34, 35], we hypothesize that caspase-1 could indirectly enhance IL-1α maturation. In agreement, IL-1α secretion is impaired in caspase-1-deficient mice [36], and secretion of pro-IL-1α requires efficient caspase-1 activity [37].

In accordance with a previous study showing the involvement of geranylgeranylation on IL-1β production by LPS-activated PBMCs (20), simvastatin, GGTI-298, but not FTI-277, strongly induced the secretion of IL-1α, IL-1β and IL-18 by LPS-stimulated PBMCs. These results also suggest the involvement of geranylgeranylation in the simvastatin-induced activation of caspase-1. Geranylgeranylation seems to be associated with the activity of Rac-1, a small G-protein of the Rho family reported to play a critical role in caspase-1 activation [26, 27]. Rac-1-dependent phosphorylation of p38 was also demonstrated [28]. Under simvastatin treatment, Rac-1 was partially relocalised from the plasma membrane to the cytoplasm, but surprisingly, the level of GTP-bound Rac-1 is enhanced. In agreement, a simvastatin-induced activation of Rac-1 has also been recently reported in the vascular wall [38] and in THP-1 cells [39] and, independently, a simvastatin-reduced expression of membranous Rac-1 has been observed in smooth muscle cells [40]. The increased Rac-1 signaling activity was supported by the enhancement of p38 phosphorylation. Such an increase of p38 phosphorylation after 24 h simvastatin treatment has been reported in H2O2-treated smooth muscle cells, but not in unstimulated cells [40]. In the absence of LPS-stimulation of THP-1 cells, simvastatin does not enhance p38 phosphorylation (data not shown), as reported [39]. Although the link between Rac-1 geranylgeranylation and caspase-1 activation remains to be further explored, these results clearly show the increase Rac-1 GTP loading activity under simvastatin stimulation. In accord with our observations, it has been recently and independently demonstrated that dysregulated isoprenoid biosynthesis activates the Rac-1/PI3K pathway, resulting in caspase-1 activation and increased IL-1β release [39].

The stimulatory effect of simvastatin on the enhancement of IL-1α, IL-1β and IL-18 production by macrophages in vitro is largely mediated via the activation of caspase-1. On the other hand, we and others have demonstrated that statins inhibit the release of cytokines such as IFN-γ, IL-2, IL-10 and IL-4 by in vitro activated-T lymphocytes [41, 42] or IL-10 by monocytes. In vivo, treatment with statins have anti-inflammatory effects associated with ex vivo inhibition of IL-1β, TNF-α and IL-6 production [14], suggesting that the inhibitory effect of statins on cytokine synthesis predominates over caspase-1 activation. These effects could account for the beneficial effect of statins as lipid-lowering agents in the reduction of atherosclerotic cardiovascular events.

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