Sufficient production of geranylgeraniol is required to maintain endotoxin tolerance in macrophages

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Abstract  Endotoxin tolerance allows macrophages to produce large quantities of proinflammatory cytokines immediately after their contact with lipopolysaccharides (LPSs), but prevents their further production after repeated exposure to LPSs. While this response is known to prevent overproduction of proinflammatory cytokines, the mechanism through which endotoxin tolerance is established has not been identified. In the current study, we demonstrate that sufficient production of geranylgeraniol (GGOH) in macrophages is required to maintain endotoxin tolerance. We show that increased synthesis of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) protein following LPS treatment is required to produce enough GGOH to inhibit expression of Malt1, a protein known to stimulate expression of proinflammatory cytokines, in macrophages repeatedly exposed to LPSs. Depletion of GGOH caused by inhibition of HMGCR led to increased Malt1 expression in macrophages subjected to repeated exposure to LPSs. Consequently, endotoxin tolerance was impaired, and production of interleukin 1-β and other proinflammatory cytokines was markedly elevated in these cells. These results suggest that sufficient production of GGOH in macrophages may cause autoinflammatory diseases. —Kim, J., J. N. Lee, J. Ye, R. Hao, R. DeBose-Boyd, and J. Ye. Sufficient production of geranylgeraniol is required to maintain endotoxin tolerance in macrophages. J. Lipid Res. 2013. 54: 3430–3437.

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Inflammatory reactions initiated by macrophages following detection of microbial infection are critical for innate immune responses. However, these reactions have to be regulated tightly in order to prevent tissue damage caused by uncontrolled inflammatory responses. A classical example of this type of regulation is endotoxin tolerance, which allows macrophages to produce large quantities of proinflammatory cytokines immediately after their contact with bacterial endotoxins such as lipopolysaccharides (LPSs), but prevents their further production after repeated exposure to LPSs (1). While the clinical significance of endotoxin tolerance has been well established (2), its underlying mechanism has yet to be identified.

Recent observations suggest that the mevalonate pathway, a biosynthetic pathway through which sterols and nonsterol isoprenoids are synthesized (Fig. 1) (3), is closely related to inflammatory reactions elicited by macrophages. It was reported that sterol regulatory element binding protein 1a, a transcription factor activating almost all genes encoding enzyme catalyzing reactions in the mevalonate pathway, also stimulated transcription of Nlrp1a, a component of inflammasome that is required for processing and secretion of interleukin 1-β (II-1β), a major proinflammatory cytokine (4). The amount of several sterols, particularly lanosterol, was elevated in macrophages treated with endotoxin (5, 6). The products of the mevalonate pathway also affect macrophage-mediated inflammatory responses. Desmosterol and some other sterols were shown to inhibit macrophage-induced proinflammatory responses through activation of the liver X receptor (7, 8). Geranylgeraniol (GGOH), a nonsterol isoprenoid produced through the mevalonate pathway (Fig. 1), also inhibits macrophage-mediated inflammatory reactions following LPS treatment (9–12), but the underlying mechanism has not been identified.

In the current study we determined that LPS treatment enhanced expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), the rate-limiting enzyme in the mevalonate pathway (3), by activating translation of the protein. The increased expression of HMGCR allowed macrophages to produce GGOH at a level that was high enough to maintain endotoxin tolerance. Inhibition of HMGCR did not affect the initial production of proinflammatory cytokines by macrophages following LPS treat-
Macrophages were collected from the intraperitoneal cavity and injected intraperitoneally with 3% thioglycollate medium, and by immunoblot analysis. Antibodies used in the current study were monoclonal anti-HMGCR (5 μg/ml) and anti-K63-specific polyubiquitin chain (1:1,000 dilution), polyclonal anti-actin (1:3,000 dilution), anti-p52 (1:1,000 dilution), RelB (1:1,000 dilution), and lysine-specific demethylase 1 (1:1,000 dilution). Horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgGs (0.2 μg/ml) were used as the secondary antibody in all immunoblot analyses. Bound antibodies were visualized by chemiluminescence using the SuperSignal substrate system (Pierce) according to the manufacturer’s instructions.

**RT-QPCR**

Real-time quantitative PCR (RT-QPCR) was performed as previously described (16). Each measurement was made in triplicate from cell extracts pooled from duplicate dishes. The relative amount of RNA was calculated through the comparative cycle threshold method by using mouse cyclophilin mRNA as the invariant control.

**Measurement of synthesis of lanosterol and GGOH**

Synthesis of lanosterol and GGOH measured by radiolabeled analysis was performed by labeling cells with [3H]acetate. For measurement of lanosterol synthesis, cells pulled from three dishes were lysed in 1 ml 0.1 M NaOH. After addition of 40 μg of nonradioactive lanosterol standard and saponification in 15% KOH and 40% ethanol at 80°C for 1 h, lipids were extracted twice with 4 ml petroleum ether. For measurement of GGOH synthesis, cells pulled from three dishes were homogenized in buffer A [50 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 100 mM KCl]. Lipids in the homogenate were extracted by 0.5 ml of chloroform/methanol (2:1; v/v), and mixed with 40 μg of nonradioactive GGOH standard. For measurement of synthesis of both lipids, lipid extracts were dried under nitrogen gas with heat, resuspended in 50 μl heptane, and analyzed by thin-layer chromatography (TLC) on plastic-backed silica gel TLC plates in a solvent system of benzene/ethyl acetate (4:1; v/v). Following visualization by exposing the TLC plates to I2 vapor, bands containing the lipid were excised, and the amount of radioactivity in them was determined by scintillation counting. The activity of synthesis of the lipids was determined by radioactivity found in the band normalized by the amount of cellular protein.

**Pulse-chase analysis**

Pulse-chase analysis of HMGCR was performed exactly as previously described (17). ImageJ was used for quantification of the result.

**Polysome fractionation analysis**

Cells pooled from five 60 mm dishes were used in polysome fractionation analysis. Cells were treated with 0.1 mg/ml cyclohexamide for 5 min, washed with PBS containing 0.1 mg/ml cyclohexamide, and lysed with 1 ml of buffer B [50 mM HEPES (pH 7.5), 250 mM KCl, 5 mM MgCl2, 2 mM DTT, and 150 μg/ml cyclohexamide] supplemented with 1 mg/ml heparin, 1% (v/v) TX-100, and 1 mM PMSF. The lysate was centrifuged for 10 min at 10,000 g. The supernatant was layered onto a 1 ml heptane, and analyzed by thin-layer chromatography (TLC) on plastic-backed silica gel TLC plates in a solvent system of benzene/ethyl acetate (4:1; v/v). The statistical analysis was performed with a one-tailed unpaired t-test.

**Fig. 1.** The mevalonate pathway. Sterols and nonsterol products of the mevalonate pathway as well as key enzymes catalyzing their synthesis are indicated. Compounds indicated in red can feed into the mevalonate pathway when added into culture medium.
ELISA
The amount of IL-1β protein secreted into culture medium was measured through ELISA using Quantikine for mouse IL-1β (R&D Systems) according to the instructions of the manufacturer.

RNA interference
Duplexes of siRNA were synthesized by Dharmacon Research. The two small interfering RNA (siRNA) duplexes siMalt1-A and siMalt1-B target nucleotide positions 1753-1769 and 999-1014 (relative to the codon for the initiating methionine) for mouse Malt1 (National Center for Biotechnology Information accession number NM_172833), respectively. Control siRNA targeting green fluorescent protein was reported previously (19). Peritoneal macrophages in suspension were transfected with 0.1 μM siRNA using Lipofectamine TM RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions, after which the cells were used for experiments as described in the figure legends.

Microarray analysis
Macrophages were treated with compactin and LPSs in the absence or presence of GGOH two times, as described in Fig. 5C.

RESULTS
Inasmuch as endotoxin treatment increased the amount of lanosterol in macrophages (5, 6), we decided to determine the effect of such treatment on expression of HMGCR that catalyzes the rate-limiting step in the mevalonate pathway (3). Treatment of mouse peritoneal macrophages with LPSs increased the amount of HMGCR protein (Fig. 2A). Elevated expression of HMGCR led to increased synthesis of lanosterol in LPS-treated cells, as inhibition of HMGCR by compactin completely abolished LPS-induced synthesis of lanosterol (Fig. 2B). These results were reproduced in RAW264.7 cells, a transformed line of mouse macrophages (Fig. 2C, D). We thus used RAW264.7 cells to further identify
the mechanism through which LPSs increase the expression of HMGCR. LPS treatment did not increase HMGCR mRNA (Fig. 2E), nor did it retard the degradation rate of HMGCR protein as determined by pulse-chase analysis (Fig. 2F, G). To determine the effect of LPSs on translation of HMGCR protein, we performed polysome fractionation analysis that was used previously to analyze translation of the protein (21). Actively translated mRNAs attach to multiple ribosomes referred to as polysomes, so they can be separated from untranslated mRNAs free of ribosomes through density centrifugation analysis (18). Compared with untreated cells, LPS treatment increased the amount of HMGCR mRNA in the polysome fractions that contained rapidly translated mRNA (Fig. 2H). Thus, LPSs induce HMGCR expression by stimulating translation of the protein.

We then determined the functional significance of LPS-induced expression of HMGCR. Treatment of peritoneal macrophages with LPSs for 8 h markedly increased expression of IL-1β mRNA, and the amount of the mRNA dropped to the basal level 24 h after the treatment (Fig. 3A, first treatment, red line). These cells were then treated with fresh LPSs again. Owing to endotoxin tolerance, treating the cells with LPSs for the second time was less efficient in increasing IL-1β mRNA expression compared with the first treatment (Fig. 3A, second treatment, red line). Cotreatment with compactin, an inhibitor of HMGCR, did not affect LPS-induced IL-1β mRNA expression during the first LPS treatment (Fig. 3A, first treatment, green line). However, this treatment led to even more expression of IL-1β mRNA in cells treated with LPSs for the second time compared with those treated for the same period of time during the first treatment (Fig. 3A, second treatment, green line). Consistent with these results, compactin did not affect LPS-induced secretion of IL-1β protein from peritoneal macrophages during the first LPS treatment, but it markedly enhanced the amount of the protein secreted into culture medium during the second LPS treatment (Fig. 3B). These results suggest that endotoxin tolerance is impaired in cells treated with compactin.

We then tried to identify the product of the mevalonate pathway required to maintain endotoxin tolerance. As shown above, compactin cotreatment significantly elevated the level of IL-1β mRNA in peritoneal macrophages subjected to repeated treatment with LPSs (Fig. 4A, 2 and 4). Supplementation of mevalonate, the direct product of HMGCR (Fig. 1), completely eliminated the effect of compactin on expression of IL-1β mRNA (Fig. 4A, lane 5).

Supplementation of GGOH, a mevalonate-derived isoprenoid that is unable to be converted to sterols (Fig. 1), produced the same result (Fig. 4A, lane 7). In contrast, supplementation of FOH, another mevalonate-derived isoprenoid, was less potent in inhibiting the effect of compactin in these cells (Fig. 4A, lane 6). Consistent with these results, supplementation of mevalonate or GGOH, but not FOH, completely inhibited the effect of compactin on secretion of IL-1β protein from macrophages repeatedly exposed to LPSs (Fig. 4B). Because only FOH, but not GGOH, can be fed into synthesis of sterols (Fig. 1), these results suggest that sterols synthesized through the mevalonate pathway may not be required to inhibit expression of IL-1β in macrophages subjected to repeated exposure to LPSs. This hypothesis is supported by the observation that supplementation of squalene or lanosterol, two sterol intermediates of the mevalonate pathway (Fig. 1), failed to inhibit the effect of compactin on secretion of IL-1β protein from macrophages treated repeatedly with LPSs (Fig. 4C). Importantly, pretreatment with GGOH did not affect secretion of IL-1β from macrophages exposed to LPSs for the first time (Fig. 4D). The effect of GGOH was not restricted to IL-1β, as the isoprenoid was also required to suppress expression of other proinflammatory cytokines such as S100a8 and tumor necrosis factor-α in macrophages subjected to repeated LPS treatment (Fig. 4E, F). These results suggest that GGOH is required to establish endotoxin tolerance.

The results shown above suggest that increased HMGCR expression in LPS-treated macrophages is required to synthesize enough GGOH so that endotoxin tolerance can be established to prevent overproduction of proinflammatory cytokines in macrophages repeatedly exposed to LPSs. To test this hypothesis, we measured GGOH synthesis by labeling peritoneal macrophages with [3H]acetate, and determined the amount of radioactivity incorporated into...
activating transcription of proinflammatory genes (22). NF-κB is retained in cytosol through interaction with NF-κB inhibitor (IκB) (23). Proinflammatory stimulations lead to nuclear translocation of NF-κB by triggering degradation of IκB (23). The amount of nuclear p52 and RelB, both of which belong to the NF-κB family of transcription factors, was low in macrophages repeatedly exposed to LPSs owing to endotoxin tolerance (Fig. 5A, lane 3). Cotreatment with compactin markedly increased the amount of these proteins in the nucleus, and this effect was completely abolished by supplementation of the cells with GGOH (Fig. 5A, lanes 4 and 5). We then examined the effect of GGOH on formation of K63-linked polyubiquitin chains that are required for phosphorylation and subsequent proteasomal degradation of IκB (23, 24). Consistent with the result of nuclear localization of NF-κB, the amount of K63-linked polyubiquitin chains was low in macrophages subjected to repeated exposure to LPSs (Fig. 5B, lane 2). Cotreatment with compactin markedly raised the amount of the polyubiquitin chains in these cells (Fig. 5B, lane 4). This increase was abolished by supplementation of the cells with mevalonate or GGOH, but not with FOH (Fig. 5B, lanes 5–7).

GGOH. Treatment with LPSs alone reduced the amount of [3H]acetate incorporated into GGOH by more than 2-fold (Fig. 4G, lane 2) even though expression of HMGCR was elevated under such treatment (Fig. 2A). Compactin alone also reduced the amount of [3H]acetate incorporated into GGOH by more than 2-fold (Fig. 4G, lane 3). Cotreatment with LPSs and compactin drastically inhibited production of GGOH as the amount of [3H]acetate incorporated into GGOH was inhibited by more than 10-fold under this condition (Fig. 4G, lane 4). These results suggest that LPS treatment causes disappearance of GGOH, but this loss is partially compensated by increased synthesis of GGOH owing to elevated expression of HMGCR. This scenario explains why compactin alone only partially inhibited GGOH synthesis, but cotreatment with compactin and LPSs depleted GGOH from macrophages.

Inasmuch as GGOH suppresses expression of multiple proinflammatory cytokines in macrophages subjected to repeated exposure to LPSs, we examined the effect of the isoprenoid on activation of nuclear factor-κB (NF-κB), a family of transcription factors that play a major role in

**Fig. 4.** GGOH is required to maintain endotoxin tolerance. A–C, E, F: On day 0, peritoneal macrophages were seeded at 5 × 10⁶ cells per 60 mm dish. On day 1, cells were switched to medium containing (+) or not containing (−) 200 ng/ml LPSs, 25 μM compactin (CPN), 250 μM mevalonate (MEV), 10 μM FOH, GGOH, squalene (SQU), and lanosterol (LAN) as indicated. On day 2, 24 h after the first treatment, cells were switched to fresh medium that was used in the first treatment. On day 3, 24 h after the second treatment, cells were harvested for quantification of indicated mRNA as described in Fig. 3A (A, E, F). The amount of IL-1β protein secreted into medium was determined as described in Fig. 3B (B, C). D: On day 0, peritoneal macrophages were seeded at 5 × 10⁶ cells per 60 mm dish. On day 1, cells were treated with 10 μM GGOH as indicated for 24 h. On day 2, cells were switched to the same medium with or without the addition of 200 ng/ml LPSs as indicated. On day 3, 24 h later, the amount of IL-1β protein secreted into medium from macrophages exposed to LPSs for the first time was determined as described in Fig. 3B. G: On day 0, peritoneal macrophages were seeded at 5 × 10⁶ cells per 60 mm dish. On day 1, cells were switched to medium containing 200 ng/ml LPSs and 25 μM compactin as indicated. Eight hours after the treatment, cells were switched to the identical medium containing 10 μCi [3H]acetate and 0.5 mM unlabeled acetate. The amount of the radioactivity incorporated into GGOH was determined as described in Experimental Procedures. A–G: Results are presented as mean ± SE from three independent experiments.
indicated siRNA, and seeded at a density of $1.25 \times 10^6$ cells per well.

On day 0, peritoneal macrophages were transfected with 0.5 μM CPN and GGOH as indicated, two times as described in Fig. 4A. Twenty-four hours after the second treatment, the amount of Malt1 mRNA was quantified by RT-QPCR as described in Fig. 3A. D: The amount of Malt1 mRNA in peritoneal macrophages subjected to the indicated treatment for the indicated time was quantified as described in Fig. 3A. E, F: On day 0, peritoneal macrophages were transfected with 0.5 μM of indicated siRNA, and seeded at a density of $1.25 \times 10^5$ cells per well in a 12-well plate. Twenty-four hours after the second treatment, cells were harvested for quantification of indicated mRNA through RT-QPCR (E), and culture medium was collected to determine the amount of IL-1β protein secreted into culture medium (F).

We then performed a microarray analysis to identify the mechanism through which GGOH inhibits synthesis of K63-linked polyubiquitin chains. This analysis revealed that supplementation with GGOH reduced expression of Malt1, which has been reported to facilitate assembly of K63-linked polyubiquitin chains (25). We transfected two different duplexes of siRNA targeting Malt1 into macrophages treated with compactin and subjected to repeated exposure to LPSs. Knockdown of Malt1 by siRNA significantly reduced the amount of IL-1β mRNA in these cells (Fig. 5E). Consistent with this result, the amount of IL-1β protein secreted into culture medium was also reduced by this treatment (Fig. 5F).

**DISCUSSION**

The current study demonstrates that sufficient production of GGOH is required to establish endotoxin tolerance in macrophages. We show that LPS treatment enhances expression of HMGCR through increasing translation of the protein so that enough GGOH can be synthesized to inhibit expression of Malt1, a protein activating NF-κB through various mechanisms including facilitating assembly of K63-linked polyubiquitin chains (25, 26), in macrophages repeatedly exposed to LPSs. Consequently, expression of prof inflammatory cytokines from macrophages (28). This result suggests that geranylgeranylation of certain proteins may play a role in inhibiting expression of Malt1.

HMGCR is subjected to feedback regulation at multiple levels including mRNA transcription, protein degradation, and protein translation (3). Our results indicate that treatment of macrophages with LPSs increased the amount of HMGCR by activating translation of the protein. Such activation led to increased synthesis of lanosterol, an observation suggesting that elevated expression of HMGCR protein also enhanced the activity of the protein. Under this circumstance, HMGCR may no longer be the rate-limiting enzyme in the mevalonate pathway. Instead, lanosterol 14α-demethylase, which is the first enzyme that converts lanosterol to cholesterol and catalyzes the rate-limiting step in the process (29), may become the rate-limiting enzyme for the entire mevalonate pathway. This scenario may explain why LPS treatment raised the amount of lanosterol more than that of cholesterol (5, 6). Exactly how LPSs activate translation of HMGCR remains unclear. It was reported previously that accumulation of an unidentified nonsterol isoprenoid inhibited translation of HMGCR, but the underlying mechanism remains unclear (30). It will be interesting...
to determine whether LPSs and the isoprenoid control HMGCR translation through a similar mechanism.

Results from the current study suggest that insufficient production of GGOH may result in autoinflammatory diseases caused by overproduction of proinflammatory cytokines from macrophages (31). Indeed, hyperimmunoglobulin D and periodic fever syndrome (HIDS), a class of autoinflammatory disease, is caused by mutations in mevalonate kinase that inhibit, but do not completely eliminate, the activity of the enzyme (32, 33). Mevalonate kinase is an enzyme involved in the mevalonate pathway that catalyzes a reaction immediately following that catalyzed by HMGCR (Fig. 1) (3). While HIDS patients suffer from recurrent inflammation as a result of deficiency in GGOH production in leukocytes, they do not appear to have gross defects in cholesterol synthesis (10, 11). This is probably because HMGCR, but not mevalonate kinase, catalyzes the rate-limiting step in the mevalonate pathway (3). Thus, mutations that inhibit the activity of the kinase may not have a significant impact on cholesterol biosynthesis in cells that have not been exposed to LPSs. The situation may be different in macrophages activated by LPSs. These cells have increased expression of HMGCR so that the enzyme may not be so rate limiting. As a result, mutations inhibiting the activity of mevalonate kinase may profoundly affect GGOH synthesis in these cells. Thus, the deficiency in endotoxin tolerance caused by insufficient production of GGOH may lead to overproduction of proinflammatory cytokines in HIDS patients. Unlike HIDS, the genetic and metabolic defects of other autoinflammatory diseases, such as systemic onset juvenile idiopathic arthritis, have not been identified (31, 34). It will be interesting to determine whether macrophages derived from these patients are defective in GGOH synthesis following activation by LPSs.

It should be emphasized that GGOH synthesis is unlikely to be inhibited by a low concentration of statins in the clinical setting. As a result, uncontrolled inflammation has not been observed as a side effect of statins for most people who have no defect in the mevalonate pathway. However, it remains unclear whether overproduction of proinflammatory cytokines is responsible for muscle and joint pains in the small percentage of the population that is statin intolerant (35). If this indeed is the case, it will be interesting to determine whether these patients have predisposed defects in GGOH synthesis, and whether supplementation of GGOH improves the outcomes of statin treatment for these patients.

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REFERENCES


