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Mandey, S.H.L.

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Saskia H.L. Mandey*, Loes M. Kuijk*, Ingrid M.M. Schellens, Paul Coffer, Ger Rijkers, Hans R. Waterham, Joost Frenkel

* Both authors contributed equally to this work.
Chapter 5

The effect of simvastatin on the LPS-stimulated secretion of IL-1β by THP-1 cells

Saskia H.L. Mandey\(^1\), Loes M. Kuijk\(^2\), Paul J. Coffer\(^3\), Ingrid M.M. Schellens\(^2\), Ger T. Rijkers\(^2\), Hans R. Waterham\(^1\), Joost Frenkel\(^2\)

\(^1\)Laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, Emma Children’s Hospital, Academic Medical Center, University of Amsterdam
\(^2\)Departments of General Pediatrics and Pediatric Immunology, Division of Pediatrics, University Medical Center, Utrecht
\(^3\)Department of Molecular Immunology, Division of Immunology, University Medical Center, Utrecht

Abstract

Mevalonate kinase deficiency (MKD) is an autosomal recessive disorder characterized by recurring episodes of fever and inflammation. Peripheral blood mononuclear cells (PBMCs) from MKD patients secrete markedly higher levels of interleukin (IL)-1β when stimulated with lipopolysaccharide (LPS) than PBMCs from control subjects. To investigate in vitro how a deficiency in mevalonate kinase (MK) leads to induced secretion of IL-1β we studied the effect of simvastatin in the monocytic cell line THP-1. Simvastatin inhibits 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the enzyme preceding MK, and thereby impairs the isoprenoid biosynthesis similar to when MK is deficient.

Simvastatin treatment leads to markedly increased secreted IL-1β levels from LPS-stimulated THP-1 cells when compared to those from cells stimulated with LPS only. This effect was reversed by addition of the non-sterol isoprenoid intermediate geranylgeranylpyrophosphate (GGPP). Simvastatin plus LPS treatment also resulted in higher IL-1β mRNA levels than LPS treatment alone, an effect which was not reversed by GGPP. The intracellular proIL-1β levels, however, did not increase by the simvastatin treatment. Simvastatin treatment alone or in combination with LPS led to secretion of the caspase-1 p20 subunit, which correlated with a decrease in intracellular caspase-1 p20.

This study indicates that impaired isoprenoid biosynthesis seems to trigger caspase-1 mediated processing of pro-IL-1β and consequently secretion of mIL-1β.

Introduction

The Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS; MIM #260920) is an autosomal recessive disorder characterized by recurrent fever attacks and elevated levels of serum IgD (>100 IU/ml) (1). 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, myalgias and arthralgias may also occur (2-4). 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(IL)-6 and interferon-γ (IFN-γ), rise during fever attacks (5;6). Also, between attacks, isolated peripheral blood mononuclear cells (PBMCs) from HIDS patients secrete large amounts of IL-1β (7).

In 1999, the genetic defect underlying HIDS was identified; patients were shown to have mutations in the MVK gene, which codes for the enzyme mevalonate kinase (MK) (8;9). Since this discovery, HIDS and a more severe presentation, mevalonic aciduria, have been jointly referred to as mevalonate kinase deficiency (MKD). MK is an important enzyme in the isoprenoid biosynthesis pathway (10). 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 (11-16). Several studies have shown that the secretion of IL-1β by activated PBMCs was stimulated by the inhibition of isoprenoid biosynthesis using inhibitors (statins) of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase the enzyme preceeding MK in the isoprenoid biosynthesis pathway. This increased secretion 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 (17;18). In PBMCs from MKD patients it also is the lack of isoprenoid products, specifically of geranylgeranylated proteins, that raises IL-1β production (19). 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 secretion.

IL-1β is synthesized in the cytosol as an inactive precursor, proIL-1β, which in order to become active requires processing by caspase-1, which cleaves proIL-1β directly after the aspartic acid residue at position 116 (20;21). Caspase-1 can also cleave IL-18 (22;23) and more recently, also IL-33 was identified as a caspase-1 substrate (24). Caspase-1 itself is synthesized as an inactive zymogen of ~45 kDa, which 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 functional enzyme. Autoactivation of caspase-1 occurs in a complex of proteins termed the inflammasome (25). Caspase-1 contains an N-terminal caspase recruitment domain (CARD), which forms a homotypic interaction (CARD-CARD interaction) with the so-called “apoptosis-associated speck-like protein containing a CARD” (ASC). This adaptor protein then recruits other members of the inflammasome (26) 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.

To gain insight into the regulation of increased IL-1β production in MKD, we performed studies with the cultured monocytic cell line THP-1 in which the isoprenoid biosynthesis pathway was impaired at the level of HMG-CoA reductase using simvastatin. We investigated the effect of this impairment on transcription and translation of caspase-1 and (pro)IL-1β and on caspase-1 enzyme activity. Our results suggest that inhibition of the isoprenoid biosynthesis pathway via simvastatin triggers caspase-1 mediated processing of pro-IL-1β and subsequent secretion of mIL-1β.
Materials and Methods

Reagents
Lipopolysaccharide (LPS; E. coli 0127:B8), geranylgeranylpyrophosphate (GGPP) and Actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO, USA). Simvastatin (a gift from Merck, Sharpe and Dohme) was prepared as previously described (27). Benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (z-VAD-FMK) was obtained from R&D Systems, Inc (Minneapolis, MN, USA). Rabbit anti-caspase-1 was from Cell Signaling (Danvers, MA, USA). Pralnacasan (an oral caspase-1 inhibitor) was a kind gift from Vertex Pharmaceuticals (Boston, U.S.A.).

Cell Culture
THP-1 cells were routinely grown in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% Fetal Calf Serum (FCS), 2 mM glutamine and 100 U/ml penicillin-streptomycin. Cells were cultured at a starting density of 1x10^6/ml in 12-well microtiter plates at 37°C in a humidified atmosphere containing 5% CO2 in air, in the presence or absence of 5 µM simvastatin. After 24 hours of incubation, LPS to a final concentration of 200 ng/ml was added to the culture medium. The incubations were continued for an additional 4 hours after which cells were pelleted by centrifugation and supernatants either frozen at -20°C or assayed immediately. Cell pellets were stored at -20°C for Western blot analysis or snap frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis and caspase-1 activity experiments.

Cytokine Measurements
Cytokine detection was carried out using commercially available ELISA kits: Pelikine-compact™ human IL-1β ELISA kit (Sanquin Amsterdam, the Netherlands), Human ProlL-1β/IL-1F2 Quantikine ELISA Kit from R&D Systems, Inc (Minneapolis, MN, USA) and human IL-18 module set from Bender MedSystems (Vienna, Austria). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate.

Quantitative real-time RT-PCR analysis
The relative expression levels of IL-1β and caspase-1 to β-actin RNA were determined with the LightCycler® system (Roche, Mannheim, Germany). To this end, total RNA was isolated from THP-1 cells with TRIzol (Invitrogen, Carlsbad, CA) extraction, after which first strand cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR (AMV) according to the manufacturer's instructions (Roche, Mannheim, Germany). 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 TGA C-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 76
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3.5 (Roche, Mannheim, Germany) and the program LinRegPCR, version 7.5 (28) 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 for the mRNA levels of the housekeeping gene β-actin.

Caspase-1 activity
Cell lysates were prepared by four consecutive freeze-thaw cycles in lysis buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM MgCl₂, 5 mM dithiotreitol (DTT), protease inhibitors (Complete Mini, Roche, Mannheim, Germany)) using 1 x 10⁸ cells/ml. Lysates were then centrifuged at 16,000 x g for 20 min at 4°C, the supernatants were collected, and protein concentration was measured. A total of 100 µg of protein in 20 µl of lysis buffer for each sample was added to 320 µl of caspase assay buffer (321.5 mM Hepes (pH 7.5), 31.25% sucrose, 0.3125% CHAPS), 100 µl 100 m DTT, 20 µl DMSO or 20 µ caspase-1 inhibitor in DMSO and 520 µl water and preincubated at 37°C for 30 minutes after which incubation in the presence of 2.5 mM acetyl-YVAD-7-amino-4-methyl coumarin (Bachem AG, Bubendorf, Switzerland), a specific fluorogenic substrate for caspase-1, was prolonged for 60 minutes. To determine background fluorescence we used the competitive caspase-1 inhibitor Ac-YVAD-CHO (Bachem AG, Bubendorf, Switzerland). This inhibitor binds to caspase-1, but cannot be cleaved because of the aldehyde-group. The emitted fluorescence was measured using an excitation wavelength of 380 nm and an emission wavelength of 465 nm. The results were expressed as the fluorescence emitted by cell lysates and corrected for the background fluorescence.

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

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.

Results

Simvastatin increases secreted mIL-1β levels from LPS-stimulated THP-1 cells
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin followed by 4 hours stimulation with LPS, after which the supernatants were assayed for IL-1β levels. Supernatants of THP-1 cells treated with a combination of simvastatin and LPS showed markedly elevated IL-1β levels when compared to those from cells treated with only LPS (figure 1). Incubation with simvastatin only did not result in detectable IL-1β levels. GGPP completely prevented the simvastatin/LPS-induced increase in IL-1β levels, confirming that this effect was caused by an impairment of non-sterol isoprenoid biosynthesis, as was shown previously for
simvastatin-treated PBMCs (29). In fact, the addition of GGPP resulted in even lower levels of IL-1β than in supernatants from cells stimulated with LPS alone (figure 1).

![Image](image-url)

**Figure 1**: IL-1β secretion by THP-1 cells incubated for 24 hours in the presence or absence of simvastatin (5 µM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Simvastatin and LPS-induced IL-1β secretion was reversed by addition of 10 µM GGPP to the culture medium. Data are represented as means ± SEM (n=4). ** denotes a significant difference (p<0.01) between the indicated conditions.

**Simvastatin-mediated increase in IL-1β levels is caspase-1 dependent**

The generation of mature IL-1β from proIL-1β normally requires caspase-1 activity (30;31). To determine whether caspase-1 is also involved in the simvastatin-mediated increase in IL-1β levels in supernatants from LPS-stimulated THP-1 cells, we cultured the cells as before in the presence or absence of simvastatin. Just prior to LPS stimulation, either the general caspase inhibitor (Z-VAD-FMK) or the specific caspase-1 inhibitor pralnacasan was added to the culture medium. After 4 hours of incubation, the IL-1β levels in the supernatants were determined. Both the incubation with general caspase inhibitor and the specific caspase-1 inhibitor lead to a decrease in IL-1β levels in a dose-dependent manner (Figure 2). Addition of 10 µM inhibitor reduced the IL-1β levels in supernatants from cells stimulated with both LPS and simvastatin to levels comparable to the levels in supernatants from cells stimulated with LPS alone. These data confirm that the simvastatin-mediated increase in IL-1β levels requires caspase-1 activity.
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Figure 2: IL-1β secretion by THP-1 cells cultured for 24 hours in the presence of simvastatin (5 µM) and stimulated for an additional 4 hours 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 the indicated concentrations.

Simvastatin treatment of LPS-stimulated cells increases IL-1β gene transcription

The simvastatin-mediated increase in IL-1β levels could be due to an increased production of proIL-1β or caspase-1 or both. We therefore determined the IL-1β- and caspase-1 mRNA levels using quantitative real-time RT-PCR analysis in THP-1 cells cultured as before with simvastatin and/or LPS. The simvastatin and LPS treatment had no significant effect on procaspase-1 mRNA levels when compared with LPS only (Figure 3A). IL-1β mRNA levels in LPS-stimulated cells were 200-fold increased compared to the levels in untreated control cells (Figure 3B)(32). The combination of LPS and simvastatin lead to a further 3-fold increase, compared to the IL-1β mRNA levels in LPS-treated cells. Thus, increased levels of IL-1β mRNA could, at least in part, be responsible for the increased mIL-1β levels observed after simvastatin treatment. Remarkably, addition of GGPP to the simvastatin and LPS-treated cells did not result in lowering of the IL-1β mRNA levels (figure 3B) in contrast to the secreted mIL-1β protein levels, which under these conditions become even lower than in cells treated with LPS alone (figure 1). This indicates that IL-1β gene transcription and maturation, c.q. secretion are, at least in part, uncoupled processes and that increased IL-1β transcription does not necessarily result in increased IL-1β secretion.
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Simvastatin effect on IL-1β secretion by THP-1 cells

Figure 4: Intracellular proIL-1β protein levels in THP-1 cells.
A. THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin and the specific caspase-1 inhibitor pralnacasan stimulated for an additional 4 hours with 200 ng/ml LPS. Data are expressed as mean ± standard error of the mean (n=2).
B. 10 Minutes prior to LPS stimulation the transcription inhibitor Actinomycin-D was added to the culture medium (2 µg/ml). After stimulation proIL-1β protein levels were determined in cell extracts. (N.D. = non-detectable). Data are expressed as mean ± SEM (n=3).

Simvastatin treatment of LPS-stimulated cells does not increase intracellular proIL-1β protein levels
We next determined whether the increase in IL-1β mRNA levels upon simvastatin treatment of LPS-stimulated THP-1 cells also leads to increased levels of proIL-1β protein in the cells. To this end, proIL-1β protein levels were determined in cell lysates using a specific proIL-1β ELISA, which does not recognize the mature processed form. In accordance with the observed rise in mRNA levels, LPS stimulation lead to a marked increase in intracellular pro-IL-1β protein levels (Figure 4A). This increase is correlated to the increased IL-1β gene transcription, because incubation with the transcription inhibitor Actinomycin-D (Act-D) reduced intracellular proIL-1β protein to an undetectable level (Figure 4B). The additional induction of IL-1β transcription by simvastatin treatment (Figure 3B) did not result in increased levels of proIL-1β protein. In contrast, the intracellular pool of proIL-1β was decreased in cells treated with both simvastatin and LPS when compared to cells treated with LPS alone (Figure 4A).

To determine whether this decrease was due to a higher conversion rate of proIL-1β into the mature, secreted IL-1β, we incubated simvastatin and LPS-treated cells with the specific caspase-1 inhibitor pralnacasan at concentrations known to inhibit IL-1β secretion (see Figure 2). Incubation with this inhibitor led to a restoration of intracellular proIL-1β protein levels in a dose-dependent manner (Figure 4A), indicating that the observed decrease was indeed due to the higher conversion.

Simvastatin affects caspase-1 activity and increases the secretion of caspase-1 p20 subunit
To determine if the simvastatin-mediated increase of IL-1β levels in supernatants from LPS-stimulated cells is regulated at the post-translational level, we investigated whether simvastatin has an effect on activation of caspase-1 or on the export of IL-1β
protein or on both. If simvastatin stimulates the export of mIL-1β, without affecting caspase-1 activation, then inhibition of caspase-1 in the presence of simvastatin might lead to increased export of inactive proIL-1β. THP-1 cells cultured in the presence or absence of the inhibitors Z-VAD-FMK and pralnacasan, however, did not show an increase in secreted proIL-1β (data not shown), suggesting that simvastatin treatment does not exclusively target export of IL-1β. However, although it was previously shown that proIL-1β can be released by intact cells independent of processing by caspase-1 (33), caspase-1 activation and subsequent export of mIL-1β have been described to be very closely linked processes (34).

To determine if simvastatin induces mIL-1β secretion through activation of caspase-1, we measured caspase-1 activity in extracts of THP-1 cells cultured for 24 hours in the absence or presence of simvastatin followed by 18 hours LPS stimulation. This indicates that incubations with LPS and simvastatin alone or in combination resulted in a small increase in the intracellular caspase-1 activity. Co-incubations with GGPP reversed the LPS plus simvastatinmediate increase in intracellular caspase-1 activity to levels observed in non-stimulated cell (figure 5).

As another measure for the activation of caspase-1 we also examined the autoprocessing of caspase-1. Since caspase-1 subunits have been shown to be readily secreted upon activation (35), autocleavage of caspase-1 was determined by measuring caspase-1 p20 in the culture supernatants. Incubation with simvastatin resulted in a time-dependent (figure 6A) and dose-dependent increase of caspase-1 p20 subunits in the culture supernatants (figure 6B). Incubations with LPS also induced some caspase-1 p20 release, whereas co-incubation with both simvastatin and LPS resulted in the strongest release (figure 6B). GGPP completely blocked the simvastatin-induced, but not the LPS-induced release of caspase-1 p20.

**Figure 5**: Caspase-1 activity in cell lysates of THP-1 cells. THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 µM) and/or GGPP (10 µM) and stimulated for an additional 4 hours with 200 ng/ml LPS. The results are expressed as the percentage of the control incubation and represented as means ± SEM (n=4).
Simvastatin effect on IL-1β secretion by THP-1 cells

Figure 6: Caspase-1 p20 levels in supernatants of THP-1 cells.
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) at the indicated concentrations. After 24 hours the cells were stimulated with 200 ng/ml LPS for 4 hours. N.D. = non-detectable. Data are represented as means ± SEM (n=2).

Simvastatin treatment of LPS-stimulated cells results in higher levels of secreted IL-18
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 (36). To determine IL-18 processing, we treated THP-1 cells as before with or without simvastatin for 24 hours and stimulated for an additional 4 hours with LPS, after which the supernatants were analyzed for the presence of IL-18. Similar as with IL-1β, LPS stimulation alone induced a moderate increase whereas coincubation with LPS and simvastatin resulted in a marked increase in secreted IL-18 levels (Figure 7).

Figure 7: IL-18 secretion by THP-1 cells cultured for 24 hours in the presence or absence of simvastatin (5 µM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Simvastatin, but not LPS-induced IL-18 secretion was reversed by addition of 10 µM GGPP to the culture medium. Data are represented as means ± SEM (n=4). * denotes a significant difference (p<0.05) between the indicated conditions.

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Furthermore, also treatment with simvastatin alone induced a moderate increase in secreted IL-18 levels similar as observed for IL-1β (figure 1). The simvastatin-mediated elevation of secreted IL-18 levels could be completely reversed by coincubation with GGPP.

**Discussion**

Previous studies have shown that inhibition of non-sterol isoprenoid biosynthesis can induce IL-1β secretion by simvastatin-treated PBMCs (18;19;37-39). In the current study we show that monocytic THP-1 cells pre-treated with simvastatin and subsequently stimulated with LPS also have an increased mIL-1β secretion. Both simvastatin and LPS had no or very little effect on IL-1β secretion when administered alone. The increased mIL-1β secretion could be completely reversed by addition of geranylgeranylpyrophosphate (GGPP). The simvastatin-induced increase of IL-1β secretion is specifically mediated by caspase-1 and appears to be regulated at transcriptional and post-transcriptional levels. Caspase-1 mRNA levels are not significantly influenced by LPS or simvastatin treatment, the IL-1β mRNA levels showed a dramatic increase upon LPS treatment, while additional treatment with simvastatin even led to a further increase. This increase in IL-1β mRNA levels could not be reversed by GGPP, which indicates that the effect of GGPP on the secretion of mIL-1β must be post-transcriptional.

The increased IL-1β mRNA levels resulted in elevated secreted mIL-1β levels but did not result in elevated intracellular proIL-1β protein levels. In contrast, these were even somewhat reduced in the presence of simvastatin. Thus, our data indicate that in addition to IL-1β gene transcription simvastatin stimulates both the conversion of pro-IL-1β into mIL-1β and the secretion of the latter. Although we did not find conclusive evidence for this, our data suggest that this stimulation may be related to increased caspase-1 activity.

We only observed small increases in intracellular caspase-1 activities as a result of simvastatin and/or LPS treatment. However, simvastatin incubation led to a marked increase in levels of extracellular p20. Because activation of procaspase-1 into its active form requires processing into p20 and p10 units, this finding suggests that active caspase-1 becomes secreted when cells are incubated with simvastatin. Such secretion of active caspase-1 subunits has been reported previously (35).

Taken together, our data suggest a two-step model where LPS stimulation results in efficient transcription of the IL-1β gene leading to high levels of intracellular proIL-1β protein, whereas inhibition of the isoprenoid biosynthesis pathway via simvastatin triggers caspase-1 mediated processing of pro-IL-1β and subsequent secretion of mIL-1β. A similar mechanism is probably responsible for the marked increase in IL-18 secretion after simvastatin treatment.

If proven correct in future studies, this model would imply that therapies aimed at blocking caspase-1 activity may prove to be very beneficial for MKD patients.

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