Excess phosphate promotes SARS-CoV-2 N protein-induced NLRP3 inflammasome activation via the SCAP-SREBP2 signaling pathway

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Abstract. Hyperphosphatemia or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection can promote cardiovascular adverse events in patients with chronic kidney disease. Hyperphosphatemia is associated with elevated inflammation and sterol regulatory element binding protein 2 (SREBP2) activation, but the underlying mechanisms in SARS-CoV-2 that are related to cardiovascular disease remain unclear. The present study aimed to elucidate the role of excess inorganic phosphate (PI) in SARS-CoV-2 N protein-induced NLRP3 inflammasome activation and the underlying mechanisms in vascular smooth muscle cells (VSMCs). The expression levels of SARS-CoV-2 N protein, SREBP cleavage-activating protein (SCAP), mature N-terminal SREBP2, NLRP3, procaspase-1, cleaved caspase-1, IL-1β and IL-18 were examined by western blotting. The expression levels of SREBP2, HMG-CoA reductase, HMGCS1, low density lipoprotein receptor, proprotein convertase subtilisin/kexin type 9 (PCSK9), SREBP1c, fatty acid synthase, stearyl coenzyme A desaturase 1, acetyl-CoA carboxylase α and ATP-citrate lyase were determined by reverse transcription-quantitative PCR. The translocation of SCAP or NLRP3 from the endoplasmic reticulum to the Golgi was detected by confocal microscopy. The results showed that excess PI promoted SCAP-SREBP and NLRP3 complex translocation to the Golgi, potentially leading to NLRP3 inflammasome activation and lipogenic gene expression. Furthermore, PI amplified SARS-CoV-2 N protein-induced inflammation via the SCAP-SREBP pathway, which facilitates NLRP3 inflammasome assembly and activation. Inhibition of phosphate uptake with phosphonoformate sodium alleviated NLRP3 inflammasome activation and reduced SREBP-mediated lipogenic gene expression in VSMCs stimulated with PI and with SARS-CoV-2 N protein overexpression. Inhibition of SREBP2 or small interfering RNA-induced silencing of SREBP2 effectively suppressed the effect of PI and SARS-CoV-2 N protein on NLRP3 inflammasome activation and lipogenic gene expression. In conclusion, the present study identified that PI amplified SARS-CoV-2 N protein-induced NLRP3 inflammasome activation and lipogenic gene expression via the SCAP-SREBP signaling pathway.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection leads to transient hyperphosphatemia (1), which is associated with a higher risk of death from atherosclerosis in both the general population and patients with chronic kidney disease (2-4). Hyperphosphatemia on admission has been associated with late acute kidney injury in patients with coronavirus disease 2019 (COVID-19) (5). Likewise, an increased risk of death from severe cases of COVID-19 has been observed in patients with chronic kidney disease (6). Therefore, the present study aimed to clarify the mechanism by which hyperphosphatemia promotes the progression of SARS-CoV-2-related cardiovascular disease, particularly the effect of excess inorganic phosphate (PI) on SARS-CoV-2-induced cytokine storm.

Sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP) serves a central role in regulating lipid homeostasis and inflammasome activation. Low serum cholesterol concentration increases the activity of SREBP2 in patients with COVID-19 (7,8). In addition, SARS-CoV-2 activates SREBP2 and can lead to a cytokine storm in the peripheral blood monocytes of patients with

COVID-19 (9). Moreover, an inhibitor of SCAP-SREBP, 25-hydroxycholesterol, has been shown to suppress SARS-CoV-2 replication and excessive inflammatory responses in patients with COVID-19 (10). Therefore, the SCAP-SREBP signaling pathway has been suggested to serve a crucial role in SARS-CoV-2-induced cytokine storm and organ damage.

Serum phosphate levels are positively correlated with SCAP-SREBP2-mediated cholesterol synthesis (11). PI has been reported to accelerate foam cell and atherosclerotic plaque formation in an apolipoprotein E (ApoE) knockout (KO) mouse model (12) through enhanced SCAP-SREBP2 activation (13). In addition, it has been demonstrated that SCAP overexpression in vascular smooth muscle cells (VSMCs) induces NLRP3 inflammasome activation and atherosclerosis (14). Recently, Pan *et al* (15) found that the SARS-CoV-2 N protein can induce NLRP3 inflammasome activation, indicating that NLRP3 may be a coronavirus inflammatory target. However, the underlying effects of PI on SARS-CoV-2 N protein-induced cytokine storm remain unclear.

SARS-CoV-2 N protein is responsible for condensation of the viral genome and promotes NLRP3 inflammasome activation to induce hyperinflammation (15). The present study aimed to investigate the effect of PI and SARS-CoV-2 N protein on NLRP3 inflammasome activation. Furthermore, this study investigated whether PI was able to amplify SARS-CoV-2 N protein-induced NLRP3 inflammasome activation and lipogenesis through SCAP-SREBP signaling pathways.

Materials and methods

Cell culture. Mouse VSMCs (MOVAS cell line; CRL-2797) were purchased from the American Type Culture Collection, and were cultured in DMEM/F-12 (HyClone; Cytiva) supplemented with 10% fetal bovine serum (Shanghai ExCell Biology, Inc.) as described previously (14). Thereafter, the VSMCs were cultured at $2x10^6$ cells/dish for 24 h at 37˚C and then starvation was induced with serum-free DMEM for 24 h at 37˚C before the experimental treatment. In some experiments, the VSMCs were pretreated with lycorine (20 μM; MedChemExpress) for 12 h before 3 mmol/l PI treatment for 24 h at 37˚C. For some experiments, SARS-CoV-2 N protein-overexpressing VSMCs were pretreated with 1.0 mmol/l phosphonoformate sodium (PFA; CAS. no. 63585-09-1; Shanghai Aladdin Biochemical Technology Co., Ltd.) for 24 h before PI treatment for 24 h at 37˚C. PFA is not only an antiviral drug, but also a specific cell membrane sodium phosphate transporter (pit1/2) inhibitor that prevents extracellular phosphate from entering the cell (13). Phosphate stock solution contained NaH_2PO_4 and Na_2HPO_4 (pH 7.40).

Lentivirus-mediated SARS-CoV-2 N protein transduction. VSMCs were transduced with 50 μl SARS-CoV-2 N protein lentivirus or negative control empty vector (Lenti-EF1α-2019-nCOV-N-Flag/His-CMV-Puro; $1x10^8$ IU/ml; cat. no. C3021; Beyotime Institute of Biotechnology) for 24 h with a multiplicity of infection of 50 at 37˚C. A total of 48 h post-transduction, the VSMCs were cultured for 4-7 days with 2 μg/ml puromycin (Beyotime Institute of Biotechnology) at 37˚C. Subsequently, the VSMCs were lysed and the specific protein expression in VSMCs was confirmed by western blotting.

RNA interference. The VSMCs or SARS-CoV-2 N protein-overexpressing VSMCs were transfected with 100 pmol SREBP2 small interfering RNA (siRNA) or negative control (NC) siRNA using 5 μl Lipo8000™ (cat. no. C0533; Beyotime Institute of Biotechnology) for 24 h at 37˚C, according to the manufacturer's instructions. Subsequently, the SARS-CoV-2 N protein-overexpressing VSMCs were further cultured for 12 h and then treated with or without 0.2 mmol PI for 24 h at 37˚C. The sequence of the SREBP2-targeted siRNA was 5'-GCGGACAACACACAAUAUCAU-3' and the sequence of the scrambled siRNA control was 5'-UUCUCCGAACGUGUCACGUTT-3'. SREBP2 siRNA and the scrambled siRNA were constructed by Sangon Biotech Co., Ltd.

Western blotting. Total protein was extracted from VSMCs using ice-cold RIPA Lysis Buffer (cat. no. P0013B; Beyotime Institute of Biotechnology), containing a protease and phosphatase inhibitor cocktail (cat. no. P1045; Beyotime Institute of Biotechnology). Protein concentrations were measured using a BCA protein assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology) and 80 μg protein/lane was separated by SDS-PAGE on a 10 or 12% gel. The separated proteins were then transferred to a PVDF membrane and blocked for 2 h. The PVDF membranes were incubated with the following antibodies overnight at 37˚C: SARS-CoV-2 N protein (cat. no. AF0325; 1:500; Beyotime Institute of Biotechnology), SCAP (cat. no. NBP2-04113; 1:1,000; Novus Biologicals, LLC), mature N-terminal SREBP2 (N-SREBP2; 1:1,000; 70 Kd, cat. no. ab30682; Abcam), NLRP3 (cat. no. WL02635; 1:1,000; Wanleibio Co., Ltd.), procaspase-1 (cat. no. WL02996; 1:800; Wanleibio Co., Ltd.), cleaved caspase-1 (cat. no. WL03450; 1:800; Wanleibio Co., Ltd.), IL-1β (cat. no. 16806-1-AP; 1:600; Wuhan Sanying Biotechnology), IL-18 (cat. no. 10663-1-AP; 1:500; Wuhan Sanying Biotechnology) and β-actin (cat. no. 81115-1-RR; 1:5,000; Wuhan Sanying Biotechnology). After overnight incubation, the blots were incubated with goat anti-rabbit IgG-HRP antibody (1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 2 h at 37˚C. Finally, the bands were visualized using the ECL reagent kit (cat. no. WBKlS0100; MilliporeSigma) and the densities of the bands were analyzed using ImageJ 1.47i software (National Institutes of Health) for semi-quantification.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). The total RNA was extracted from the cells with TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using a high-capacity cDNA synthesis kit (cat. no. BL696A; Biosharp Life Sciences). The RT protocol was as follows: 25˚C for 10 min, 55˚C for 15 min and 85˚C for 5 min. qPCR analysis was performed using the SYBR Green PCR Mix kit (cat. no. BL697A; Biosharp Life Sciences) and the ABI7500 System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min; followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec, with a final extension at 72˚C for 30 sec. The specific primer sequences are listed in Table I. The

Table I. Reverse transcription-quantitative PCR primer sequences.

Mouse gene name	Forward, 5'-3'	Reverse, 5'-3'
SREBP2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
HMGCoAR	AGCTTGCCCGAATTGTATGTG	TCTGTTGTGAACCATGTGACTTC
HMGCS1	GCCGTGAACTGGGTCGAA	GCATATATAGCAATGTCTCCTGCAA
LDLR	TGACTCAGACGAACAAGGCTG	ATCTAGGCAATCTCGGTCTCC
PCSK9	CAGCGGCACCCTCATAGG	CCCGAGGGCTGGATTAGC
SREBP1c	ATCGGCGCGGAAGCTGTCGGGGTAGCGTG	ACTGTCTTGGTTGTTGATGAGCTGGAGCCAT
FASN	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
SCD1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
ACACA	TGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
ACLY	GCCAGCGGGAGCACATC	CTTTGCAGGTGCCACTTCATC
NLRP3	TGTGAGAAGCAGGTTCTACTCT	GACTGTTGAGGTCCACACTCT
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
B-actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

SREBP, sterol regulatory element binding protein.

relative fold changes in gene expression were normalized to the mRNA expression levels of β-actin and were quantified using the $2^{-\Delta\Delta Cq}$ method (16).

Confocal microscopy. VSMCs were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 for 5 min at 37˚C. Thereafter, the VSMCs were treated overnight with anti-SCAP (1:100; cat. no. NBP2-04113; Novus Biologicals, LLC), anti-NLRP3 (1:200; cat. no. NBP1-97601; Novus Biologicals, LLC) and anti-Golgin 97 (1:150; cat. no. PA5-30048; Thermo Fisher Scientific, Inc.) at 37˚C. Then, the VSMCs were stained with goat anti-rabbit IgG (TRITC; 1:100; cat. no. SA00007-2; Proteintech Group, Inc.) or goat anti-mouse IgG (FITC; 1:100; cat. no. SA00003-1; Proteintech Group, Inc.) for 45 min at 37˚C. The nuclei were stained with DAPI for 8 min at 37˚C and the images were viewed with a confocal laser scanning microscope (Zeiss GmbH).

Statistical analysis. Data are presented as the mean ± SD. The significance between two groups was assessed using unpaired Student's t-test. Differences among multiple groups were analyzed by one-way analysis of variance with Tukey's post hoc test. All statistical analyses were performed using SPSS software (version 18, SPSS, Inc.). All of the experiments were performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PI amplifies SARS-CoV-2 N protein-induced lipogenesis and NLRP3 inflammasome activation through SCAP-SREBP2. SCAP transports SREBP2 to the Golgi apparatus, and then SREBP2 becomes transcriptionally active when its N-terminal DNA-binding fragment is proteolytically cleaved to its mature, active form (mature N-terminal SREBP2, N-SREBP2) (17,18). Subsequently, the cytoplasmic N-SREBP2 translocates to the nucleus to bind to DNA and initiate gene transcription. This process can be assessed by measuring the mature N-SREBP2 protein in the whole cell lysate (17-19). Our previous study indicated that SARS-CoV-2 N protein activates the dissociation of SCAP from the endoplasmic reticulum (ER), resulting in SREBPs activation, increased lipogenic gene expression and NLRP3 inflammasome activation (20). To examine the effect of PI on the activation of SCAP-SREBP2, SARS-CoV-2 N protein-overexpressing VSMCs were incubated with PI (3 mmol/l) for 24 h. Firstly, SARS-CoV-2 N protein was significantly increased in SARS-CoV-2 N protein-overexpressing VSMCs (Fig. 1A). Secondly, compared with those in the SARS-CoV-2 N protein group, PI increased the mRNA expression levels of SREBP2-targeted genes, i.e. HMG-CoA reductase (HMGCoAR), low-density lipoprotein receptor (LDLR) and proprotein convertase subtilisin/kexin type 9 (PCSK9) in SARS-CoV-2 N protein-overexpressing VSMCs (Fig. 1B). In addition, the mRNA expression levels of SREBP1c-targeted genes, i.e. recombinant fatty acid synthase (FASN), stearyl coenzyme A desaturase 1 (SCD1), acetyl-CoA carboxylase α (ACACA) and ATP-citrate lyase (ACLY) were significantly increased in SARS-CoV-2 N protein-overexpressing VSMCs (Fig. 1C). In addition, the results indicated that PI significantly increased SCAP and SREBP2 protein expression levels in SARS-CoV-2 N protein-overexpressing VSMCs compared with in the non-phosphate-treated control (Fig. 1D). Likewise, PI significantly increased the relative mRNA expression levels of NLRP3 and IL-1β (Fig. 1E), and the protein expression levels of NLRP3, procaspase-1, cleaved caspase-1 and IL-1β compared with those in the control group (Fig. 1F). These results indicated that PI could amplify SARS-CoV-2 N protein-induced NLRP3 inflammasome activation and lipogenesis, which may be mediated by SCAP-SREBPs.

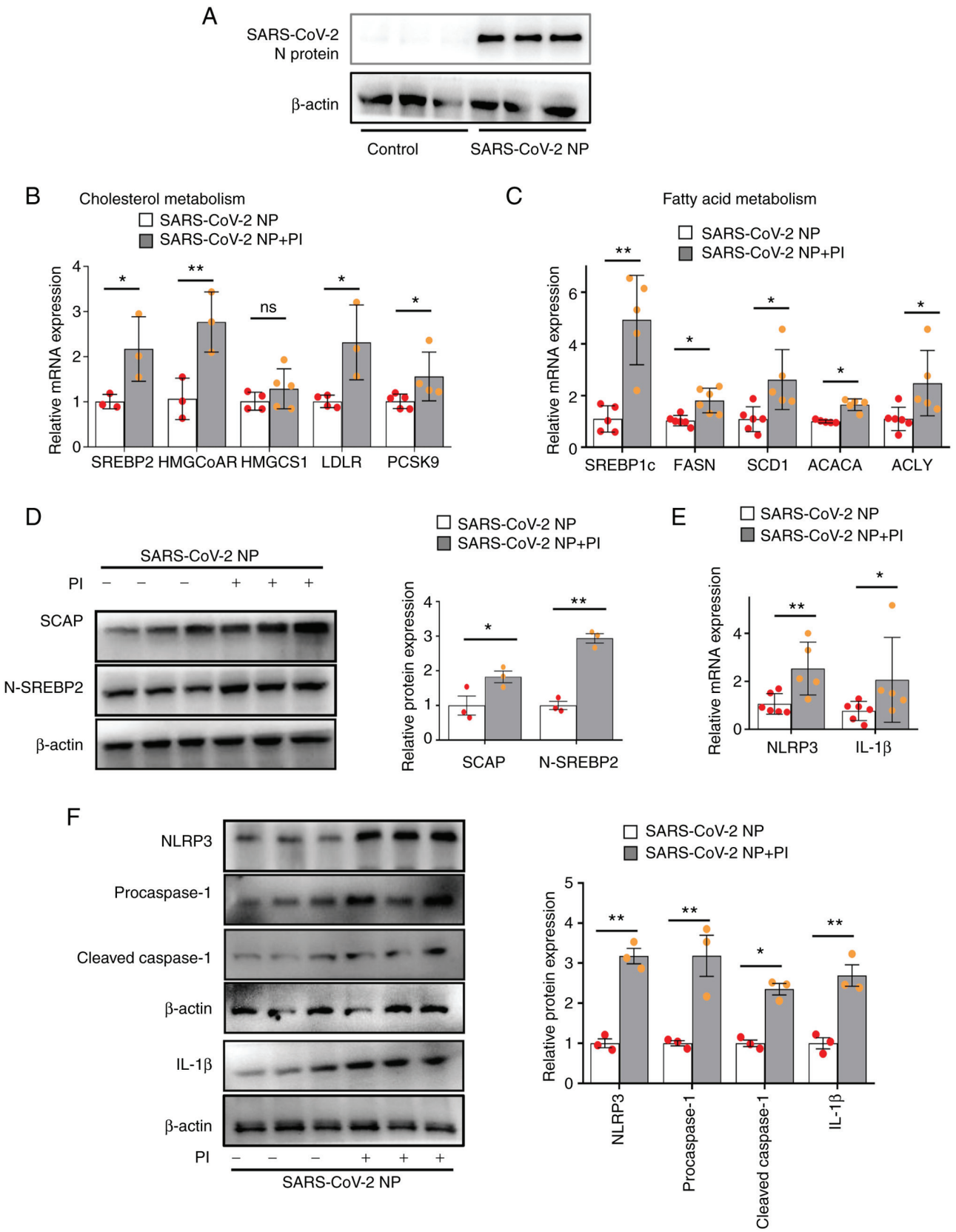

Figure 1. PI promotes SCAP-SREBP-induced lipogenesis and NLRP3 inflammasome activation in VSMCs overexpressing the SARS-CoV-2 N protein. The SARS-CoV-2 N protein-overexpressing VSMCs were treated with 3 mmol/l PI for 24 h. (A) Representative western blot images of SARS-CoV-2 N protein. (B) Quantification of SREBP2, HMGCoAR, HMGCS1, LDLR and PCSK9 mRNA expression levels. (C) Quantification of SREBP1c, FASN, SCD1, ACACA and ACLY mRNA expression levels. (D) Western blot analysis of SCAP and N-SREBP2. (E) Quantification of NLRP3 and IL-1β mRNA expression levels. (F) Western blot analysis of NLRP3, procaspase-1, cleaved caspase-1, and IL-1β expression (n≥3). *P<0.05, **P<0.01. PI, inorganic phosphate; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SREBP, sterol regulatory element binding protein; SCAP, SREBP cleavage-activating protein; N-SREBP2, mature N-terminal SREBP2; NP, N protein; HMGCoAR, HMG-CoA reductase; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; FASN, recombinant fatty acid synthase; SCD1, stearyl coenzyme A desaturase 1; ACACA, acetyl-CoA carboxylase α; ACLY, ATP-citrate lyase; VSMCs, vascular smooth muscle cells; ns, not significant..

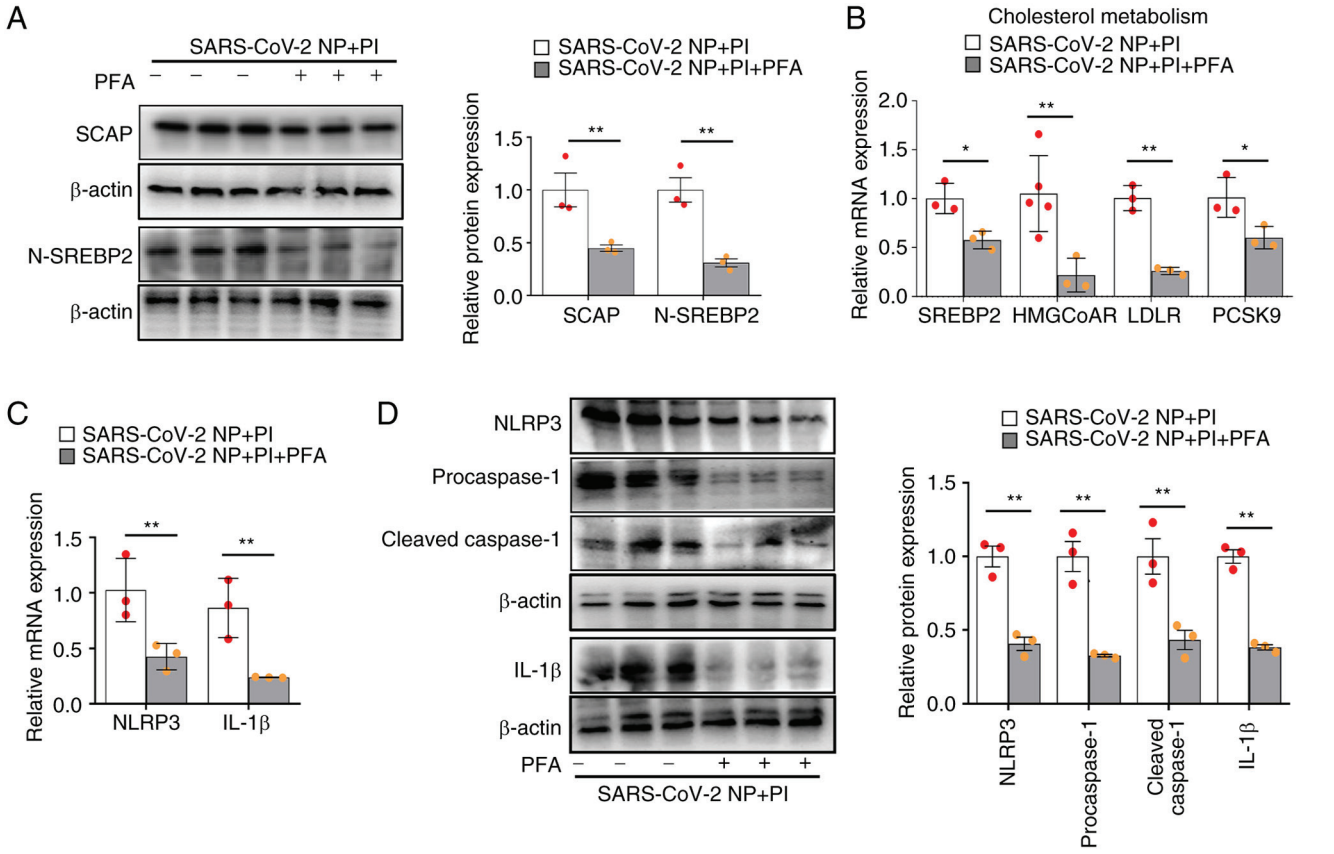

Figure 2. PFA inhibits lipogenesis and NLRP3 inflammasome activation in VSMCs stimulated with PI and overexpressing the SARS-CoV-2 N protein. The SARS-CoV-2 N protein-overexpressing VSMCs were treated with PI and 1.0 mmol/l PFA for 24 h. (A) Western blot analysis of SCAP and N-SREBP2. (B) Quantification of SREBP2, HMGCoAR, LDLR and PCSK9 mRNA expression levels. (C) Quantification of NLRP3 and IL-1β mRNA expression levels. (D) Western blot analysis of NLRP3, procaspase-1, cleaved caspase-1, and IL-1β expression (n≥3). *P<0.05, **P<0.01. PI, inorganic phosphate; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SREBP, sterol regulatory element binding protein; SCAP, SREBP cleavage-activating protein; N-SREBP2, mature N-terminal SREBP2; NP, N protein; VSMCs, vascular smooth muscle cells; HMGCoAR, HMG-CoA reductase; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; PFA, phosphonoformate sodium.

PFA reverses the effect of PI and SARS-CoV-2 N protein on SCAP-SREBP-induced lipogenesis and NLRP3 inflammasome activation. PFA is a specific inhibitor of intracellular phosphate uptake (21). In VSMCs overexpressing SARS-CoV-2 N protein, PFA significantly decreased the relative protein expression levels of SCAP and N-SREBP2 compared with those in the PI-treated group, suggesting that PFA reversed PI-induced SCAP-SREBP2 activation (Fig. 2A). Likewise, PFA significantly decreased the mRNA expression levels of SREBP2-targeted genes, i.e. HMGCoAR, LDLR and PCSK9 in PI-treated and SARS-CoV-2 N protein-overexpressing VSMCs (Fig. 2B). In addition, PFA significantly decreased the relative mRNA expression levels of NLRP3 and IL-1β (Fig. 2C), and the relative protein expression levels of NLRP3, procaspase-1, cleaved caspase-1 and IL-1β (Fig. 2D) compared with those in the phosphate-treated controls. These data indicated that PFA ameliorated the effect of PI and SARS-CoV-2 N protein on SCAP-SREBP-induced lipogenesis and NLRP3 inflammasome activation.

PI induces NLRP3 inflammasome activation through increased SCAP and NLRP3 translocation to the Golgi. It has previously been shown that SCAP-SREBP2 binds with NLRP3 to form a ternary complex that promotes its translocation to the Golgi, thereby facilitating NLRP3 inflammasome activation (22). Consistent with previous studies (14), we found that NLRP3 (Fig. 3A) or SCAP (Fig. 3B) proteins were separately localized to Golgin 97 after PI stimulation, compared with that in the non-phosphate-treated controls.

Furthermore, the relative protein expression levels of NLRP3, procaspase-1, cleaved caspase-1, IL-1β and IL-18 were significantly increased by PI compared with those in the non-phosphate-treated control group (Fig. 3C). Therefore, these data demonstrated that PI could induce NLRP3 inflammasome activation by promoting SCAP and NLRP3 translocation to the Golgi.

PI promotes cholesterol metabolism and fatty acid metabolism via the SCAP-SREBP pathway in VSMCs. It was demonstrated that the SCAP-SREBP inhibitor lycorine decreased the protein expression levels of SCAP and N-SREBP2 in VSMCs treated with PI (Fig. 4A). Compared with in the control group, PI also increased SREBP2-mediated cholesterol biosynthesis (i.e. SREBP2, HMGCoAR, LDLR and PCSK9 mRNA expression) (Fig. 4B) and SREBP 1c-mediated fatty acid synthesis (i.e. SREBP1c, FASN, ACACA and SCD1 mRNA expression) (Fig. 4C). Moreover, compared with in the PI-treated VSMCs, lycorine significantly decreased SEBP2-mediated cholesterol

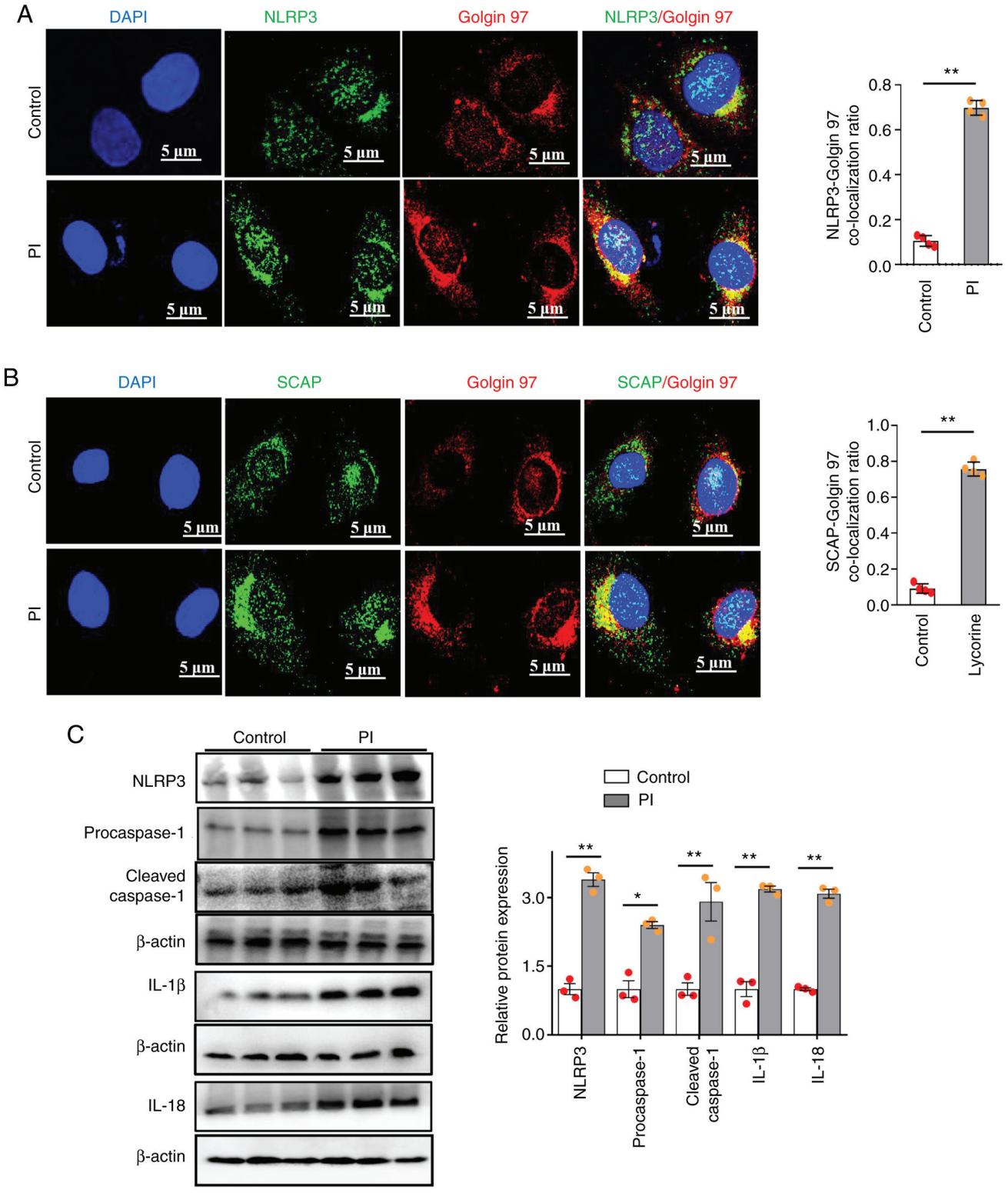

Figure 3. PI induces NLRP3 inflammasome activation via increased SCAP translocation to the Golgi in VSMCs. The VSMCs were treated with 3 mmol/l PI for 24 h. (A) NLRP3 and (B) SCAP co-localization with Golgin 97, as visualized via immunofluorescence staining. (C) Western blot analysis of NLRP3, procaspase-1, cleaved caspase-1, IL-1β and IL-18 (n=3). *P<0.05, **P<0.01. PI, inorganic phosphate; SCAP, sterol regulatory element binding protein cleavage-activating protein; VSMCs, vascular smooth muscle cells.

biosynthesis (i.e. SREBP2, HMGCoAR, LDLR and PCSK9 mRNA expression) and SREBP 1c-mediated fatty acid synthesis (i.e. SREBP1c, FASN, ACACA and SCD1 mRNA expression) (Fig. 4B and C). These results indicated that PI induced lipogenesis through SCAP-SREBPs.

PI promotes NLRP3 inflammasome activation via the SCAP-SREBP pathway in VSMCs. VSMCs were treated with lycorine to confirm whether SCAP was involved in PI-induced NLRP3 inflammasome activation, it was shown that in PI-treated VSMCs, lycorine significantly decreased the

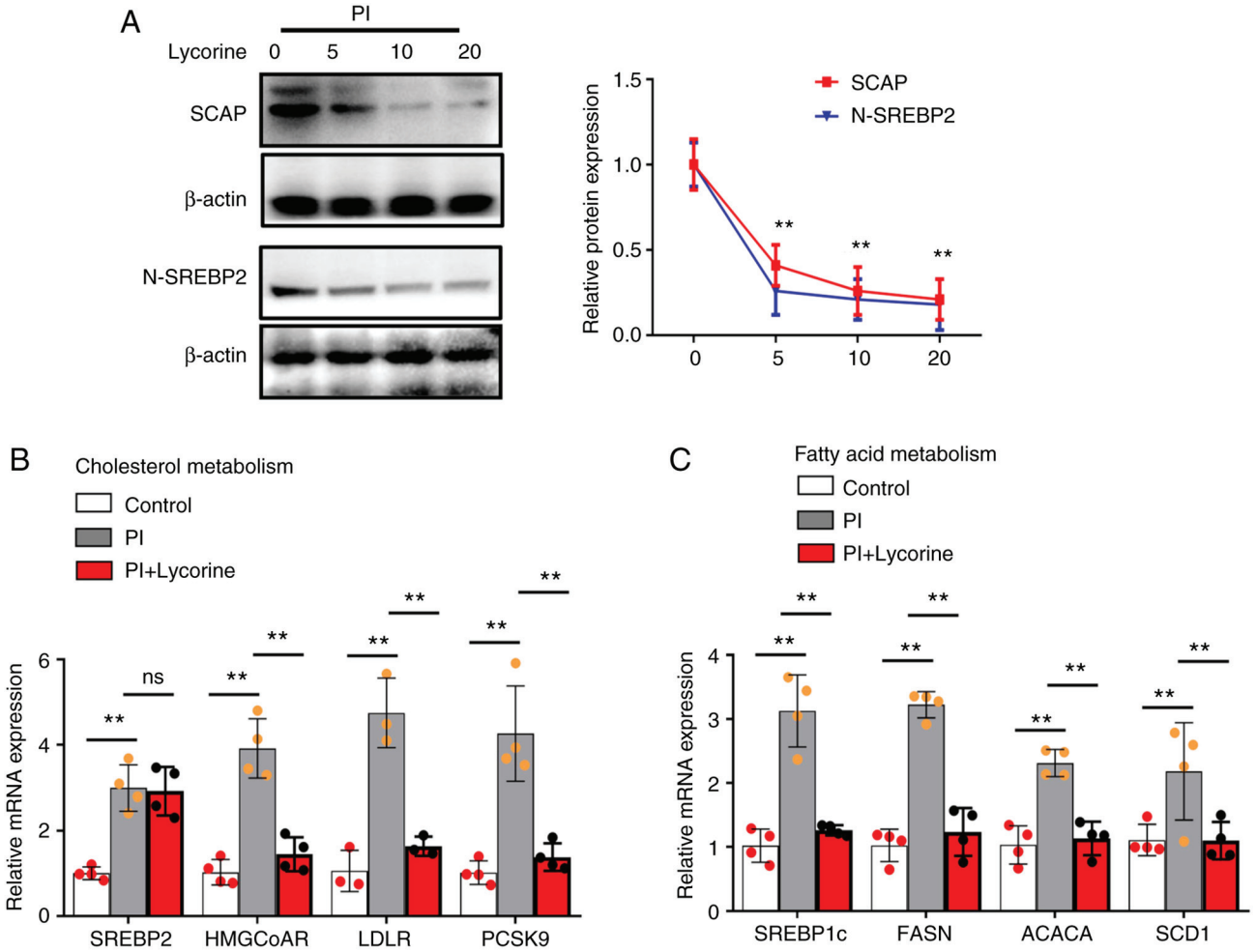

Figure 4. PI induces cholesterol metabolism and fatty acid metabolism via the SCAP-SREBP pathway in VSMCs. The VSMCs were treated with 3 mmol/l PI for 24 h with or without lycorine. (A) Western blot analysis of SCAP and N-SREBP2. (B) Quantification of SREBP2, HMGCoAR, LDLR and PCSK9 mRNA expression levels. (C) Quantification of SREBP1c, FASN, ACACA and SCD1 mRNA expression levels (n≥3). **P<0.01. PI, inorganic phosphate; SREBP, sterol regulatory element binding protein; N-SREBP2, mature N-terminal SREBP2; SCAP, SREBP cleavage-activating protein; HMGCoAR, HMG-CoA reductase; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; FASN, recombinant fatty acid synthase; ACACA, acetyl-CoA carboxylase α; SCD1, stearyl coenzyme A desaturase 1; VSMCs, vascular smooth muscle cells; ns, not significant.

NLRP3 (Fig. 5A) and SCAP (Fig. 5B) Golgi co-localization compared with the PI-only control.

Likewise, lycorine significantly decreased the relative protein expression levels of NLRP3, procaspase-1, cleaved caspase-1, IL-1β and IL-18 compared with those in the PI-only control (Fig. 5C). Therefore, PI was predicted to induce NLRP3 inflammasome activation by promoting SCAP-SREBP ER-to-Golgi translocation.

Inhibitor of SCAP or knockdown of SREBP2 suppresses PI and SARS-CoV-2 N protein-induced lipogenesis synthesis and inflammasome formation in VSMCs. SCAP-SREBP2 has previously been identified as serving a key role in inflammation and cholesterol metabolism (22). The present results demonstrated that lycorine significantly decreased SREBP2-mediated cholesterol biosynthesis (i.e. SREBP2, HMGCoAR, LDLR and PCSK9 mRNA expression) and SREBP1c-mediated fatty acid synthesis (i.e. FASN and ACLY mRNA expression) (Fig. 6A and B), compared with in PI-treated SARS-CoV-2 N-protein overexpressing VSMCs. Furthermore, SREBP2 siRNA significantly decreased the protein expression levels of SREBP2 in untreated VSMCs compared with those in the control siRNA group (Fig. 6D). Likewise, it was demonstrated that in PI-treated SARS-CoV-2 N-protein overexpressing VSMCs, lycorine and SREBP2 siRNA markedly reduced NLRP3, procaspase-1, cleaved caspase-1 and IL-1β expression compared with those in the control groups (Fig. 6C and E). These findings suggested that SCAP-SREBPs are necessary for lipogenesis and NLRP3 inflammasome activation in PI and SARS-CoV-2 N protein-overexpressing VSMCs.

Discussion

The risk of mortality in patients hospitalized with COVID-19 infection is strongly influenced by chronic kidney disease (CKD) (23). COVID-19-related mortality is ~10-times higher than that of patients with CKD and without COVID-19 (24). These studies indicated the incidence of COVID-19 in patients with CKD was higher than that in the community (24,25), and the risk of death from COVID-19 has been reported to be increased by ~6-fold in patients with chronic kidney disease (26). In addition, SARS-CoV-2 infection increases

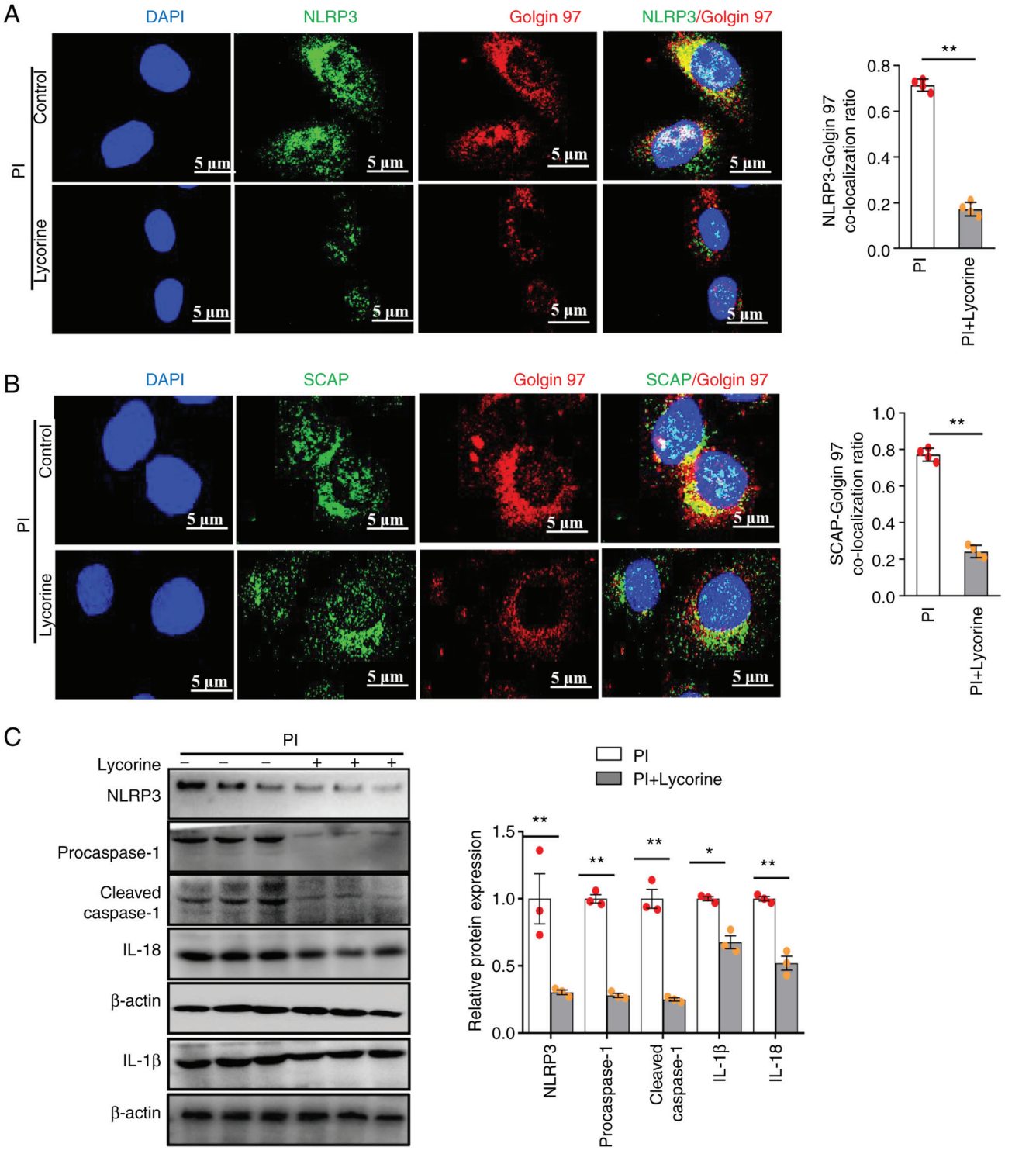

Figure 5. Lycorine blocks PI-induced NLRP3 inflammasome activation by preventing SCAP translocation to the Golgi. The vascular smooth muscle cells were treated with 3 mmol/l PI for 24 h with or without lycorine. (A) NLRP3 and (B) SCAP co-localization with Golgi97, as visualized via immunofluorescence staining. (C) Western blot analysis of NLRP3, procaspase-1, cleaved caspase-1, IL-1β and IL-18 (n=3). *P<0.05, **P<0.01. PI, inorganic phosphate; SCAP, sterol regulatory element binding protein cleavage-activating protein.

the risk of cardiovascular complications and death in patients with acute kidney injury (27) and preexisting chronic kidney disease (28). Therefore, COVID-19 may be linked to increased cardiovascular risk and mortality in patients with CKD (29-31); however, few studies have examined the nature or frequency of cardiovascular outcomes in patients with COVID-19 and chronic kidney disease (27,32). In our previous study, SARS-CoV-2 N protein promoted NLRP3 inflammasome activation by increased SREBP activation (20). However, the role of the SARS-CoV-2 N protein on NLRP3 inflammasome activation was not tested in this study, The present study was the first to demonstrate that PI amplified SARS-CoV-2 N protein-induced lipogenesis and NLRP3 inflammasome activation via the SCAP-SREBP signaling pathway (Fig. 7),

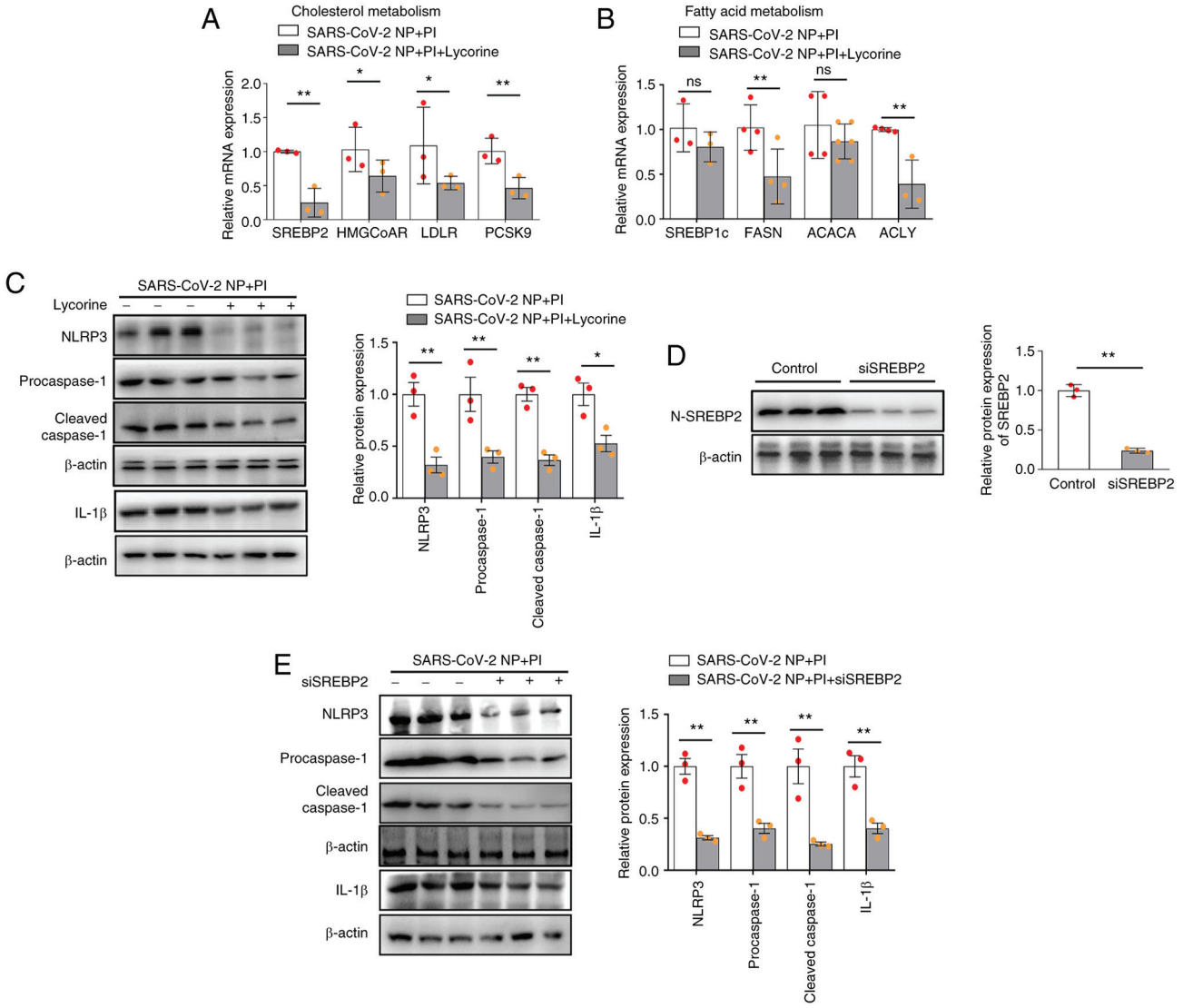

Figure 6. Inhibition of SREBP cleavage-activating protein or knockdown of SREBP2 alleviates inflammasome formation in VSMCs stimulated with PI and SARS-CoV-2 N protein. The SARS-CoV-2 N protein-overexpressing VSMCs were treated with lycorine or transfected with siSREBP2. (A) Quantification of SREBP2, HMGCoAR, LDLR and PCSK9 mRNA expression levels. (B) Quantification of SREBP1c, FASN, ACACA and ACLY mRNA expression levels. (C) NLRP3, procaspase-1, cleaved caspase-1 and IL-1β protein expression levels in SARS-CoV-2 N protein-overexpressing VSMCs treated with lycorine were examined via western blotting. (D) Western blot analysis of N-SREBP2 protein expression in VSMCs transfected with siSREBP2). (E) Western blot analysis of NLRP, procaspase-1, cleaved caspase-1, and IL-1β level in SARS-CoV-2 N protein-overexpressing VSMCs transfected with siSREBP2. *P<0.05, **P<0.01. PI, inorganic phosphate; si, small interfering; OE, overexpression; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; NP, N protein; SREBP, sterol regulatory element binding protein; N-SREBP2, mature N-terminal SREBP2; HMGCoAR, HMG-CoA reductase; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; FASN, recombinant fatty acid synthase; SCD1, stearyl coenzyme A desaturase 1; ACACA, acetyl-CoA carboxylase α; ACLY, ATP-citrate lyase; VSMCs, vascular smooth muscle cells; ns, not significant.

which suggested that PI could potentially act alongside the SARS-CoV-2 N protein to enhance the hyperinflammatory response. These results could provide novel insights into the comorbidities in which PI may increase the risk of mortality in patients with COVID-19 and chronic kidney disease, and could provide new therapeutic targets for COVID-19-related cardiovascular disease.

Hyperphosphatemia is highly prevalent and is associated with increased mortality in patients with COVID-19 (33). Hyperphosphatemia accelerates the occurrence and development of atherosclerosis (12), which is associated with an increased cardiovascular disease risk in the community (34). Until now, it has been widely accepted that adequate control of hyperphosphatemia is important in the clinical management of patients with CKD (35-37). Treatment with the phosphate binders lanthanum carbonate and sevelamer-HCl (35,36), or dietary phosphate restriction (37), has been reported to markedly ameliorate vascular calcification and atherosclerosis in an apolipoprotein E-deficient mouse model. Notably, it has been reported that specific inhibition of phosphate uptake reduces SREBP2 mRNA levels and intracellular cholesterol accumulation (13). Previous studies have indicated that SCAP-SREBPs accelerate the accumulation of cholesterol in cells by increasing intracellular cholesterol synthesis (38). Moreover, SREBP2-mediated cholesterol biosynthesis is a necessary step for SARS-CoV-2 exocytosis and replication (39). SARS-CoV-2 also regulates host lipid metabolism to facilitate viral replication (40). The present study demonstrated that PI increased

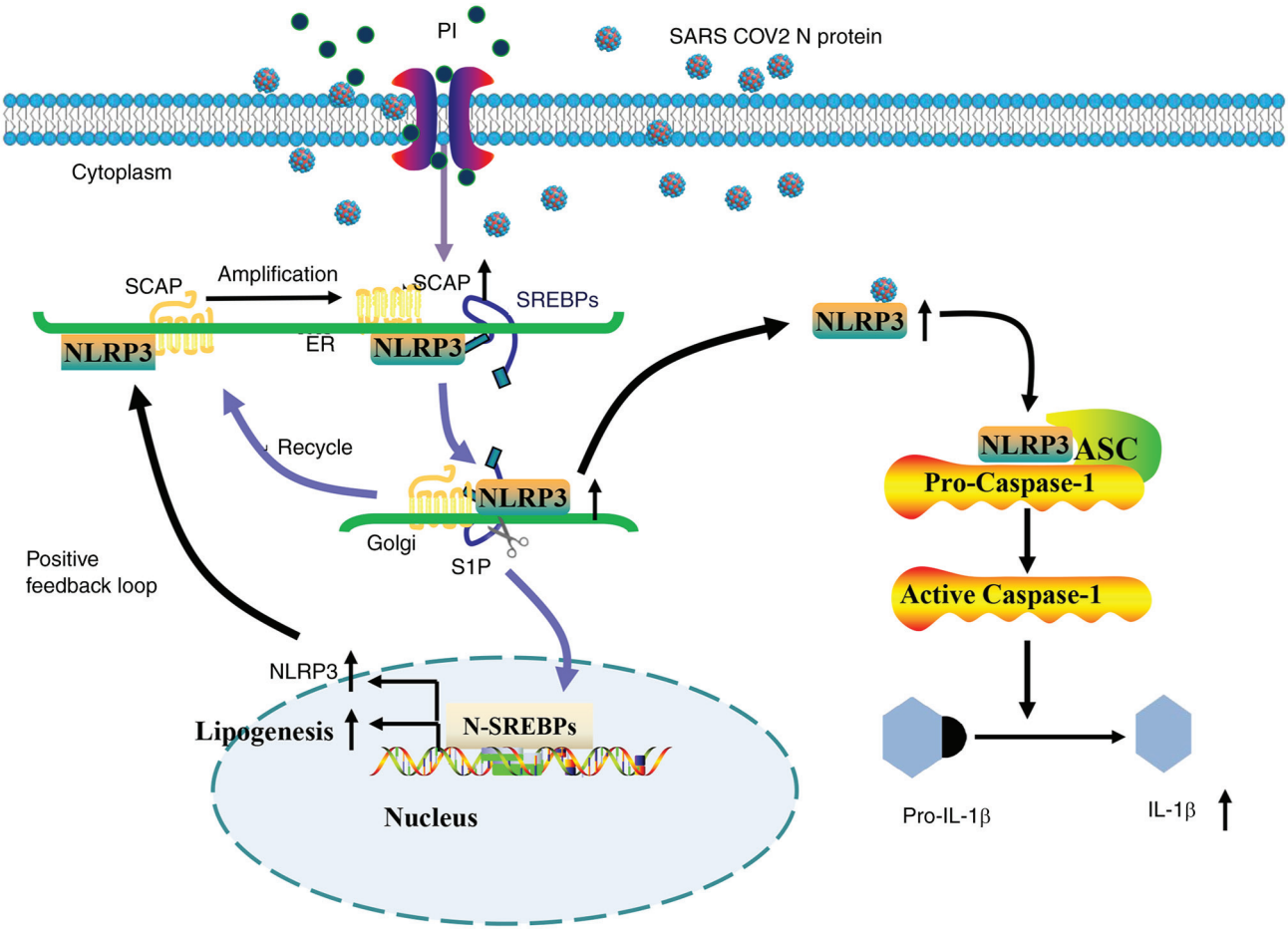

Figure 7. PI amplified SARS-CoV-2 N protein-induced NLRP3 inflammasome activation via increased SCAP-SREBP2/NLRP3 ternary complex translocation to the Golgi. On the one hand, SCAP-SREBPs directly facilitate NLRP3 inflammasome assembly and activation; on the other hand, PI upregulates NLRP3 levels through SCAP-SREBPs, thereby forming an inflammatory positive feedback loop. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SREBP, sterol regulatory element binding protein; SCAP, SREBP cleavage-activating protein; ER, endoplasmic reticulum; N-SREBPs, mature, active forms SREBPs (including N-SREBP1 and N-SREBP2).

SCAP-SREBP and enhanced the expression of lipogenic-associated mRNAs in SARS-CoV-2 N protein-overexpressing VSMCs. The SCAP-SREBPs inhibitor AM580 has been shown to block SREBP-mediated lipid biosynthesis and subsequent double-membrane vesicle formation (41), thus inhibiting SARS-CoV-2 replication (42). Therefore, PI could function synergistically with the SARS-CoV-2 N protein to increase intracellular cholesterol synthesis and SCAP-SREBPs could be used as potential therapeutic targets to inhibit viral infection and decrease the severity of COVID-19.

SCAP-SREBPs serve as a potential mediator of NLRP3 inflammasome activation in sterile inflammation and atherosclerosis (43-45). Previous research (14,22) has indicated that SCAP-SREBPs serve a role in NLRP3 inflammasome activation. Thus, the present study aimed to evaluate the potential effect of SCAP-SREBPs on SARS-CoV-2 N protein-induced NLRP3 inflammasome activation. It was demonstrated that PI in SARS-CoV-2 N protein-overexpressing VSMCs promoted SCAP or NLRP3 proteins separately localizing to the Golgi, thus leading to robust SCAP-SREBP-mediated NLRP3 inflammasome activation. SREBP2 has also been reported to upregulate NLRP3 expression and to thus enhance hemodynamic-induced endothelial inflammation and atherosclerosis (46).

Consistent with a previous report (46), the present study demonstrated that PI led to an increase in NLRP3 protein and mRNA expression levels, thus potentially resulting in an inflammatory positive feedback loop in NLRP3 inflammasome activation (20). Combined with our previous study, it may be hypothesized that the SARS-CoV-2 N protein promotes NLRP3 inflammasome activation via the SCAP-SREBP signaling pathway (20). Furthermore, the results further verified that inhibition of SCAP with lycorine or siRNA-induced silencing of SREBP2 almost completely abolished the promoting effect of PI and SARS-CoV-2 N protein on the activation of the NLRP3 inflammasome and the levels of IL-1β, further supporting an amplified action of SCAP-SREBPs in SARS-CoV-2 N protein-induced NLRP3 inflammasome activation. A recent report has shown that COVID-19-activated SREBP2 disturbs cholesterol biosynthesis and leads to cytokine storm (9). Moreover, the SARS-CoV2-N protein can directly interact with the NLRP3 protein, promote the binding of NLRP3 with ASC, and facilitate NLRP3 inflammasome assembly, thus induces excessive inflammatory responses (22). Therefore, these results indicated that PI may amplify SARS-CoV-2 N protein-induced NLRP3 inflammasome

activation via a SCAP-SREBP/NLRP3 positive feedback loop.

In summary, the present study provided new insight into the effect of PI on SCAP-SREBP-mediated NLRP3 inflammasome activation in SARS-CoV-2 N protein-induced cytokine storms. Recently, it has been reported that the mortality of patients with COVID-19 is linked to cytokine storm, which is triggered by the overproduction of proinflammatory cytokines (47). Inhibition of the SREBP pathway prevents SARS-CoV-2 replication (48) and cytokine storms (9). Therefore, SCAP-SREBPs may serve a role in inducing lipogenesis and NLRP3 inflammasome activation in COVID-19 (41) and targeting SCAP-SREBPs may be a promising strategy for the treatment of patients with COVID-19.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MHL collected and analyzed the data and wrote the manuscript. MHL and LLX performed the experiments. XLL contributed to the study design and data analyses and revised the manuscript. MHL and LLX conducted the final verification and proofreading of the article and confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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