Resveratrol inhibits the acetylated α-tubulin-mediated assembly of the NLRP3-inflammasome

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Abstract
With its adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), Nod-like receptor family, pyrin domain containing 3 (NLRP3) forms the inflammasome and mediates inflammatory innate immune responses. Development of an anti-inflammatory drug targeting the NLRP3-inflammasome is urgently required because its aberrant activation often causes inflammatory diseases, including gout. We show that resveratrol, a natural polyphenol in grapes and wine, is a safe and effective phytochemical that inhibits NLRP3-inflammasome activation. Resveratrol inhibits the accumulation of acetylated α-tubulin caused by mitochondrial damage in macrophages stimulated with inducers of the NLRP3-inflammasome. Consequently, resveratrol inhibits the acetylated-α-tubulin-mediated spatial arrangement of mitochondria and their subsequent contact with the endoplasmic reticulum (ER), causing insufficient assembly of ASC on the mitochondria and NLRP3 on the ER. These findings indicate that resveratrol targets the generation of an optimal site for the assembly of NLRP3 and ASC, thus inhibiting NLRP3-inflammasome activation. Therefore, resveratrol could be an effective medication for the treatment of NLRP3-related inflammatory diseases.

Keywords: cytokine, inflammasome, innate immunity, microtubule

Introduction
Pattern-recognition receptors (PRRs) specifically detect microbial components or stimulatory factors and induce inflammation. Nod-like receptors and AIM2 are intracellular PRRs that form a protein complex, the so-called inflammasome, upon exposure to various stressors or infections. Inflammasome activation triggers the caspase-1-dependent maturation of the inflammatory cytokines Interleukin (IL)-1β and IL-18. NLRP3 (also known as NALP3 or cryopyrin), a Nod-like receptor family member, recruits caspase-1 via its adaptor protein ASC and forms the NLRP3-inflammasome (1, 2), which plays a pivotal role in host defense against pathogens such as Candida albicans, Listeria monocytogenes, Mycobacterium tuberculosis and Influenza A virus. However, the excessive and persistent activation of the NLRP3-inflammasome often leads to the development of severe inflammatory diseases, including gout, arteriosclerosis, type 2 diabetes, Alzheimer’s disease and pneumoconiosis (3–9). This is because the NLRP3-inflammasome is activated by host-derived stimulatory substances such as monosodium urate (MSU) crystals, islet amyloid polypeptides, cholesterol crystals, amyloid β, and environmental irritants such as asbestos and silica nanoparticles. Therefore, the identification of compound(s) that regulate the NLRP3-inflammasome is important in developing therapeutic methods with which to treat infectious and inflammatory diseases.

Microtubules, rope-like polymers of α- and β-tubulin, form a platform that allows the intracellular transportation of cargo and which mediate a variety of cellular events (10, 11). Microtubules undergo diverse post-translational modification to regulate the distribution of intracellular organelles, such as mitochondria and ER. We previously demonstrated that mitochondrial damage, followed by a reduction in intracellular nicotinamide adenine dinucleotide+ (NAD+), induced the accumulation of acetylated α-tubulin in macrophages stimulated with inducers of the NLRP3-inflammasome (12). Acetylated α-tubulin mediates the dynein-dependent transportation of the mitochondria to an area near the ER, which in turn enhances the contact of ASC on mitochondria with the NLRP3 on the ER, resulting in the assembly of the NLRP3-inflammasome (12). Colchicine, a tubulin polymerization
Resveratrol inhibits NLRP3-inflammasome assembly

inhibitor that is used as a treatment drug for gout, consistently disrupts the assembly of the NLRP3-inflammasome to suppress inflammation induced by MSU crystals. Therefore, the microtubule system is a promising therapeutic target for the treatment of NLRP3-related inflammatory diseases. Acetylated α-tubulin is a particularly attractive molecular target for an inhibitor of NLRP3-inflammasome activation, because the genetic loss of MEC-17, the acetyltransferase for α-tubulin, did not cause a severe phenotype in mice (13–15).

Resveratrol is a naturally occurring compound produced by many types of plants and is one of the most intensely investigated phytochemicals. Previous studies have reported that resveratrol exerts a variety of beneficial effects by regulating protein acetylation, including anti-aging, anti-cancer, and anti-inflammatory effects in animal models (16–19). However, it remains unclear whether resveratrol affects the microtubule-mediated activation of the NLRP3-inflammasome. In this study, we have shown that resveratrol treatment prevents the accumulation of acetylated α-tubulin, thereby suppressing NLRP3-inflammasome assembly and subsequent inflammatory responses.

Methods
Reagents and cells
Butylated hydroxyanisole (BHA), colchicine, resveratrol, nigericin, poly(dA–dT), ethylene glycol bis(succinimidyl succinate) (EGS), and antibodies directed against acetylated tubulin (6-11B-1), tubulin (DM1A) and FLAG (M2) were purchased from Sigma. Disuccinimidyl suberate (DSS) was purchased from Tokyo Chemical Industry Co. Ltd. Complete Mini protease inhibitor tablets were purchased from Roche. Ciliobrevin D was purchased from Xcess Biosciences. Mitotracker Green, Mitotracker Red, MitoSox, Lipofectamine 2000, Hoechst 33342, Alexa-Fluor-488-conjugated phalloidin and Alexa-Fluor-labeled secondary antibodies were purchased from Invitrogen. LPS, Pam3CSK4, and flagellin were purchased from Enzo Life Science. Anti-ASC, anti-NLRP3 (Cryo-2), and anti-Caspase-1 p20 (Casper-1) antibodies were purchased from Adipogen. Silica and MSU were purchased from Nacalai Tesque. Perfect P1 was purchased from Targeting Systems. An anti-IL-1β antibody and an ELISA kit for mouse IL-1β were purchased from R&D Systems. An anti-Tom20 antibody was purchased from Santa Cruz Biotechnology. The J774 mouse macrophage cell line was purchased from RIKEN. Plat-E cells were kindly donated by Dr T. Kitamura (The University of Tokyo).

Mice
Eight-week-old female C57BL/6 mice were purchased from CLEA Japan. The mice were treated in accordance with the guidelines of Osaka University.

Macrophage preparation and stimulation
Mouse bone-marrow cells were cultured in the presence of macrophage colony-stimulating factor (M-CSF) for 6 days to prepare primary macrophages. Unless otherwise stated, M-CSF-induced bone-marrow macrophages (BMMs) were used in all the experiments. The BMMs were seeded in 48-well plates and primed with Pam3CSK4 (100 ng ml⁻¹) for 4 h. The primed BMMs were pretreated with colchicine (5 μM), ciliobrevin D (5 μM), BHA (5 μM) or resveratrol (0.5–100 μM) for 1 h, and then stimulated with nigericin (2 μM), ATP (2 mM), silica (250 μg ml⁻¹), MSU (500 μg ml⁻¹), flagellin (5 μg ml⁻¹) or poly(dA–dT) (1 μg ml⁻¹).

Plasmid constructs
The One-StrEP-tag (OST-tag)-encoding sequence was amplified by PCR and cloned into pMRX-IRES-bsr-FLAG vector (a kind gift from Dr S. Yamacka, Tokyo Medical and Dental University), generating pMRX-IRES-bsr-OST. The FLAG-encoding sequence was cloned into pMRX-IRES-bsr-OST, generating pMRX-IRES-bsr-FLAG-OST. cDNA corresponding to the full-length mouse ASC gene was amplified by PCR and subcloned into pMRX-IRES-bsr-FLAG-OST. A fragment of the mouse MEC-17 coding sequence (5′-ggtacagagaagctctttg-3′) was targeted by RNA interference and cloned into the pSUPER.retro vector. Recombinant retrovirus was prepared as previously described (20).

Cell viability measurement
The viability of the BMMs was measured with Cell Titer-Glo (Promega), according to the manufacturer’s instructions.

Quantitative RT–PCR
Total RNA was isolated with ZR RNA MicroPrep (Zymo Research), according to the manufacturer’s instructions. Reverse transcription was performed with ReverTra Ace reverse transcriptase (Toyobo), in accordance with the manufacturer’s instructions. For quantitative PCR, cDNA fragments were amplified with Real-Time PCR Master Mix (Toyobo), in accordance with the manufacturer’s instructions. The fluorescence from the TaqMan probe was detected with a 7500 Real-Time PCR System (Applied Biosystems). The level of MEC-17 mRNA expression was normalized to the expression of 18S ribosomal RNA. The experiments were repeated at least three times and the results are given as means ± SD.

Immunocytochemistry
Cells cultured on coverslips were fixed with 3% paraformaldehyde and then processed for immunocytochemistry as previously described (20). Genomic DNA was stained with Hoechst 33342. The samples were examined using an LSM 780 confocal laser-scanning microscope (Carl Zeiss).

In situ proximity ligation assay
In situ proximity ligation assays were performed with a Duolink In Situ PLA Kit (Olink Bioscience), according to the manufacturer’s instructions. Actin filaments were stained with phalloidin to observe cell shape. Cells containing >30 dots were defined as proximity ligation positive. To calculate the percentage of proximity ligation-positive cells, at least 10 fields were observed per sample.

ASC oligomerization assay
Cells were lysed with NP-40 lysis buffer (20 mM HEPES, 150 mM KCl, 1% NP-40 and protease inhibitor). Lysates of
BMMs or J774 cells were incubated with EGS (1 mM) or DSS (2 mM), respectively, for 2 h at 37 °C. The cross-linked insoluble pellets were dissolved directly in SDS sample buffer.

Affinity purification assay
J774 cells stably expressing OST-FLAG-tagged ASC were primed with LPS for 6 h. The primed cells were washed with PBS and treated with resveratrol, colchicine or ciliobrevin D for 1 h. The cells were then stimulated with nigericin for 1 h, collected and incubated with DSS for 1 h on ice before they were lysed with NP-40 lysis buffer. The insoluble pellets were removed by centrifugation. The cell lysates were incubated with Strep-Tactin Sepharose beads for 2 h at 4 °C. The beads were washed three times with NP-40 lysis buffer and incubated with desthiobiotin for 30 min at 4 °C. The bound proteins were dissolved in SDS sample buffer.

Immunoblotting
Immunoblotting was performed as previously described (20).

Enzyme-linked immunosorbent assay
IL-1β levels were measured by ELISA, in accordance with the manufacturer’s instructions.

Flow-cytometric analysis
BMMs were stained with Mitotracker Green, Mitotracker Red and MitoSox (Invitrogen), in accordance with the manufacturer’s instructions. Cells extracted from peritoneal cavities of mice were stained with FITC rat anti-mouse CD11b (M1/70, BD Biosciences) and APC rat anti-mouse Ly-6G and Ly-6C (RB6-8C5, BD Biosciences), in accordance with the manufacturer’s instructions. The cells were suspended in 1% PBS containing FCS and then analyzed on a FACSCalibur flow cytometer (BD Biosciences).

NAD+ measurement
Intracellular NAD+ levels were measured with an NAD/NADH Quantitation Colorimetric Kit (BioVision), according to the manufacturer’s instructions. The NAD+ level in each sample of BMMs was divided by the NAD+ level in the untreated control BMMs to determine the relative NAD+ concentration (arbitrary units).

Results
Resveratrol treatment suppresses activation of the NLRP3-inflammasome
We first examined the effect of resveratrol on the activation of the NLRP3-inflammasome in primary mouse macrophages. Resveratrol treatment successfully suppressed the maturation of both IL-1β and caspase-1 in response to various inducers of the NLRP3-inflammasome, with no cytotoxicity (Fig. 1A–D). Resveratrol did not affect the production of IL-1β mediated by the NLRC4-inflammasome or the AIM2-inflammasome (Fig. 1A and B).

It is well known that two signals are required to fully activate the NLRP3-inflammasome in macrophages. The first step, termed the ‘priming step’, is induced by TLR ligands, leading to the activation of transcription factor nuclear factor (NF)-κB, which enhances the expression level of NLRP3 and pro-IL-1β. Then, a second signal induced by stimulation with nigericin or MSU crystals promotes the activation of the NLRP3-inflammasome and the subsequent maturation of IL-1β. Although resveratrol is reported to inhibit the activation of NF-κB (21, 22), we could discriminate the effect of resveratrol on the activation of NLRP3 in macrophages treated with resveratrol after priming with Pam3CSK4. Consistently, resveratrol treatment did not alter the expression levels of NLRP3, ASC, procaspase-1 or pro-IL-1β (Fig. 1B and E).

Investigation of the effect of resveratrol on a mouse model of acute gout indicated that resveratrol reduced the amount of mature IL-1β and the subsequent recruitment of neutrophils into the peritoneal cavities of mice after challenge with MSU crystals (Fig. 2A–C). These findings indicate that resveratrol suppresses the activation of the NLRP3-inflammasome both in vitro and in vivo.

Low-dose resveratrol does not prevent mitochondrial damage
We next investigated the mechanism underlying the action of resveratrol on NLRP3-inflammasome activation. NLRP3-inflammasome inducers cause aberrant mitochondrial damage, which in turn induces the production of reactive oxygen species (ROS) and a reduction in intracellular NAD+ levels leading to the accumulation of acetylated α-tubulin (23–25). Consistently, resveratrol treatment did not alter the expression level of mature IL-1β and the subsequent recruitment of neutrophils into the peritoneal cavities of mice after challenge with MSU crystals (Fig. 2A–C). These findings indicate that resveratrol suppresses the activation of the NLRP3-inflammasome both in vitro and in vivo.

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the NLRP3-inflammasome. Therefore, we next examined the effects of resveratrol on the levels of acetylated α-tubulin. Resveratrol treatment suppressed the nigericin- or MSU crystal-induced accumulation of acetylated α-tubulin, without affecting the microtubule structure (Fig. 4A–C), whereas colchicine treatment suppressed the accumulation of acetylated α-tubulin by disrupting the microtubule structure (Fig. 4A–C). Resveratrol treatment also inhibited the nigericin- or MSU crystal-induced accumulation of acetylated α-tubulin.
Fig. 3. Effect of resveratrol on mitochondrial damage. (A) Flow-cytometric analysis of primed BMMs pre-treated with or without resveratrol (5 μM) and then left unstimulated or stimulated with various ligands. The cells were stained with the dyes MitoTracker Red and Mitotracker Green. (B) Immunocytochemical analysis of mitochondrial morphology in primed BMMs pre-treated with or without resveratrol (5 μM) and then left unstimulated or stimulated for 1 h with various ligands. (C) Flow-cytometric analysis of BMMs pre-treated with resveratrol (5 μM) or BHA (5 μM) and then left unstimulated or stimulated with various ligands. The cells were stained with the dye MitoSOX. (D) Intracellular NAD⁺ levels in primed BMMs pre-treated with or without resveratrol (5 μM) and then left unstimulated or stimulated with various ligands for 1 h. Data are presented in arbitrary units relative to the amount of NAD⁺ in untreated BMMs. The results are presented as means ± SD. SSC-H, side scatter height. The numbers above the plots (A,C) indicate the percentages of cells with low membrane potential (A) or producing mitochondrial ROS (C). The data (A–C) are representative of three independent experiments. The scale bar represents 15 μm. Blue, nuclei.
crystal-induced transportation of mitochondria to the perinuclear region (Fig. 5A and B). Using an in situ proximity ligation assay, a technique that visualizes the spatial proximity of two molecules, we found that resveratrol treatment suppressed the proximate localization of ASC and NLRP3 in the perinuclear region (Fig. 5C and D). However, BHA, a ROS scavenger, failed to suppress the accumulation of acetylated α-tubulin and the subsequent proximate localization of ASC and NLRP3 (Fig. 5E–H), indicating that ROS is dispensable for the regulation of α-tubulin acetylation and subsequent proximity of ASC and NLRP3. Taken together, resveratrol treatment prevents the accumulation of acetylated α-tubulin and thus inhibits the proximate localization of ASC and NLRP3 in macrophages.

Resveratrol treatment suppresses the assembly of NLRP3 and ASC

NLRP3 binds with its adaptor protein ASC to activate caspase-1. As resveratrol inhibits the acetylated α-tubulin-mediated proximity of ASC and NLRP3, we next examined the effect of resveratrol on the assembly of NLRP3 and ASC. Treatment with colchicine or ciliobrevin D, a specific inhibitor of dynein motor protein (30), inhibited the nigericin-induced interaction between NLRP3 and ASC in J774 immortalized mouse macrophage cells (Fig. 6A). Furthermore, the short hairpin RNA (shRNA)-mediated knockdown of MEC-17, an α-tubulin acetyltransferase involved in activation of the NLRP3-inflammasome (12, 13), inhibited the

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**Fig. 4.** Effect of resveratrol on α-tubulin acetylation. (A) Immunoblot analysis of acetylated α-tubulin in primed BMMs pretreated with resveratrol (upper panel, 5 μM) or colchicine (lower panel, 5 μM) and then left unstimulated or stimulated with nigericin or MSU crystals. β-Actin was used as the loading control. Ac-α-tubulin, acetylated α-tubulin. (B, C) Immunocytochemical analysis of the subcellular location of acetylated α-tubulin (magenta) and α-tubulin (green) in primed BMMs pre-treated with resveratrol (5 μM) or colchicine (5 μM) and then left unstimulated or stimulated with nigericin or MSU crystals (B). The percentages of BMMs with elevated acetylated α-tubulin levels were calculated from at least 10 fields per sample (C). The data (A, B) are representative of three independent experiments. The results are presented as means ± SD. Statistical significance was determined with the Student’s t-test. **P < 0.01. The scale bar represents 10 μm. Blue, nuclei.
Fig. 5. Effect of resveratrol on the proximate localization of ASC and NLRP3. (A,B) Immunocytochemical analysis of the subcellular location of Tom20 (magenta) and α-tubulin (green) in primed BMMs pre-treated with resveratrol (5 μM) or colchicine (5 μM) and then left unstimulated or stimulated with nigericin or MSU crystals (A). The percentages of BMMs with clustered mitochondria in the perinuclear region were calculated (B). (C,D) Proximity ligation assay of ASC and NLRP3 in primed BMMs pre-treated with resveratrol (5 μM) or colchicine (5 μM) and then left unstimulated or stimulated with nigericin or MSU crystals (C). The percentages of BMMs with positive proximity ligation signals were calculated (D). (E) Immunoblot analysis of acetylated α-tubulin in primed BMMs pre-treated with BHA (5 μM) and then left unstimulated or stimulated with nigericin or MSU crystals. β-Actin was used as the loading control. Ac-α-tubulin, acetylated α-tubulin. (F,G) Proximity ligation assay of ASC and NLRP3 in primed BMMs pre-treated with BHA (5 μM) and then left unstimulated or stimulated with nigericin or MSU crystals (F). Percentages of BMMs with positive proximity ligation signals were calculated (G). (H) ELISA of IL-1β in the culture supernatants of primed BMMs pre-treated with BHA (5 μM) and then left unstimulated or stimulated with various ligands. To calculate the percentages of BMMs with clustered mitochondria in the perinuclear region (B) or BMMs with positive proximity ligation signals (D,G), at least 10 fields were observed per sample. The data (A, C, E, F) are representative of three independent experiments. The results are presented as means ± SD. Statistical significance was determined with the Student’s t-test. *P < 0.05, **P < 0.01, n.s., not significant. The scale bar represents 10 μm. Blue, nuclei.
Fig. 6. Effect of resveratrol on the assembly of ASC and NLRP3. (A) LPS-primed J774 cells stably expressing OST-FLAG alone or OST-FLAG-tagged ASC were pre-treated with resveratrol (5 μM), colchicine (5 μM) or ciliobrevin D (5 μM) and then left unstimulated or stimulated with nigericin. The cell lysates were subjected to an affinity purification assay and immunoblotted with the indicated antibodies. (B) The levels of MEC-17 mRNA in J774 cells stably expressing OST-FLAG alone or OST-FLAG-tagged ASC, together with control shRNA or MEC-17-directed shRNA, were determined by quantitative RT–PCR. (C) LPS-primed J774 cells stably expressing OST-FLAG alone or OST-FLAG-tagged ASC, with or without shRNA directed against MEC-17, were left unstimulated or stimulated with nigericin. The cell lysates were subjected to an affinity purification assay and immunoblotted with the indicated antibodies. (D) Primed BMMs were pre-treated with resveratrol (5 μM), colchicine (5 μM) or ciliobrevin D (5 μM) and then left unstimulated or stimulated with nigericin. The cell lysates were incubated with EGS, and the cross-linked pellets were immunoblotted for ASC. (E) The levels of MEC-17 mRNA expression in J774 cells stably expressing the control shRNA or MEC-17-directed shRNA were measured by quantitative RT–PCR. (F) LPS-primed J774 cells stably expressing control shRNA or MEC-17-directed shRNA were left unstimulated or stimulated with nigericin. The cell lysates were incubated with DSS, and the cross-linked pellets were immunoblotted for ASC. The results are presented as means ± SD. The data (A,C,D,F) are representative of three independent experiments.
nigericin-induced interaction between NLRP3 and ASC in J774 cells, confirming that the dynein-dependent transportation of cargos, together with acetylated α-tubulin, is required for the assembly of the NLRP3-inflammasome complex (Fig. 6B and C). Consistent with this, resveratrol treatment suppressed the acetylated-α-tubulin-mediated interaction of NLRP3 and ASC (Fig. 6A). After the NLRP3-inflammasome is stimulated, ASC oligomerizes to activate caspase-1 (31, 32). The inhibition of tubulin polymerization, dynein and MEC-17 suppressed the nigericin-induced oligomerization of ASC, indicating that the dynein-dependent transportation of cargos on acetylated microtubules is required for the formation of ASC oligomers (Fig. 6D–F). Consistent with this, resveratrol treatment suppressed the acetylated-α-tubulin-mediated oligomerization of ASC (Fig. 6D). Collectively, these findings indicate that resveratrol treatment prevents the accumulation of acetylated α-tubulin, thus inhibiting the microtubule-mediated formation of the NLRP3-inflammasome complex and oligomerization of ASC.

Discussion

We have shown that acetylated α-tubulin mediates the spatial arrangement of the mitochondria and promotes the assembly of NLRP3 and ASC. Although colchicine, a powerful medication for gout, inhibits the microtubule-mediated assembly of NLRP3 and ASC, it is severely toxic to cells and tissues and causes many adverse effects (33). Therefore, colchicine is not an appropriate drug for the treatment of NLRP3-related inflammatory diseases, such as atherosclerosis or type 2 diabetes, which persist for a long time. In this study, we have shown that resveratrol is a phytochemical that can inhibit the assembly of NLRP3 and ASC. Because resveratrol can be consumed safely, even at high doses, by humans and rodents, it could be a more effective medication than colchicine for the treatment of NLRP3-inflammasome-related inflammatory diseases.

The mechanism by which resveratrol suppresses the accumulation of acetylated α-tubulin is still unclear. We previously demonstrated that acetyltransferase MEC-17 is required for the acetylation of α-tubulin in macrophages and is involved in NLRP3-inflammasome activation (12). Because a method for the measurement of enzymatic activity of MEC-17 has not been established yet, it is difficult to investigate the effect of resveratrol on MEC-17. We have also demonstrated that the NAD+-dependent deacetylase SIRT2 regulates the acetylation of α-tubulin. Inactivation of SIRT2 by its specific inhibitor AGK2 enhanced the α-tubulin acetylation and subsequent activation of the NLRP3-inflammasome in macrophages (12). Because resveratrol is reported to regulate the enzymatic activity of SIRT family members including SIRT2 (34), we speculate that resveratrol promotes the activity of SIRT2 via an unknown mechanism, thereby suppressing acetylated α-tubulin-mediated NLRP3-inflammasome activation. Further study is needed to clarify the molecular mechanism underlying the action of resveratrol on SIRT2.

Resveratrol has multiple targets, which will ultimately prove relevant to health, and some resveratrol targets have been implicated in regulating the activation of the NLRP3-inflammasome. At high doses, resveratrol is reported to activate adenosine monophosphate-activated protein kinase, resulting in the induction of autophagy, an intracellular clearance system for damaged organelles, which is involved in the negative regulation of the NLRP3-inflammasome (8, 16, 17, 20, 28, 35, 36). Consistently, an increasing dose of resveratrol effectively suppressed mitochondrial ROS production and subsequent NLRP3-inflammasome activation. Therefore, it will be interesting to investigate the effects of high-dose resveratrol on NLRP3-related inflammatory diseases in a future study.

Supplementary data

Supplementary data are available at International Immunology Online.

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Conflict of interest statement: The authors declare that they have no competing financial interests.

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