Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Forskolin attenuates the NLRP3 inflammasome activation and IL-1β secretion in human macrophages

Yan Chen[1,2], Jian Guo Wen[1], Jin Jin Feng[1], Yi He Wang[1], Tian Fang Li[3], Katariina Nurmi[2], Kari K. Eklund[2,4] and Dong Xing[1]

BACKGROUND: The treatment of nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) inflammasome-mediated pediatric inflammatory diseases is challenging. Here we studied whether cyclic adenosine monophosphate (cAMP) elevator forskolin could attenuate the nigericin-induced NLRP3-inflammasome activation and interleukin-1β (IL-1β) secretion in human macrophages.

METHODS: The proteins and messenger RNA (mRNA) levels of inflammasome structural proteins and proinflammatory cytokines were measured in forskolin-stimulated nigericin-activated human THP-1 macrophages and primary macrophages.

RESULTS: Activation of THP-1 macrophages with nigericin increased the mRNA expression of NLRP3, IL-1β, and caspase-1 ($P < 0.01$). Forskolin stimulation had no effect on the mRNA expression of NLRP3, caspase-1, or IL-1β in nigericin-activated cells ($P > 0.05$), while their protein levels were significantly decreased ($P < 0.05$). Forskolin-mediated increase in cytoplasmic cAMP in non-activated cells was attenuated in nigericin-activated macrophages ($P < 0.05$). Basal IL-1β secretion increased from 584 to 2696 pg/mL ($P < 0.01$) in nigericin-activated macrophages; forskolin dose-dependently reduced the nigericin-induced secretion of mature IL-1β ($P < 0.01$). Forskolin also inhibited the IL-1β secretion from activated human primary macrophages.

CONCLUSIONS: Forskolin inhibits the NLRP3 inflammasome activation and the secretion of mature IL-1β, in human macrophages. Forskolin and other cAMP elevator drugs could represent a novel approach for treatment of diseases associated with excessive inflammasome activation, like pediatric inflammatory diseases.

Pediatric Research (2019) 86:692–698; https://doi.org/10.1038/s41390-019-0418-4

INTRODUCTION

Severe pediatric infections, such as sepsis, which potentially leads to systemic inflammatory response, and multiple organ failure, are associated with activation of canonical nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) and noncanonical inflammasomes.[1,2] NLRP3 inflammasome activation is also implicated in various other diseases such as severe airway inflammation and inflammation caused by human metapneumovirus, identified in 5–10% of young children hospitalized with acute respiratory tract infection,[3] and also in acute lung injury.[4] Inflammasomes are mainly expressed in monocytic cells, and their activation critically controls the responses of innate and adaptive immunity. Inflammasomes are large intracellular structures that regulate the secretion of proinflammatory cytokines interleukin-1β (IL-1β) and IL-18.[5] The NLRP3 inflammasome is the most widely studied inflammasome. NLRP3 inflammasome consists of the sensor protein NLRP3, an adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the proform of caspase-1. Pro-caspase 1 is autocatalytically cleaved into its active form in the inflammasome complex, and the active caspase-1 further catalyzes the cleavage of the inactive precursors of pro-IL-1β and pro-IL-18 into their mature forms that are secreted from the cell.[6–8]

NLRP3 inflammasome is activated by a variety of factors, including endogenous factors and exogenous infections, involved in immunological and inflammatory diseases in infants and children.[7,8] NLRP3 inflammasome can be activated directly by bacteria, viruses, mycoplasma, parasites, and their toxins, such as nigericin.[9] The activation of the NLRP3 inflammasome requires two steps, the first priming step initiates the expression of the inflammasome sensor protein NLRP3 and the pro-forms of IL-1β and IL-18, and the second step induces the assembly of the inflammasome complex and the cleavage of pro-caspase-1.[8,10] Mature caspase-1 then mediates the cleavage of the proforms of the cytokines. However, the mechanisms that negatively regulate the NLRP3 inflammasome are less well known; the study of the pathways that regulate or resolve the inflammasome activation can provide treatment option for the severe systematic inflammatory diseases and autoimmune diseases.

Forskolin is extracted from *Coleus forskohlii* that mainly grows in India, China, Thailand, and Brazil. Forskolin activates adenylyl cyclase in eukaryotic cells, and it is often used to increase the intracellular level of the second messenger cyclic adenosine monophosphate (cAMP).[11] While the article was under review Du et al.[12] showed that forskolin inhibited the lipopolysaccharide (LPS)-induced activation of Toll-like receptor 4 (TLR4) and subsequent expression of several

[1]Pediatric Urodynamic Center and Department of Urology, Institute of Clinical Medicine, The First Affiliated Hospital of Zhengzhou University, 450052 Zhengzhou, China; [2]Helsinki Rheumatic Diseases and Inflammation Research Group, Institute of Clinicum, University of Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland; [3]Department of Medicine, Division of Rheumatology, The First Affiliated Hospital of Zhengzhou University, 450052 Zhengzhou, China and [4]Department of Medicine, Division of Rheumatology, Helsinki University Hospital, Haartmaninkatu 2, 000290 Helsinki, Finland
Correspondence: Yan Chen (chenyan.abby@163.com, yan.chen@helsinki.fi)
Co-first authors: Yan Chen, Jian Guo Wen

Received: 21 November 2018 Revised: 1 April 2019 Accepted: 18 April 2019
Published online: 13 May 2019

proinflammatory cytokines in human mononuclear cells.[12] Furthermore, cAMP has been previously shown to bind the NLRP3 to promote its degradation in mouse macrophages and in human primary macrophages.[13,14] The effect of forskolin on NLRP3 inflammasomes in human monocytes/macrophages is still poorly known. Here we investigated the effects of forskolin on the nigericin-induced NLRP3 inflammasome activation and secretion of IL-1β in human THP-1 macrophages and primary macrophages, which could provide novel perspectives on the use of existing drugs to treat pediatric inflammatory diseases.

MATERIALS AND METHODS

Cell culture

Human acute monocytic leukemia cell line THP-1 (TIB-202) was obtained from American Type Culture Collection and maintained at 37 °C in a humidified 5% CO_2 in air. First, the cells were cultured in RPMI-1640 (Invitrogen #11875-093, Invitrogen/Thermo Fisher Scientific, Shanghai, China) supplemented with 100 g/L fetal bovine serum (Gibco #10099-141), 1% penicillin and streptomycin (P/S) (Gibco #15140-122), 2 mmol/L glutamine (Gibco® GlutaMAX™ Supplement, 35050061), and 10 mmol/L hydroxyethylpiperazine ethylsulfonic acid (HEPES). The media were changed to the THP-1 monocytes every 2–3 days. Once the cell density reached about 8×10^5 cells/mL, the suspension cells were directly aspirated, centrifuged, re-suspended, and divided into new flasks to passage the cells. The passage concentration was 3×10^5 cells/mL. Second, THP-1 monocytes were differentiated into macrophages by seeding the cells in 24-well plates in culture media containing 100 nmol/L phorbol 12-myristate 13-acetate (PMA, CAS 16561-29-8-Calbiochem, Calbiochem/Merck) and cultured for 72 h. Third, Experimental medium: on the day of the experiment, THP-1 macrophages were washed twice with phosphate-buffered saline with/without Ca^{2+}/Mg^{2+} and then placed in the experimental medium containing RPMI-1640 supplemented with 1% P/S, 2 mmol/L L-glutamine, and 10 mmol/L HEPES.

Human monocytes were isolated from buffy coats (from Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by centrifugation in Ficoll-Paque gradient as described by Yeo et al.[15] Washed cells were suspended in Dulbecco's modified Eagle's medium and seeded on 24-well plates (1.5×10^6 cells/well). After 1 h, non-adherent cells were removed and the medium was replaced with serum-free macrophage medium supplemented with 10 ng/mL granulocyte–macrophage colony-stimulating factor. The media were then replaced every 48 h until the cells were used for experiments after 6–7 days of culture.

Ethics statement. Buffy coats were obtained from healthy blood donors who had signed an informed consent document. The buffy coats were by-products from the preparation of blood products for clinical use. The use of buffy coats in monocyte isolation was approved by the Finnish Red Cross Blood Service.

Cell activation and stimulation

THP-1 macrophages were activated with 4 μmol/L nigericin (N7143, Sigma-Aldrich) for 1 h, simultaneously with or without forskolin (F3917, Sigma-Aldrich) for 1 h, in the indicated concentrations. Cell experimental groups: control group (inactivated group) and nigericin group (activated group), and three stimulating groups, nigericin+forskolin 10 μmol/L group, nigericin+forskolin 50 μmol/L group, and nigericin+forskolin 100 μmol/L group.

Human primary macrophages were primed by 1 μg/mL LPS (L3012, Sigma-Aldrich) for 3 h, and then by the addition of three activators, adenosine triphosphate (ATP, A6419, Sigma-Aldrich) 5 μmol/L for 45 min, monosodium urate (MSU, U2875, Sigma-Aldrich) 100 μg/mL for 4 h, nigericin 4 μmol/L for 1 h, simultaneously with or without forkolin 100 μmol/L. Primary macrophage experimental groups: control group, LPS group, LPS + forskolin group, ATP group, MSU group, and nigericin group, three activated groups, LPS + ATP, LPS + MSU, and LPS + nigericin groups, and three stimulating groups, LPS + ATP + forskolin, LPS + MSU + forskolin, and LPS + nigericin + forskolin groups.

Quantitative real-time reverse transcription-PCR analysis

THP-1 macrophages lyzed in RLT and total cellular RNA was purified using the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA). Quantitative reverse transcription-PCR was performed with 10 ng first-strand complementary DNA using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in an iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA copy numbers were determined in triplicates and normalized against the β-actin gene as an endogenous control, and the relative units were calculated using the comparative Ct method. The following primers were designed using the NCBI Primer Blast program and purchased from Sangon Biotech (Shanghai, China):

NLRP3 sense primer (F, forward): 5′-AGAGACCTTTATGAGAAAG CAA-3′, anti-sense primer (R, reverse): 5′-GCTGTCTTCCTGGCATAT CACA-3′;

IL-1β sense primer (F, forward): 5′-TTACAGTGGCAATGAGGA TGAC-3′, anti-sense primer (R, reverse): 5′-GTCGGAGATTCGTAGCT GGAT-3′;

caspase-1 sense primer (F, forward): 5′-CCGAAGGTGATCATCA TCCA-3′, anti-sense primer (R, reverse): 5′-ATAGCATCATCCTCA AACTCTTCTG-3′;

IL-6 sense primer (F, forward): AGGAGACTTGCCTGGTGAAA, anti-sense primer (R, reverse): GAGGTGCCCATGCTACATTT;

Tumor necrosis factor-α (TNFα) sense primer (F, forward): GACA AGCCTGTAGCCCATGT, anti-sense primer (R, reverse): TTGATG GCAGAGAGGAGGTT

β-Actin sense primer (F, forward): 5′- CACCCAGCACAATGAAG ATCAAGAT-3′, anti-sense primer (R, reverse): 5′-CCAGTTTTTA AATCCTGAGTCAAGC-3′.

Western blotting

Cells were lysed in 200 μL of Mammal Cell Protein Extraction Reagent (CWBiotech, Beijing, China). The total protein concentration was determined with a Pierce BCA Protein Assay Kit (Trans, Beijing, China). For each sample, 20 μg of protein was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis consisting of 5% stacking gel and 12% resolving gel, and the proteins were electrophoretically transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST). The membranes were incubated overnight at 4 °C with anti-rabbit polyclonal antibodies from the Inflammasome Antibody Sampler Kit (#32961, Cell Signaling, Shanghai, China) against caspase-1 (D7F10, Rabbit mAb 3866S, 20 kDa, 48 kDa), IL-1β (3A6, mouse mAb #12242S, 20 kDa, 48 kDa), NLRP3 (rabbit mAb #15101), and apoptosis-associated speck-like protein containing a CARD (ASC, D154049, anti-PYCARD rabbit polyclonal antibody, Sangon Biotech, Shanghai, China) and anti-cAMP Protein Kinase Catalytic subunit antibody (ab76238, Abcam, Shanghai, China) in TBST with 5% nonfat milk. The secondary antibody goat anti-mouse immunoglobulin G (IgG), horseradish peroxidase (HRP)-conjugated (CW0102S, CWBiotech, Beijing, China), and mouse anti-rabbit IgG-HRP (sc-2357, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to incubate the membrane for 2 h at room temperature. Labeling was performed using an enhanced chemiluminescence system (Thermo Fisher Scientific, Thermo, USA). The density and area of the bands were quantitated.

Enzyme-linked immunosorbent assay

The culture medium supernatant for IL-1β, IL-6, and TNFα concentrations were quantified by enzyme-linked immunosorbent

assay (ELISA) following the manufacturer's instruction (Human IL-1β/IL-1F2 DuoSet ELISA, DY201, R&D Systems; Human IL-6/IL-6 ELISA Kit, D710322, Sangon Biotech, Shanghai, China; Human TNFα/TNFα ELISA Kit, D710599, Sangon Biotech, Shanghai, China, respectively).

Statistical analysis

Calculations were performed using GraphPad InStat3 for Macintosh software. Overall, significance levels between the stimulated and control groups or between stimulated and inhibited groups were analyzed using one-way analysis of variance, and with the Dunnett and Tukey–Kramer multiple comparisons tests when appropriate. Statistical significance was set at $P < 0.05$. The data are shown as mean ± SD.

RESULTS

Effect of forskolin on the mRNA expression of NLRP3, caspase-1, IL-1β, IL-6, and TNF in activated THP-1 macrophages

THP-1 monocytes were differentiated to macrophages by using 100 nmol/L PMA for 72 h (Fig. 1a, b). Neither nigericin nor forskolin affected the mRNA expression of IL-6 or TNFα in PMA-differentiated THP-1 macrophages ($P > 0.05$) (Fig. 1c–d). However, nigericin increased the expression of NLRP3, IL-1β, and caspase-1 by 7.6-folds ($P < 0.01$), 579.1-folds ($P < 0.001$), and 3.6-folds ($P < 0.01$), respectively, compared to those of the non-activated cells. Stimulation of the cells with forskolin had no effect on the nigericin-induced expression of NLRP3, IL-1β, or caspase-1 mRNA ($P > 0.05$) (Fig. 1e–g).

Effects of forskolin on the expression of pro-caspase 1, pro-IL-1β, NLRP3, IL-1β, caspase-1, and ASC protein levels in activated THP-1 macrophages

Activation of the THP-1 macrophages with nigericin increased the protein level of pro-IL-1β, pro-caspase-1, NLRP3, active caspase-1, and ASC ($P < 0.05$ or $P < 0.01$). Prior treatment with forskolin had no effect on the protein levels of pro-IL-1β or pro-caspase-1 ($P > 0.05$), but significantly reduced the levels of NLRP3, mature IL-1β (17 kDa), and active caspase-1 (20 kDa) in a concentration-dependent fashion ($P < 0.05$ or $P < 0.01$). Forskolin also reduced the protein expression of ASC ($P < 0.05$), but the reduction was not dose dependent (Fig. 2).

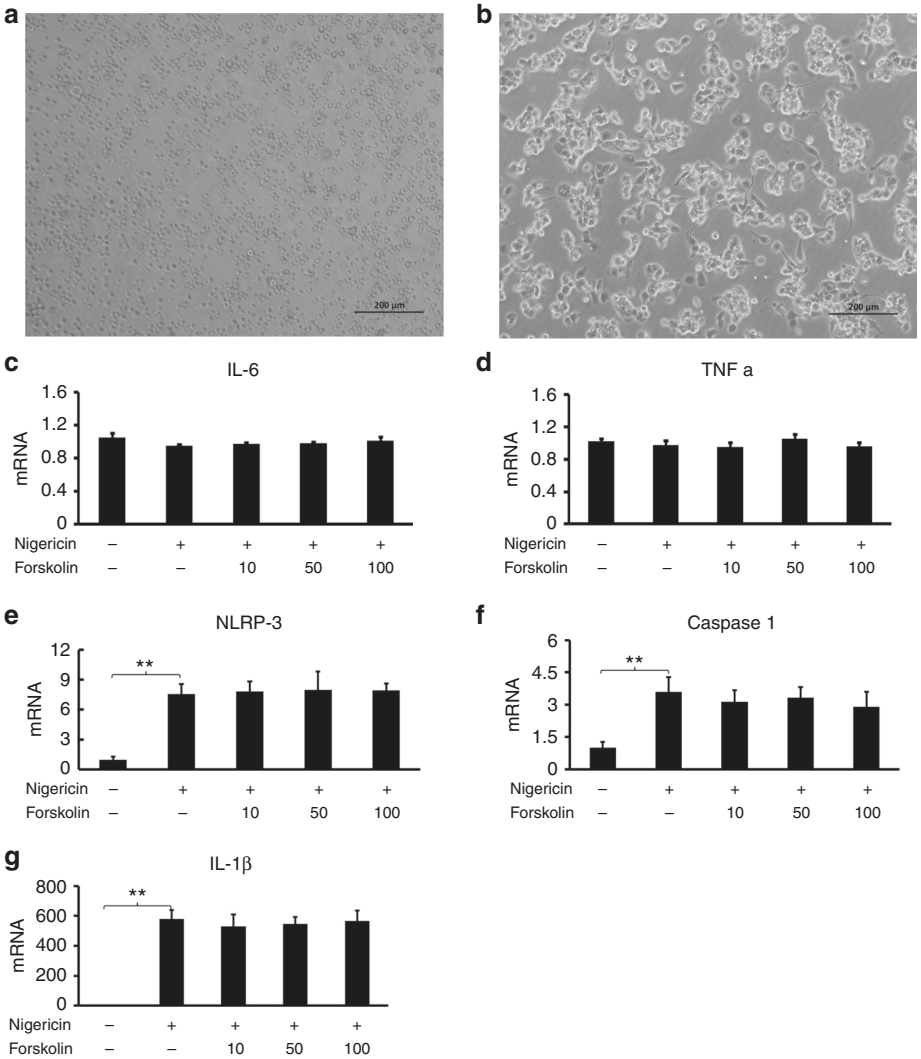

Fig. 1 The effect of forskolin on the expression of interleukin-6 (IL-6), tumor necrosis factor (TNF), nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3), caspase-1, and IL-1β in activated THP-1 macrophages. **a** THP-1 monocytes, scale bar 200 μm; **b** THP-1 macrophages after 72 h phorbol 12-myristate 13-acetate (PMA) incubation), scale bar 200 μm; **c–g** mRNA expression of IL-6, TNF, NLRP3, caspase-1, and IL-1β in THP-1 macrophages activated for 1 h with nigericin (4 μmol/L) with/without forskolin in the indicated concentrations (10, 50, and 100 μmol/L). After comparing with β-actin house keeping gene, then normalized each control group = 1; data represent the means ± SD of 12 samples (three repeated experiments of each for four samples); $^{**}P \leq 0.01$

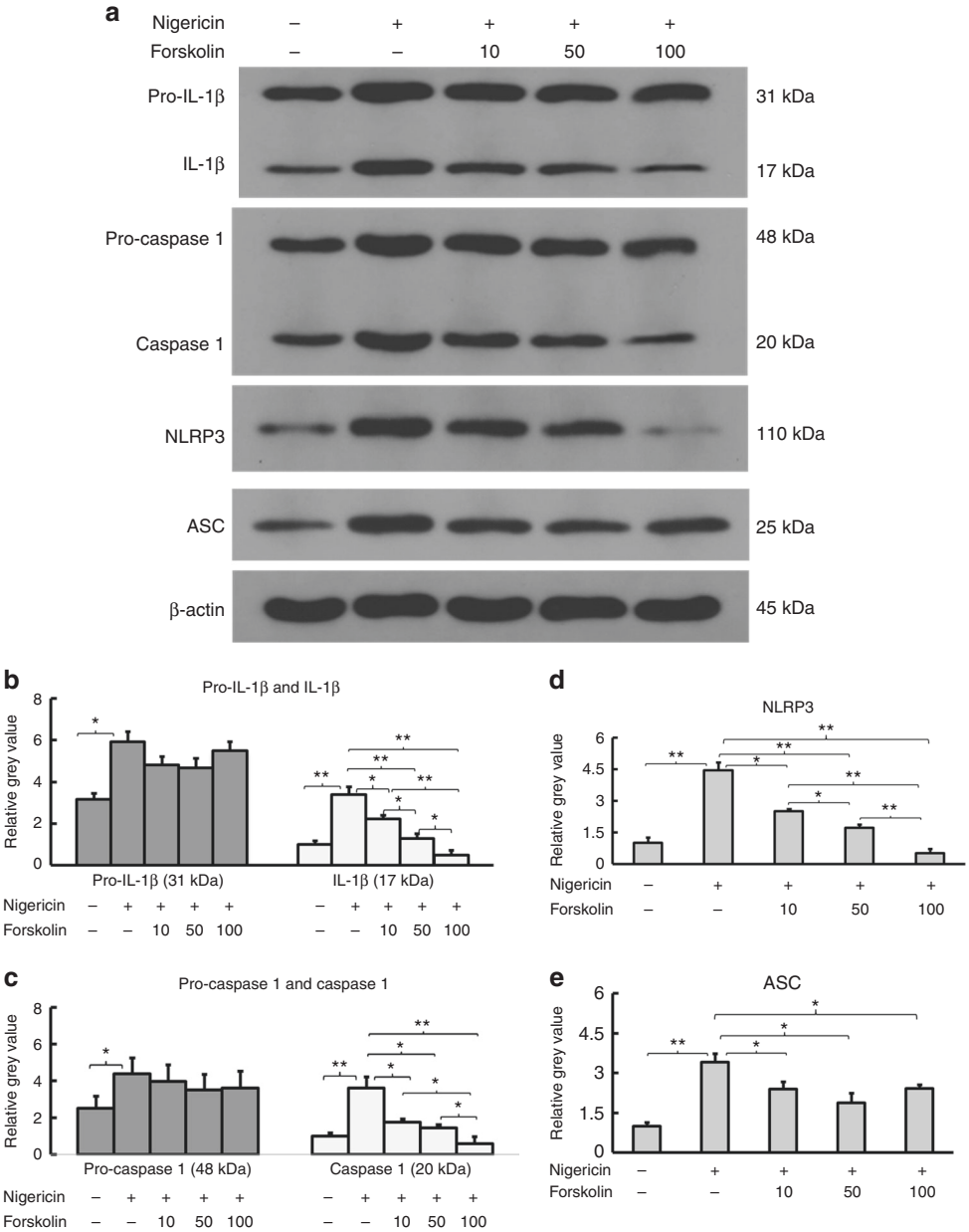

Fig. 2 The effect of forskolin on nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) inflammasome components in activated THP-1 macrophages. **a** The expression of interleukin-1β (IL-1β), caspase-1, NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and actin was assessed from cell lysates. The western blots shown are the representative of four individual experiments; **b**–**e** The normalized protein expressions to β-actin. Data represent the means ± SD of four individual experiments; *$P \le 0.05$; **$P \le 0.01$

Effect of forskolin on the level cAMP in activated THP-1 macrophages

Forskolin increased the level of cytoplasmic cAMP dose-dependently in non-activated THP-1 macrophages ($P < 0.05$). However, activation of THP-1 macrophages with nigericin reduced cAMP concentration. The reason for this is not entirely clear, but one possibility is that cAMP is bound to the NLRP3, which is then directed to degradation together with cAMP as described by Yan et al.,[13] thus explaining the reduced level of cAMP ($P < 0.05$) (Fig. 3).

Effects of forskolin on the secretion of proinflammatory cytokines in activated THP-1 macrophages

The PMA differentiation primed THP-1 macrophages to secrete IL-6 and TNFα without a further stimulus. Forskolin had no effect on the secretion of IL-6 and TNFα in the presence or absence of nigericin (Fig. 4a, b). However, secretion of the inflammasome-dependent cytokine IL-1β was increased by nigericin activation from basal 584 to 2695.6 pg/mL ($P < 0.001$). Forskolin dose-dependently reduced the secretion of IL-1β in nigericin-activated THP-1 macrophages to 1858.4 pg/mL ($P < 0.05$), 1467.9 pg/mL ($P < 0.05$), and 1246.7 pg/mL ($P < 0.01$), respectively, at concentrations of 10, 50, and 100 µmol/L (Fig. 4c).

Effects of forskolin on the secretion of IL-1β in activated human primary macrophages

The LPS primed human primary macrophages, followed by ATP, MSU, or nigericin stimulus, respectively. Secretion of the inflammasome-dependent cytokine IL-1β was increased by ATP,

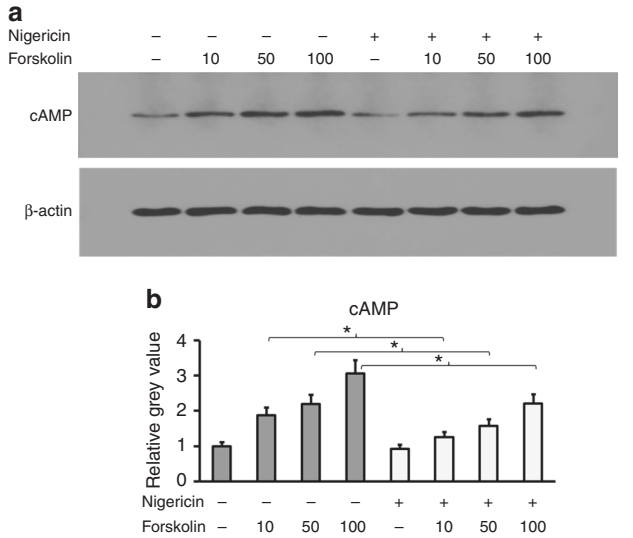

Fig. 3 Effect of forskolin on the level cyclic adenosine monophosphate (cAMP) in activated THP-1 macrophages. **a** The expression of cAMP in THP-1 macrophages treated with indicated concentrations of forskolin (10, 50, and 100 μmol/L) in or treated with indicated concentrations of forskolin and activated with nigericin (4 μmol/L, 1 h). The western blots shown are the representative of four individual experiments; **b** the normalized protein expressions to β-actin. Data represent the means ± SD of 12 samples (three repeated experiments of each for four samples); *$P \le 0.05$

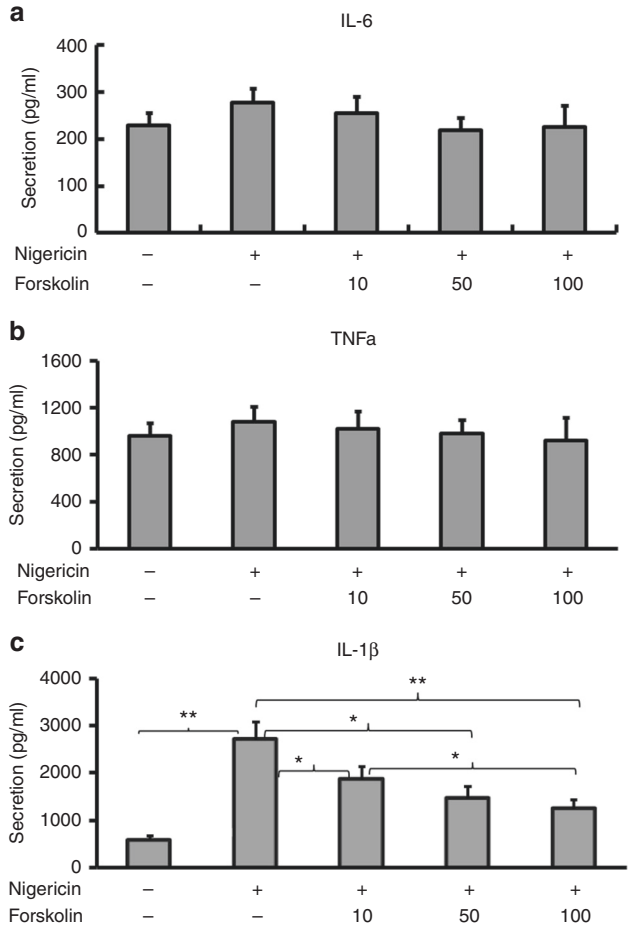

Fig. 4 Effects of forskolin on the secretion of proinflammatory cytokines in activated THP-1 macrophages. Effects of indicated concentrations of forskolin (10, 50, and 100 μmol/L) on the secretion of **a** interleukin-6 (IL-6), **b** tumor necrosis factor (TNF), and **c** mature IL-1β in nigericin (4 μmol/L, 1 h) activated THP-1 macrophages. The secretion of cytokines was measured by enzyme-linked immunosorbent assay (ELISA). Data represent the means ± SD of 12 samples (three repeated experiments of each for four samples); *$P \le 0.05$; **$P \le 0.01$

MSU, and nigericin activation from basal 0.04 pg/mL to 37.7, 57.4, and 25.9 pg/mL, respectively ($P < 0.001$). Forskolin reduced the secretion of IL-1β in ATP-, MSU-, and nigericin-activated primary macrophages to 25.2 pg/mL ($P < 0.05$), 23.9 pg/mL ($P < 0.01$), and 10.5 pg/mL ($P < 0.01$), respectively (Fig. 5).

DISCUSSION

IL-1β is an important proinflammatory cytokine that plays a role in addition to inflammatory diseases also in metabolic diseases. Excessive or aberrant secretion of IL-1β is the key factor in the pathogenesis of the rare autoinflammatory diseases,[16] in sepsis, and multiple organ failure in children, and also in common chronic diseases with low-grade inflammation,[17] such as type 2 diabetes mellitus and cardiovascular diseases.[1,7] IL-1β and IL-18 are secreted in particular by cells of monocyte–macrophage lineage upon activation of the NLRP3 inflammasome. Human THP-1 monocyte cell line is the most commonly used model for inflammasome activation studies, as after PMA differentiation they express inflammasome components abundantly. By using THP-1 macrophages, the effect of forskolin could be studied on inflammasome activation without a prior induction of the priming step, that is, the activation of nuclear factor-κB (NF-κB) signaling via TLR4 or cytokine receptors.[16,17]

Nigericin is a bacterial pore-forming toxin that acts as a K^+ ionophore and activates the NLRP3 inflammasome by stimulating an intracellular K^+ efflux through pannexin-1-dependent pathway that induces the assembly of the NLRP3 inflammasome and its subsequent activation.[18,19] In PMA-differentiated THP-1 macrophages, nigericin alone activates the NLRP3 inflammasome and the secretion of IL-1β.

Different compounds that increase the intracellular levels of cAMP have been shown to reduce NF-κB signaling[12] and the activation of the NLRP3 inflammasome.[13,14] Also, prostaglandin E_2 (PGE_2) is an activator of cAMP and has been shown to inhibit NLRP3 inflammasome activation in human primary macrophages. The effect of PGE_2 was shown to be mediated via the phosphorylation of NLRP3 sensor protein by protein kinase A.[14,20] However, the effect of PGE_2 is transient,[20] and in addition PGE_2 increases the transcription of pro-IL-1β and adds to the proinflammatory effects of LPS.[21] Therefore, in human monocytes, in which LPS alone is sufficient for NLRP3 inflammasome activation, PGE_2 increases the LPS-induced inflammasome activation.

We studied the effect of adenylate cyclase activator forskolin on inflammasome activation in THP-1 macrophages. As expected, the treatment of THP-1 macrophages with forskolin increased the concentration of intracellular cAMP. Forskolin treatment had no effect on the expression of several NF-κB-regulated cytokines, TNF, IL-6, or IL-1β, and also the level of constantly expressed caspase-1 was not affected. However, forskolin reduced the protein expression of NLRP3, which is also dependent of the NF-κB.[22] Forskolin significantly inhibited the nigericin-induced activation of the NLRP3 inflammasome, reflected by dose-dependent reduction of cleaved IL-1β and caspase-1. In addition, the secretion of mature IL-1β into the culture medium was dose-dependently reduced. It is conceivable that the reduced NLRP3 inflammasome

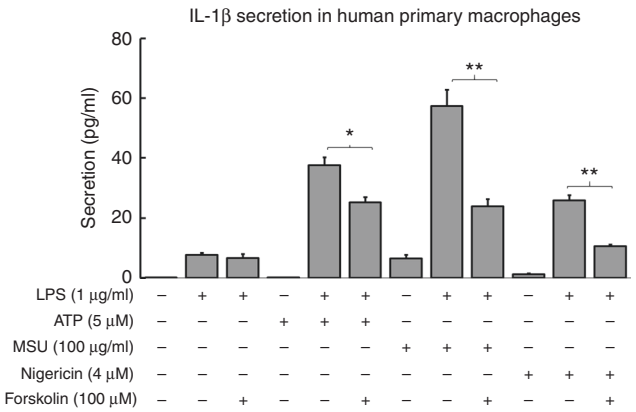

Fig. 5 Effects of forskolin on the secretion of interleukin-1β (IL-1β) in activated human primary macrophages. Effects of indicated concentrations of forskolin (100 μmol/L) on the secretion of **a** mature IL-1β in nigericin (4 μmol/L, 1 h) activated human primary macrophages. The secretion of cytokines was measured by enzyme-linked immunosorbent assay (ELISA). Data represent the means ± SD of 12 samples (three repeated experiments of each for four samples); *$P \le 0.05$; **$P \le 0.01$

activation was partly due to the reduced expression of its components NLRP3 and ASC.

We also measured the effect of nigericin activation on forskolin-induced concentrations of cytoplasmic cAMP and found that the nigericin activation reduced the levels of cAMP, although a clear dose-dependent increase of cAMP was still detected after inflammasome activation. The reduction of cAMP could be due to its binding to NLRP3 inflammasome, which results in the ubiquitination and degradation of NLRP3, and most likely also cAMP.[13,14]

It is conceivable that the mechanism of forskolin-induced inhibition of inflammasome activation proceeds via increased production of cAMP, which then binds to the NLRP3 protein and targets it for degradation, thus interrupting the function of the inflammasome, and secretion of IL-1β in human macrophages. This mechanism is further supported by our results of the mRNA expression of NLRP3, which was not reduced by forskolin, indicating that the NLRP3 was expressed but the protein was degraded. However, unlike the effect of PGE_2, the effect of forskolin was not transient, as the THP-1 macrophages were stimulated for 1 h with forskolin. Whether the mechanisms that mediate the inhibitory effects of forskolin in human macrophages are consistent with mouse macrophages and whether the increase of cAMP is the main mediator of the inhibition still require further investigation.

In conclusion, our results support the findings that forskolin, along with other compounds that increase the level of cAMP such as apremilast, negatively regulate NLRP3 inflammasome activation and subsequent secretion of IL-1β. Thus, increasing the levels of cAMP with substances such as forskolin could represent a novel approach for treatment of patients with NLRP3 activation-related severe systemic inflammatory diseases.

ACKNOWLEDGEMENTS

We thank Xiaoping Shang at the Medical Records Department for the statistical assistance. This work was supported by the National Natural Science Foundation of China (81500588) and an independent research grant from the Finnish Cultural Foundation (00130167). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

Y.C. got the financial supports from the Foundations, conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures, reviewed drafts of the paper, redid the experiments, and revised the paper. J.G.W. performed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper, and revised the paper. J.J.F. performed the experiments, analyzed the data, prepared figures, reviewed drafts of the paper, and revised the paper. Y.H.W. performed the experiments, reviewed drafts of the paper, and revised the paper. T.F.L. contributed reagents and some materials, reviewed the draft of the paper, and revised the paper. K.N. contributed analysis protocols, helped to design the experiments, reviewed the manuscript, and revised the paper. K.K.E. contributed reagents, helped to design the experiments, reviewed the manuscript, and revised the paper. D.X. helped redo some experiments and reviewed the revised paper.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. Scott, H. F. et al. The prevalence and diagnostic utility of systemic inflammatory response syndrome vital signs in a pediatric emergency department. *Acad. Emerg. Med.* **22**, 381–389 (2015).
2. Pfalzgraff, A. & Weindl, G. Intracellular lipopolysaccharide sensing as a potential therapeutic target for sepsis. *Trends Pharm. Sci.* **40**, 187–197 (2019).
3. Malmo, J. et al. Cytokine profiles in human metapneumovirus infected children: identification of genes involved in the antiviral response and pathogenesis. *PLoS ONE* **11**, e0155484 (2016).
4. Li, D. et al. Regulation of the NLRP3 inflammasome and macrophage pyroptosis by the p38 MAPK signaling pathway in a mouse model of acute lung injury. *Mol. Med. Rep.* **18**, 4399–4409 (2018).
5. Elliott, E. I. & Sutterwala, F. S. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunol. Rev.* **265**, 35–52 (2015).
6. Guo, H. T., Callaway, J. B. & Ting, J. P. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat. Med.* **21**, 677–687 (2015).
7. Cuenca, A. G. et al. Role of innate immunity in neonatal infection. *Am. J. Perinatol.* **30**, 105–112 (2013).
8. Man, S. M. & Kanneganti, T. D. Regulation of inflammasome activation. *Immunol. Rev.* **265**, 6–21 (2015).
9. Shenker, B. J. et al. Aggregatibacter actinomycetemcomitans cytolethal distending toxin activates the NLRP3 inflammasome in human macrophages, leading to the release of proinflammatory. *Infect. Immun.* **83**, 1487–1496 (2015).
10. Jo, E. K. et al. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell. Mol. Immunol.* **13**, 148–159 (2016).
11. Daems, C. et al. MEF2 cooperates with forskolin/cAMP and GATA4 to regulate star gene expression in mouse MA-10 leydig cells. *Endocrinology* **156**, 2693–2703 (2015).
12. Du, X. et al. Isoforskolin and forskolin attenuate lipopolysaccharide-induced inflammation through TLR4/MyD88/NF-κB cascades in human mononuclear leukocytes. *Phytother. Res.* **33**, 602–609 (2019).
13. Yan, Y. et al. Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. *Cell* **160**, 62–73 (2015).
14. Sokolowska, M. et al. Prostaglandin E2 inhibits NLRP3 inflammasome activation through EP4 receptor and intracellular cyclic AMP in human macrophages. *J. Immunol.* **194**, 5472–5487 (2015).
15. Yeo, C. et al. Ficoll-Paque versus Lymphoprep: a comparative study of two density gradient media for therapeutic bone marrow mononuclear cell preparations. *Regen. Med.* **4**, 689–696 (2009).
16. Torre-Minguela, C., Mesa Del Castillo, P. & Pelegrín, P. The NLRP3 and Pyrin inflammasomes: implications in the pathophysiology of autoinflammatory diseases. *Front. Immunol.* **8**, 43 (2017).
17. Mangan, M. S. J. et al. Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat. Rev. Drug Discov.* **17**, 688 (2018).
18. Perregaux, D. & Gabel, C. A. Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J. Biol. Chem.* **269**, 15195–15203 (1994).

19. Pelegrin, P. & Surprenant, A. Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway. *J. Biol. Chem.* **282**, 2386–2394 (2007).
20. Mortimer, L. et al. NLRP3 inflammasome inhibition is disrupted in a group of auto-inflammatory disease CAPS mutations. *Nat. Immunol.* **17**, 1176–1186 (2016).
21. Zasłona, Z. et al. The induction of pro-IL-1β by lipopolysaccharide requires endogenous prostaglandin E_2 production. *J. Immunol.* **198**, 3558–3564 (2017).
22. Bauernfeind, F. G. et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* **183**, 787–791 (2009).