NLRP3 inflammasome links vascular senescence to diabetic vascular lesions

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Abstract

Vascular senescence is inextricably linked to the onset and progression of cardiovascular diseases (CVDs), which are the main cause of mortality in people with Type 2 diabetes (T2DM). Previous studies have emphasized the importance of chronic aseptic inflammation in diabetic vasculopathy. Here, we found the abnormal activation of NLRP3 inflammasome in the aorta of both old and T2DM mice by immunofluorescence and Western Blot analysis. Histopathological and isometry tension analysis showed that the presence of T2DM triggered or aggravated the increase of vascular aging markers, as well as age-associated vascular impairment and vasomotor dysfunction, which were improved by NLRP3 deletion or inhibition. Differential expression of aortic genes links to senescence activation and vascular remodeling supports the favorable benefits of $NLRP3^{-/-}$ during T2DM. *In vitro* results based on primary mice aortic endothelial cells (MAECs) and vascular smooth muscle cells (VSMCs) demonstrate that NLRP3 deficiency attenuated premature senescence and restored proliferation and migration capability under-stimulation, and partially ameliorated replicative senescence. These results provide an insight into the critical role of NLRP3 signaling in T2DM-induced vascular aging and loss of vascular homeostasis, and provide the possibility that targeting NLRP3 inflammasome might be a promising strategy to prevent

diabetic vascular senescence and associated vascular lesions.

Keywords: T2DM; NLRP3 inflammasome; vascular senescence

1. Introduction

Type 2 diabetes mellitus (T2DM) has emerged as a global health issue, with the number of patients increasing at an exponential rate in recent decades[1]. Cardiovascular diseases (CVDs) are the leading causes of mortality and disability of T2DM, and patients with T2DM have a higher prevalence of CVDs than those non-diabetic people [2]. Various structural and functional alternations occurred in the large elastic arteries with older age, including endothelial dysfunction, arterial wall thickening, extracellular matrix changes in collagen and elastin, and arterial stiffening[3-5], which contribute to age-related diseases, such as hypertension, atherosclerosis, stroke, and acute myocardial infarction[6, 7]. Recently, a 5-year longitudinal study conducted in adolescents has shown that the presence of diabetes accelerates the early vascular aging process associated with several key risk factors[8]. Connecting the evidence that hyperglycemia could trigger premature senescence of both ECs and VSMCs, it suggests that diabetes may be a driving factor for vascular senescence[9, 10]. Furthermore, the accumulation of senescent vascular cells was found in diabetic vascular lesions, which is positively related to the pathological process and, inhibition of vascular aging ameliorated diabetic atherosclerosis considerablely[11, 12]. Though

evidence reveals the centrality of vascular aging to diabetic vasculopathy, the mechanism by which diabetes accelerates vascular senescence is not well established.

Chronic, sterile, low-grade inflammation is a hallmark of the aging process ("inflammaging"). Meanwhile, the rate of progression of inflammaging is currently recognized as the main force driving aging, indicating a cross-link between inflammation and aging[13]. Type 2 diabetes is a metabolic condition with the involvement of inflammation in the pathogenesis. Recent investigation reported that inhibition of inflammation and oxidative stress may protect against aortic injury and vascular senescence in streptozotocin (STZ)-induced diabetic mice[14], revealing the potential of vasoprotection in diabetes by targeting inflammation. NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome is a cytoplasmic multiprotein complex that responds to a variety of stimuli, activates phases of assembly and activation, and eventually leads to caspase-1-dependent release of interleukin-1β (IL-1β), and interleukin-18 (IL-18)[15]. It has been confirmed that NLRP3 inflammasome is highly activated during T2DM, which may be associated with increased reactive oxygen species (ROS) and the pro-inflammatory transcription factor NF-kB[16, 17]. Moreover, NLRP3 inflammasome has been linked to various diabetic cardiovascular complications, and its activation and related IL-1β production have lately been postulated as possible indicators of

cardiovascular risk[18]. Interestingly, a recent investigation indicates that ablation of NLRP3 inflammasome can attenuate the cardiac aging process, extend lifespan and improve the metabolic characteristics in aged mice[19], revealing the potential role of NLRP3 inflammasome in the aging process. Additionally, bleomycin has been shown to induce premature senescence of EC through activating NLRP3 inflammasome and increasing the secretion of mature IL-1β, which was reversed by knocking down NLRP3 expression[20]. ROS-NLRP3 signaling pathway has also been reported to be involved in D-galactose-induced EC senescence[21]. Although these findings indicate a relationship between NLRP3 inflammasome and both aging and diabetic vasculopathy, little has been investigated about the role of NLRP3 inflammasome diabetic vascular senescence.

In the present study, we aimed to clarify the contribution of T2DM to vascular aging and determine whether NLRP3 inhibition may prevent aortic injuries and vascular senescence during T2DM, and provide insight into the underlying mechanisms.

2. Material and methods

2.1 Animal experiments

5 weeks old male (wide type) WT and NLRP3$^{-/-}$ mice on a C57BL/6J background were obtained from GemPharmatech Company, Nanjing, China. 11 months old male C57BL/6 mice were provided by the Laboratory

Animal Center of Nanjing Medical University, which were bred normally until 16 months old for subsequent experiments. Mice were allowed to adapt to the new environment for 1 week. All mice were maintained under controlled conditions (20–22°C, 55%–60% humidity, and regular 12 h light/dark cycle) and fed with standard food and water. All animal experimental procedures were approved by the ethics committee of China Pharmaceutical University, in accordance with the Guide for Care and Use of Laboratory Animals published by the European Parliament (Directive 2010/63/EU).

T2DM mice were generated according to the method previously described[22]. Briefly, aged (16 months old), WT (6 weeks old), and NLRP3 KO (6 weeks old) mice were fed with high-fat diet (HFD, 10% saccharose, 10% lard, 10% sugar, 5% egg yolk powder, 0.5% cholesterol, 74.5% basal chow), and the age-matched control mice with a standard chow diet for 4 weeks. Subsequently, mice fed with HFD were intraperitoneally injected with STZ (65 mg/kg, dissolved in cold citrate buffer, pH 4.5) for 5 consecutive days, and the other mice were sham-treated with citrate buffer. One week after the final injection, the mice were fasted for 12 hours to test fasting blood glucose (FBG), and mice with FBG levels higher than 11.1 mmol/l were regarded as type 2 diabetic mice. The old diabetic mice were then randomly separated into three groups, two of which were administered with MCC950 (10mg/kg every 2 days, MedChem

Express, Shanghai, China) and Metformin (200mg/kg every day, Shanghai yuanye BioTechnology Co., Ltd, China) respectively for 10 weeks. All mice were maintained on HFD or NFD after injection with STZ or citrate buffer till sacrifice.

2.2 Senescence-associated beta-galactosidase (SA-β-Gal) staining

SA-β-Gal staining was performed by using β-Galactosidase Staining Kit (Solarbio, Beijing, China) according to the manufacturer's instructions. Mice were anesthetized through isoflurane inhalation (2%) before euthanasia by cervical dislocation. The freshly isolated aorta was immersed in fixation solution for 30 mins at room temperature after removing the fatty tissue and connective tissue, washing the aorta three times with PBS and then covering the aorta with staining solution containing X-Gal in a CO_2-free incubator at 37°C overnight for 16 h. For in vitro experiments, cells were washed twice with enough PBS and then incubated with fixation solution for 5 min at room temperature. After washing steps, cells were incubated with freshly prepared staining solution under the same conditions mentioned above for incubation.

2.3 Immunofluorescence staining

Mice thoracic aortas were separated, optimal cutting temperature compound (OCT, CellPath, U.K.) embedded aorta frozen sections were prepared, and immunofluorescence staining was performed as described previously[23]. Frozen sections were fixed by 4% paraformaldehyde for

15 min, then permeabilized by 0.3% (v/v) Triton X-100 (Biosharp, Hefei, China) in PBS for 15 min and blocked with 5% (w/v) bovine serum albumin (BSA, Solarbio, Beijing, China) for 1 h, then incubated with primary antibodies against NLRP3 (1:100, Abways Technology, Inc., Shanghai, China), PYCARD (ASC, 1:100, Abways Technology, Inc., Shanghai, China), Caspase-1 p20 (1:100, Affinity Biosciences, OH, USA) and tert (1:100, Affinity Biosciences, OH, USA) at 4°C overnight, followed by Cy3–conjugated Goat Anti-Rabbit IgG (H + L) (1:100, Proteintech™, Wuhan, China) and Goat Anti-Rabbit IgG (H + L) Alexa Fluor 488 (1:100, Abways Technology, Inc., Shanghai, China) for 2 h, respectively. DAPI was used to stain the nucleus.

Treated VSMCs and ECs were fixed and blocked like the above procedures, and then incubated with γ-H2AX (1:100, Abways Technology, Inc., Shanghai, China), followed by Cy3, and then counterstained with DAPI.

2.4 Immunohistochemistry

The fixed thoracic aorta was embedded with paraffin and 4-6 μm thick sections were prepared. After the slides were deparaffinized and hydrated, antigen retrieval was conducted, then permeabilized and the peroxidase was inactivated by 3% H_2O_2. Tissue was blocked by 5% goat serum (Beyotime Biotechnology, Shanghai, China) in PBS for 1 h and incubated with primary antibodies of p53 (1:200, Wanleibio, Shengyang, China),

$p21^{Cip1}$ (1:200, Affinity Biosciences, OH, USA), γ-H2AX (1:100, Abways Technology, Inc., Shanghai, China) at 4 °C for 12 h, after washing, horseradish peroxidase-conjugated rabbit anti-goat IgG (1:500, Abways Technology, Inc., Shanghai, China) was applied for 30 min at room temperature. Finally, the staining was performed by using DAB kit (ZSGB-BIO, Beijing, China), followed by counterstaining with hematoxylin.

2.5 Histopathological analysis

After deparaffinization and rehydration, the sections were stained by Hematoxylin (hematoxylin 0.5g, sodium iodate 0.1g, aluminum chloral hydrate 25g, citric acid 0.5g, potassium sulfate 25g, distilled water added to 500 ml) for 8 min, followed by alcoholic eosin (eosin 2.5g, distilled water 500ml, hydrochloric acid 10ml, filtered product dissolved in 1000ml 95% alcohol, dilute twice before use) for 30 s. According to the manufacturer's instructions, the Verhöeff Van Gieson staining kit (VVG, Sbjbio, Nanjing, China) and Masson staining kit (Sbjbio, Nanjing, China) were used to examine the elastin integrity and the collagen content in aortic rings, respectively. Elastin breaks were defined as the fracture in the continuity of the fibers where the end of the break was observable.

2.6 Isometric vascular function

The constriction and relaxation function of the aorta was examined through a tension detection system (BL-420S, TaiMeng, Chengdu, China) as described previously[24]. After mice were anesthetized, descending

thoracic aorta was dissected and removed the fat and connective tissue carefully in Krebs Henseleit solution (mM) (KH, pH 7.4, 119.0 NaCl, 25.0 $NaHCO_3$, 11.1 Glucose, 2.4 $CaCl_2$, 4.7 KCl, 1.2 KH_2PO_4, 1.2$MgSO_4$, 0.024 Na_2EDTA). The arteries were cut into approximately 3mm length rings, then suspended in a water-jacketed tissue bath to test the tension. The solution was maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. The baseline load placed on the aortic rings was 1.0 g. The aortic rings were washed with KH solution every 10 mins until the tension returns to the basic level. After stabilization, the rings were contracted with 60 mM KCl to obtain a maximal response, and then a cumulative dose-response curve to phenylephrine (Phe, 1×10^{-9}-10^{-4} M), acetylcholine (1×10^{-9}-10^{-4} M), sodium nitroprusside (SNP, 1×10^{-9}-10^{-5} M) was conducted. The MCC950 (10μmol) was incubated with aortic segments for 30 min before the construction of the dose-response curve.

2.7 Plasma analysis

Blood samples were collected after mice fasted for 12 h, and immediately centrifuged at 2500 g for 15 min and extracted plasma was stored at -80°C. Plasma levels of fasting insulin, triglyceride (TG), IL-18, IL-1β, and IL-6 were measured by using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute and Proteintech Group (96T, Proteintech™, Wuhan, China), and the procedures specified by the manufacturer were followed. The absorbance was determined using

Multiskan FC (Thermo Scientific, USA) and concentrations were calculated according to the instructions.

2.8 Intraperitoneal injection glucose tolerance test (IPGTT)

The IPGTT was conducted as previously described[25]. In brief, after the mice fasted overnight for 12 h, the fasting blood glucose was measured and then intraperitoneally injected glucose (1g/kg). Blood glucose concentration at 15, 30, 45, 60, and 90 mins after injection were collected using Sinocare blood glucose meter and test strips. The area under the curve for glucose (AUC_{glc}) during the IPGTT was calculated.

2.9 RNA-sequencing of adventitia-removed aortic tissue

Sample quality control, library preparation and RNA-sequencing were executed by Shanghai Biotechnology Corporation, China. Sequencing raw reads were preprocessed and then Hisat2 (version:2.0.4) was used to map the cleaned reads to the human GRCh38 reference genome with two mismatches. Differentially expressed genes were identified using edgeR. (filter criteria: *p*-value ≤0.05 and fold-change ≥2)

Then the functional enrichment was performed with the differentially expressed genes by Metascape, and several annotation categories were found. A heatmap was constructed for representative genes expression, using pheatmap in the R package and considering normalized values by z-scores.

2.10 Primary MAECs and VSMCs culture

Then primary MAECs were isolated as previously described[26]. MAECs were maintained in RPMI-1640 medium containing 10 % FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The purity of the cultures was identified by immunofluorescence assay with anti-CD31 (1:50, Santa Cruz, Heidelberg, Germany) as the primary antibody.

Primary VSMCs were isolated according to the prior study[27]. VSMCs were cultured in DMEM with 10 % fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin at 37 °C in a 5% CO_2 humidified incubator. Primary VSMCs were characterized by immunofluorescence analysis for the expression of specific marker α-sma (1:100, Proteintech™, Wuhan, China).

2.11 Cell treatment

Mannitol was utilized as a high glucose osmotic control in the preliminary experiment, and it was confirmed that mannitol had no effect on the associated indicators, in accord with the normal culture medium. To simulate the diabetes microenvironment, cells were incubated with medium containing 30 mM glucose or 100μg/ml AGE-BSA for 72 h, and cells with normal culture medium or 100μg/ml BSA were utilized as controls.

To induce replicative senescence, cells were continuously cultured up to

passage 25th as the senescent cells and passage 5th used as a young comparison. For stress-induced premature senescence (SIPS), the hydrogen peroxide (H_2O_2) treatment protocol was conducted in primary MAEC and VSMC [28, 29]. Briefly, passage 3th-5th cells at approximately 60-70% confluence were exposed to 10 μM H_2O_2 diluted in growth medium for 72 h.

Recombinant mouse interleukin-1 receptor antagonist protein (IL-1RA, 100 ng/mL, Novoprotein) and recombinant mouse interleukin-18 binding protein (IL-18BP, 50 ng/ml, Novoprotein) were used in MAECs or VSMCs during high glucose.

2.13 Plasmids transfection

VSMCs and MAECs were transfected with plasmids expressing NLRP3 or plasmids vector for 24 h using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions, then the medium was replaced with fresh medium and processed for indicated treatments.

2.12 Western blotting

The protein expression of the cells and the aorta was analyzed using western blot as described previously[30]. Protein concentrations were quantified by using a BCA protein quantity kit (Beyotime, Shanghai, China). Equal amounts of cell lysate or tissue protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to 0.45 μm polyvinylidene difluoride membrane

(PVDF, Millipore, USA). After being blocked with 5% non-fat milk diluted in Tris-buffered saline with Tween-20 (TBST, 100 mM Tris-HCL, pH 7.4) for 2 h, the membranes were incubated with each primary antibody (1:100-1:5000 dilutions) overnight at 4°C. After washing three times with TBST, the membranes were incubated with goat anti-rabbit IgG (1:5000, Abways Technology, Inc., Shanghai, China) for 1.5 h. The blots were detected using automatic chemiluminescence image analysis system (5200 Multi, Tanon, Shanghai, China) with LumiGlo and Peroxide (1:1, Tanon, Shanghai, China), and staining intensity was analyzed by Image J software (NIH, Littleton, CO, USA).

2.14 BrdU assay

Cell proliferation capacity was determined by BrdU assay as described previously[31]. Briefly, MAECs and VSMCs were seeded on coverslips in six-well plates with normal medium to 50-60% confluences, and then cells were incubated with serum-deprived medium with or without high glucose (30mM), advanced glycosylation end products (AGE-BSA, 100μg/ml, BioVision, US), BSA (100μg/ml, BioVision, US) and H_2O_2 (10μm) for 68 h, and subsequently labeled with BrdU (0.03mg/mL, Solarbio, Beijing, China) for 4 h at 37℃. After fixed by 70% ethanol for 5 min, perforated with 0.3% (v/v) Triton X-100 for 15 min and denatured by 2 M HCl for 30 min, then renatured with 0.1M sodium tetraborate. After blocked, cells were incubated with mouse anti-BrdU (1:200, Proteintech™, Wuhan,

China) overnight at 4℃, and followed by Cy3 for 2 h, then counterstained with DAPI.

2.15 Scratch wound assay

Cells were cultured in six-well plates, grown until confluence, then incubated with serum-deficient medium with or without HG, AGE-BSA, BSA, H_2O_2 for 48 h. The sterile pipette tip was used to make a gentle scratch, after washing with PBS, and then added the same supplements as before into the medium for another 24 h. Photographs of the wounded area were taken immediately (0 h) and 24 h after scratch. The wound areas were calculated by using Image J (NIH, Littleton, CO, USA), and the closure rate is quantified as the percentage of recovery area to the initial wound area.

2.16 Statistical analysis

Data are expressed as mean ± standard error (SE) and were obtained in three or more independent experiments. Statistical analysis was carried out using GraphPad Prism 8.2.0. The significant differences between the two groups were evaluated using Student's t-test and for more than two groups, with one-way or two-way ANOVA followed by post hoc Tukey's multiple comparisons. Kolmogorov-Smirnov test was used to check the normality of the data. The $p<0.05$ was considered statistically significant.

3. Results

3.1 Systemic inflammation in T2DM and aging mice, accompanied by activation of NLRP3 inflammasome in the aorta

Most elderly individuals develop inflammageing, which is characterized by increased levels of blood inflammatory markers[32]. The plasma biochemical parameters showed that compared with control and young mice, T2DM and old mice displayed significantly elevated IL-1β, IL-6 and IL-18 levels (Table S1), which also termed senescence-associated secretory phenotypes (SASPs)[33, 34], revealing sustained inflammatory response during T2DM and aging. In addition, old mice showed increased insulin levels similar to T2DM mice, accompanied by a trend of impaired glucose tolerance (Table S1).

It has been confirmed that abnormal activation of NLRP3 inflammasome in the pathological process of T2DM[35] and aging process[32]. Therefore, colocalization of NLRP3 *vs*.ASC or caspase-1 p20, the critical markers of NLRP3 inflammasome activation were detected by immunofluorescence. The yellow dots merged by green (NLRP3) and red (ASC or caspase-1 p20) represent the formation of NLRP3 inflammasome in the aorta, which is increased in T2DM and old mice in comparison with control and young mice (Fig 1B). The levels of caspase-1 p20/caspase-1 and IL-1β/proIL-1β in aortic tissue were also notably elevated in T2DM and old mice (Fig 1C-D), corroborating the activation of NLRP3 inflammasome in the aorta

during pathological conditions or aging.

3.2 T2DM drove vascular senescence and triggered loss of vascular homeostasis

Senescence is an irreversible form of long-term cell-cycle arrest and exhibits recognized biomarkers including enhanced activity of senescence-associated β-galactosidase (SA-β-Gal) and elevated expression of cyclin-dependent kinase inhibitors, as well as telomere dysfunction[36-38]. The induction of p53 is critical for the establishment of senescence, and p21 has been identified as an essential downstream target of p53 that mediates p53-dependent cell-cycle arrest[39]. Compared with young control, the presence of T2DM augmented the positive area of SA-β-Gal staining and the expression of p53 (increased by 26.3%, $p<0.001$), p21 (increased by 30.2%, $p<0.001$) in the aortic tissue, accompanied by impaired telomerase reverse transcriptase (tert) activity (Fig 2B, C-E). In addition, DNA damage also plays a pivotal role in triggering pro-senescence responses, which leads to the accumulation of γ-H2AX at the damaged site[40]. We also found the elevated expression of γ-H2AX (increased by 31.6%, $p<0.0001$) in T2DM mice, indicating the contribution of T2DM to DNA damage (Fig 2B, F). Moreover, the presence of T2DM further reduced tert expression and elevated p53 levels (increased by 16.1%, $p<0.01$) of aging mice, and p21 levels also tended to increase relatively ($p=0.1481$) (Fig 2B-F). Connecting the augmented senescence phenotypes observed in old

diabetic mice versus young diabetic mice, it is indicated that T2DM may not only drive vascular senescence but can also aggravate it.

Vascular stiffening, the hallmark of vascular aging, is predominantly due to the loss and fragmentation of elastin and the enhanced collagen deposition. Compared with age-matched young control, T2DM mice displayed increased aortic wall thickness, augment of elastin breakage and collagen deposition (Fig 2G-J). In addition, the endothelium-dependent dilation (EDD) to Ach (decreased by 19.9%, p=0.02) and vasoconstriction to Phe (increased by 36.2%, p<0.0001) observed in T2DM mice indicating that T2DM induced endothelial dysfunction and enhanced smooth muscle contractility (Fig 2K-L, Fig S1A). However, the vasorelaxation responses to SNP had no significant difference among groups showing that the endothelial-independent dilation was not impaired by T2DM (Fig 2K-L). Furthermore, the presence of T2DM intensified aortic injury in old mice, and the arterial wall thickness and elastin breakage were also augmented in old diabetic mice compared to young diabetic mice (Fig 2G-L), suggesting its harmful effect on the artery regardless of age. The plasma levels of IL-1β, IL-6 and IL-18 were elevated to varying degrees in old diabetic mice compared to old mice or young diabetic mice, indicating that T2DM further amplified the levels of SASPs in old individuals (Table S1).

3.3 NLRP3$^{-/-}$ ameliorated vascular senescence and aortic damage during T2DM

To evaluate the impact of NLRP3 deficiency on vascular senescence during T2DM, NLRP3$^{-/-}$ and littermate control (WT) mice were used to develop T2DM. The current results showed that NLRP3$^{-/-}$ restored aortic tert expression and diminished senescent staining area under diabetic conditions (Fig 3B, C), and besides reduced the percentage of p53 (decreased by 15.6%, p=0.002), p21 (decreased by 17.8%, p=0.004), and γ-H2AX (decreased by 12.75%, p=0.003) positive cells (Fig 3B, D-F), implicating a critical role of NLRP3 inflammasome in the vascular aging process during T2DM. There was no significant difference between WT and NLRP3$^{-/-}$ mice in senescence markers.

In addition, NLRP3 deletion significantly reduced arterial wall thickness and decreased aortic collagen content in the context of T2DM (Fig 3G, I-J). However, the improvement in elastin rupture was not observed in NLRP3$^{-/-}$ diabetic mice (Fig 3G, H). Meanwhile, ablation of NLRP3 ameliorated the EDD (increased by 13.1%, p=0.03) and vasoconstriction (decreased by 17.6%, p=0.03) during T2DM (Fig 3K-L, Fig S1B). There were no significant differences among groups in vasodilation responses to SNP through dose-response curves and maximum relaxation analysis (Fig 3K, L). The effect of MCC950 preincubation (a selective NLRP3 inflammasome inhibitor) on vascular tone *in vitro* was further assessed and

the results showed that diabetic aortic segements exposed to MCC950 displayed improved vasomotor function, further corroborating the regulatory role of the NLRP3 inflammasome in vascular function (Fig S1D-E).

Plasma analysis demonstrated that NLRP3 depletion during T2DM not only downregulated the levels of FBG, insulin, and TG, but even ameliorated glucose tolerance (Table S2). The increase in IL-1β, IL-6, and IL-18 levels induced by T2DM was also mitigated by NLRP3 deficiency (Table S2). Our data indicated that the absence of NLRP3 may be of benefit to diabetic phenotypes and can partly diminish SASPs levels.

To better define the regulatory pathway underlying the vascular protection effect in the absence of NLRP3, RNA sequencing analysis was performed on adventitial-removed aortic tissue. The differential gene data were loaded into Metascape for gene ontology (GO) enrichment analysis (Fig S2A, B), and selected genes expression was represented in a heatmap (Fig S2C). The enrichment analysis showed that T2DM could affect mitotic cell cycle phase transition, extracellular organization, vasculature development and inflammatory response. Notably, upregulation of cyclin-dependent kinase inhibitor 1A (CDKN1A, also termed p21) was observed in T2DM mice but not $NLRP3^{-/-}$ diabetic mice when compared to WT mice. Moreover, CTD Small Phosphatase 2 (CTDSP2), which could result in a decreased number of cells in S-phase and cell cycle arrest[41], and a tumor

suppressor BRCA1 that linked to transcriptional activation of p21[42], were upregulated in T2DM mice but not NLRP3$^{-/-}$ diabetic mice. Additionally, the gene expression of ITGB3 (Integrin β3) and MAPK13 (mitogen-activated protein kinase 13) was increased in T2DM mice as compared to WT mice, while downregulated by NLRP3$^{-/-}$. Several studies have reported the contribution of integrin β3 and p38MAPK to cellular senescence [43, 44]. Accordingly, the increased expression of integrin β3 and elevated ratio of p-p38/p38 was observed in the aorta of T2DM mice, which was reversed by NLRP3$^{-/-}$ (Fig S2D). These findings may explain the improvement of NLRP3 exhaustion on vascular senescence during T2DM.

Furthermore, the expression of integrin alpha X (ITGAX), which is associated with diabetic atherosclerosis[45], and collagen type III alpha 1 (COL3A1) linked to the pathogenesis of vascular lesions were increased in T2DM mice but downregulated by NLRP3$^{-/-}$. Nuclear factor of activated T cells 5 (NFAT5) is related to diabetic arterial stiffness[46], can promote the development of diabetic vascular complications and induce persistent inflammation at the early stage of diabetes[47], was upregulated in T2DM mice but not in NLRP3$^{-/-}$ diabetic mice. DOCK2 (Dedicator of cytokinesis 2), a regulator of SMC phenotype[48], has recently been proposed as a predictable marker for the development of diabetic coronary artery disease[49], was also elevated in T2DM mice but not NLRP3$^{-/-}$ diabetic

mice. Collectively, these data support the vasoprotection imparted by NLRP3$^{-/-}$ during T2DM.

Several genes (IL1R1, CXCL1, CXCL9, CCL5, and CX3CR1) linked to inflammatory response, were upregulated in T2DM mice when compared to WT controls. Further, receptor-interacting serine/threonine kinase 3 (RIPK3) has been proposed to modulate inflammatory responses[50], was upregulated in T2DM mice but does not vary in NLRP3$^{-/-}$ diabetic mice. Nuclear factor erythroid 2 like 1 (NFE2L1), which performs a critical role in preventing oxidative stress and inflammation[51], is upregulated in NLRP3$^{-/-}$ diabetic mice in comparison with T2DM mice. NFE2L1 has also been implicated in glucose metabolism and insulin resistance[52].

3.4 MCC950 treatment conferred vascular protection in old diabetic mice

We further investigated the impact of NLRP3 inflammasome inhibition on old diabetic mice with exacerbated vascular aging. Therefore, MCC950, an effective tool compound for elucidating the function of NLRP3 inflammasome in a spectrum of diseases, was administrated in old diabetic mice. Besides, metformin has been shown to inhibit cellular senescence and prolong the lifespan of animal models, and reduce risk factors for CVDs under diabetic conditions[53-56], which was used for positive control. Our results showed that in aortic tissue, administration of MCC950 (10mg/kg/2d, ip) reduced SA-β-Gal staining positive area and expression

of p53, p21, and γ-H2AX, which is consistent with metformin treatment (200mg/kg/d, oral administration) (Fig 4B, C). Metformin ameliorated elastin breakage and arterial wall thickening in old diabetic mice, and decreased collagen content. Although no significant improvement in the elastin fraction was observed in the MCC950 treatment group, collagen deposition was significantly improved and there was a certain downward trend in aortic wall thickness (Fig 4D-G). Both treatment options attenuated the decline of EDD and the enhanced vasoconstriction to varying degrees (Fig 4H-I, Fig S1C).

Meanwhile, administration of metformin or MCC950 downregulated the levels of IL-1β, IL-6, and IL-18 in old diabetic mice and, MCC950 seems to have a stronger ablative effect on these inflammatory cytokines (Table S3). Furthermore, MCC950 treatment moderately reduced the plasma levels of FBG, insulin and TG, and improved glucose tolerance, verifying the benificial effect of NLRP3 inhibition on diabetic phenotype (Table S3).

3.5 Ablation of NLRP3 ameliorated cellular senescence

Higher levels of blood glucose and inflammation that accompany T2DM can hasten the binding of reducing sugars to proteins, resulting in an increase in the formation of advanced glycation end products (AGEs), which contribute to vascular damage and the onset of CVDs[57]. The present data showed that incubation with HG for 72 h significantly elevated the levels of NLRP3, caspase-1 p20/caspase-1, IL-1β/proIL-1β in the

lysate of MAECs (Fig 5A-B) and VSMCs (Fig S3A-B), as well as the levels of IL-1β and caspase-1 p20 in the culture supernatant. The expression changes of NLRP3 related components in MAECs (Fig S4A-B) and VSMCs (Fig S5A-B) stimulated by AGE-BSA, similar to HG, confirming that *in vitro* simulated diabetic microenvironment induced the activation of NLRP3 inflammasome. However, loss of NLRP3 restored the expression of tert and downregulated the expression of p53 and p21 in MAECs (Fig 5C, E-G, Fig S4C, E-G) and VSMCs (Fig S3C, E-G, Fig S5C, E-G). Furthermore, the augmented γ-H2AX foci scattered in the nucleus and the increased percentage of SA-β-Gal positive cells were blunted by $NLRP3^{-/-}$ in MAECs (Fig 5H-I, Fig S4H-I) and VSMCs (Fig S3H-I, Fig S5H-I). NLRP3 deletion also mitigated HG-induced increase in integrin β3 expression and p-p38/p38 ratio in MAECs (Fig 5J) and VSMCs (Fig S3J), which was consistent with *in vivo* results.

As the downstream products of NLRP3 inflammasome, we further investigated the role of IL-1β and IL-18 in vascular senescence. Our data showed that recombinant IL-1RA significantly ameliorated HG-induced premature senescence in MAEC/VSMC, as evidenced by decreased p53 and p21 expression, while the effect of IL-18BP was not obvious (Fig S6A-B), indicating that NLRP3- IL-1β axis may be a key signal mediating diabetic vascular senescence.

The majority of senescent cells displayed a decreased replicative capacity

and migration ability. Similarly, our data showed that the percentage of BrdU-positive cells and wound closure were markedly reduced after stimulation, which was improved by NLRP3$^{-/-}$ (Fig 5K-M, Fig S4J-L, Fig S3K-M, Fig S5J-L). These evidence together revealed that NLRP3 depletion could ameliorate cellular senescence triggered by HG or AGE-BSA.

Endothelial nitric oxide synthase (eNOS) dependent NO release is involved in several physiological processes including vasodilation and vascular remodeling [58, 59]. The ratio of p-eNOS^{Ser1177} to eNOS in MAECs cultured in the medium supplemented with HG or AGE-BSA was significantly decreased, and restored by NLRP3$^{-/-}$ (Fig 5C-D, Fig S4C-D). In addition, the aortic collagen component is predominantly derived from the secretion of VSMCs. Thus the expression of collagen III and collagen I in VSMC were examined, and NLRP3$^{-/-}$ significantly decreased the protein levels of collagen III and collagen I under stimulation (Fig S3C-D, Fig S5C-D).

Furthermore, we established the classic stress-induced premature senescence (SIPS) and replicative senescence *in vitro* to illustrate the influence of NLRP3 inflammasome on the different types of cellular senescence. As expected, incubation with H_2O_2, a classic inducer of SIPS, remarkably activated NLRP3 inflammasome in MAECs (Fig S7A-B) and VSMCs (Fig S8A-B). While NLRP3$^{-/-}$ attenuated the increase in senescent

markers caused by exposure to H_2O_2, and partially restored proliferation and migration ability in MAECs (Fig S7C-I) and VSMCs (Fig S8C-I) as well. Likewise, compared to cells with multiple successive passages (P25), lower expression of p21, γ-H2AX and a decrease in average SA-β-Gal staining positive rate were observed in NLRP3-deficient late-passage (P25) MAECs (Fig S9A-D) and VSMCs (Fig S9E-H), implicating that blockade of NLRP3 signal might alleviate replicative senescence.

3.6 Overexpression of NLRP3 exacerbated HG-stimulated cellular senescence

In order to better study the contribution of NLRP3 inflammasome to vascular senescence, MAECs and VSMCs were transfected with NLRP3 overexpression plasmid. Compared with the Vector or the NC group, the protein level of NLRP3 exhibited a noticeable up-regulation (Fig 6A-D). In addition, NLRP3 overexpression under HG stimulation enhanced the cleavage of caspase-1 and proIL-1β, and further elevated the expression of p53, p21, and γ-H2AX compared to HG-Vector group (Fig 6A-D), indicating the excessive activation of NLRP3 inflammasome exacerbated HG-induced premature senescence. Meanwhile, NLRP3 overexpression also exacerbated the decrease in the p-eNOS/eNOS ratio in MAEC and the increase of collagen I and collagen III in VSMC induced by HG (Fig 6A-D). It has not been observed that overexpression of NLRP3 in the control group can cause an increase in caspase-1 p20/caspase-1and IL-1β/proIL-

1β ratio and senescence markers, revealing that only under pathological conditions, NLRP3 overexpression can amplify the activation of the NLRP3 inflammasome complex and lead to aggravation of cellular senescence.

4. Discussion

Aging, a critical cardiovascular risk factor, is accompanied by progressive structural and functional alternations in vasculature. The accelerated vascular aging process and loss of vascular homeostasis are responsible for the increased CVD risk in T2DM patients. Here, the present study demonstrated that the presence of T2DM drives or exacerbates vascular aging and aortic physiological impairment, which can be alleviated by NLRP3 signal blockade. Our data provide the possibility to prevent diabetic vascular senescence and related vascular lesions by regulating the NLRP3 inflammasome.

There is mounting evidence that chronic inflammation appears to be the primary mechanism of age-related endothelial dysfunction and arterial damage[60]. The gene expression profile of vascular endothelial and smooth muscle cells varies with age, including the induction of inflammatory cytokines, chemokines, adhesion molecules, and other pro-inflammatory mediators[60]. Most of them are SASPs, which can in turn lead to senescence activation and inflammation through autocrine and paracrine mechanisms. Those findings indicate that chronic inflammation

may interact with vascular senescence in a vicious cycle to negatively impact vascular homeostasis and reveal the possibility of regulating inflammation to alleviate vascular aging. NLRP3 inflammasome has been widely linked to diabetic vascular diseases[32, 61], and it is highly activated during T2DM, which is mainly due to the elevated damage-associated molecular patterns (DAMPs) including ROS, free fatty acid, and cholesterol crystals[62]. Hyperglycaemia-induced ROS overproduction is a critical driver of NLRP3 inflammasome activation, with NF-kB and thioredoxin-interacting protein (TXNIP) identified as essential downstream signals of ROS [63, 64]. Further, ROS-mediated NLRP3 inflammasome activation is proved to be an important event in the pathogenesis of diabetic vascular complications[65]. Conncting the evidence that the ROS-sensitive NLRP3 signaling pathway is involved in endothelial dysfunction under HG conditions[66, 67] and mediates stress-induced EC senescence, the excessive activation of NLRP3 inflammasome observed in aortic tissue during T2DM might be associated with elevated ROS. Meanwhile, the presence of T2DM induced remarkable changes in senescence hallmarks including p21, p53, tert, SA-β-Gal activity, and γ-H2AX in young mice, which can be alleviated by $NLRP3^{-/-}$, indicating that NLRP3 inflammasome initiated vascular senescence in young diabetic individuals. The aggravated vascular senescence due to the coexistence of T2DM and aging further verified the cross-linking of chronic inflammation

and aging. Additionally, our data support the inhibitory effect of NLRP3$^{-/-}$ on integrin β3 and p38MAPK, which may be partly due to the mitigated inflammatory response by NLRP3 signal blockade. Integrin β3 has been confirmed to promote cellular senescence by activating downstream signals such as transforming growth factor β (TGF-β) and p38MAPK[43, 44]. Recently, integrin β3 was even proposed as a novel marker and mediator of senescence[68, 69]. Previous researches have clarified the involvement of p38MAPK in senescence growth arrest due to its ability to activate both the p53 and pRb/p16 pathways. However, the inhibition of integrin β3 and p38MAPK signaling might only involve part of the senescence phenotypes and besides, the downstream pathways by which integrin β3 may affect vascular senescence during T2DM requires further investigation.

Pathological changes of blood vessels are the cornerstone of CVDs. The current study indicated that the alternations of vascular function and morphology in diabetes and possibly in aged subjects may progress in a similar manner. However, NLRP3$^{-/-}$ ameliorated the aortic collagen deposition and arterial wall thickening during T2DM. NLRP3 deficiency reduced the expression of collagen I and collagen III in VSMCs under HG or AGE-BSA stimulation, which explained the decrease in collagen content in *vivo*. Furthermore, The reduction in eNOS phosphorylation in MAECs caused by stimulation was also rescued by NLRP3 depletion, indicating

blockade of NLRP3 signal during T2DM can improve EDD through amplifying the synthesis and release of NO. Intracellular Ca^{2+} in VSMCs serves as a key determinant of vasoconstriction capacity by regulating the activity of myosin-light-chain (MLC) kinase [70, 71]. Ablation of NLPR3 significantly attenuated T2DM-enhanced vasocontraction, which might be attributed to the reduction of Ca^{2+} influx and MLC20 phosphorylation. Differentially expressed genes linked to vascular remodeling and diabetic vasculopathy also corroborated the vascular protection conferred by NLRP3 exhaustion. Though improved elastin breakage has not been observed in $NLRP3^{-/-}$ diabetic mice, several studies have shown that multiple factors including mechanical fracture, calcification, glycation products, and peroxidation products jointly contribute to elastin fragility[72], indicating that the mechanisms involved in elastin fragmentation were complicated and interference with NLRP3 alone may not achieve the expected therapeutic effect.

NLRP3 inflammasome has also been reported to mediate AGEs-induced pancreatic islet damage and oxidative stress-induced pancreatic islet dysfunction[73, 74], suggesting its impact on metabolism. Additionally, previous stduies have highlighted the contribution of metabolic disorders to vascular aging[75, 76]. In the present study, the improvement of vascular senescence conferred by $NLRP3^{-/-}$ may be partly attributed to improved metabolism, but due to its relatively minor effect on metabolism, the

derived beneficial effects are limited. Senescence affects the cellular environment through the secretion of proinflammatory cytokines and chemokines, so-called SASP, and activation of NLRP3 cascade is a probable driver of SASP[77]. Our data showed that NLRP3$^{-/-}$ reduced the circulating levels of IL-1β, IL-6, and IL-18 during T2DM, suggesting the vascular protection imparted by NLRP3 deletion might be partly due to its systemic effects.

The unignorable part of T2DM population is older diabetic patients, a particularly high-risk group in primary prevention of CVDs. Metformin is the most widely-prescribed oral hypoglycemic medication for T2DM, which also can retard the aging process in model organisms and lowers the risk of age-related disorders in humans, such as neurological disease and cancer[56, 78, 79]. Here, our data revealed that MCC950 treatment, similar to the effect of metformin, can significantly decrease the aortic senescence markers and alleviate vascular damage in old diabetic mice. The improvement of MCC950 on the diabetic phenotype is much less than that of metformin. These findings indicated that even in old diabetic mice with a heavier burden on vasculature, NLRP3 inflammasome inhibition could still exert a relatively considerable vasoprotective effect, which is partly independent of its beneficial effect on metabolism.

The formation of AGEs was accelerated during T2DM due to higher amounts of circulating glucose, AGE precursors, and oxidative stress,

resulting in elevated serum and tissue levels[80]. It has been found that there is a close correlation between AGEs and the physiologic alterations observed in vascular diseases and aging[81]. The present study showed that AGE-BSA, like high glucose, is able to induce vascular cell senescence, which can be ameliorated by NLRP3$^{-/-}$. Furthermore, we found that blockade of IL-1β signaling ameliorated HG-induced vascular senescence, which supports the previous findings that IL-1β may effectively promote vascular aging[20]. Although no significant improvement in vascular aging was observed with IL-18 exhaustion, there is evidence that IL-18 promoted the lung fibroblast senescence and SASPs by blocking Klotho pathway[82]. Besides, the accumulation of IL-18 has been linked to age-related arterial inflammatory loop and atherosclerosis. Thus, it still requires further investigation to better define the role of IL-18 in vascular senescence.

Moreover, the impact of NLRP3 deficiency on H_2O_2-induced premature senescence and late-passage (P25) senescent vascular cells was also evaluated, and our results showed that NLRP3$^{-/-}$ not only attenuated the pro-senescence impact of extracellular stress, but also reduced the level of aging markers in unstimulated condition, suggesting that NLRP3 inhibition may partly counteract replicative senescence. We admitted the limitation that the unknown impact of NLRP3 inhibition on physiological vascular aging *in vivo*, although previous study indicated that NLRP3 deletion can attenuate cardiac aging and extend lifespan in elder mice, it is still

necessary to explore the role of NLRP3 inflammasome in normal aging process.

Although our current research mainly focuses on the large duct arteries rather than resistance vessels, there is evidence that the vascular lesions of the retina in old rats are surprisingly similar to that of relatively young diabetic rats, indicating that diabetes might also accelerate the aging process of microvessels[83]. However, whether the influence of T2DM or NLRP3 inhibition on the aging process and pathophysiology of microvessel are consistent with large elastic artery still needs further investigation.

Taken together, our findings reported here shed new light on the contribution of NLRP3 inflammasome to diabetic vascular senescence and associated vasculopathy. Senescence of vasculature driven by T2DM, as occurs with normal aging, is accompanied by the loss of vascular homeostasis, which can be ameliorated by NLRP3 depletion or inhibition. The differential gene expression caused by NLRP3$^{-/-}$ reinforced this conclusion, and suggested that integrin β3 and p38MAPK may be potential downstream signals affecting senescence activation. *In vitro* data were also in accord with the preceding findings that vascular cell senescence is retarded by NLRP3$^{-/-}$ and, NLRP3 depletion could partly offset replicative senescence. Thus, we concluded that targeting NLRP3 inflammasome may offer a promising goal for the prevention of diabetic vascular senescence

and related vascular lesions.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Author contributions

Guang-Jie Tai and Qing-Qing Yu contributed equally to this work. Guang-Jie Tai and Qing-Qing Yu conceived and designed this work. All authors read and approved the final manuscript for publication.

Conflict of interest

The authors declare that there is no competing of interest associated with this manuscript.

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Figure legend

Figure 1. NLRP3 inflammasome is activated in the aorta of old and T2DM mice. (A) Schematic of the study design depicting 6-weeks old male WT mice randomized to either HFD/STZ-induced type 2 diabetes or NFD/Vehicle control. Another batch of 6-weeks and 16-months-old mice maintained on NFD till sacrifice. (B) Immunofluorescence images and summarized Pearson's coefficient show the co-localization of NLRP3/ASC, NLRP3/caspase-1 p20 in aorta by confocal microscopy (n=6). (C-D) Western Blot was used to assay the protein expression of NLRP3, caspase-1 p20/caspase-1, IL-1β/proIL-1β of aortic tissue of old and T2DM mice (n=3-4). Data were analyzed using Student's t test. * denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 2. The presence of T2DM drives vascular senescence and triggers aortic impairment. (A) Schematic of the study design depicting procedure for HFD/STZ-induced type 2 diabetes in 6-weeks and 16-months old male WT mice. The other age-matched mice with NFD and vehicle were used for control. (B) Aortic SA-β-Gal activity was assayed by SA-β-Gal staining (n=3), and the expression of p53, p21, and γ-H2AX in aorta was measured by immunohistochemistry. The expression of tert was detected by immunofluorescence with anti-tert (green) and the cell nuclei were counterstained with DAPI (blue). (C) The quantitative analysis of

fluorescence intensity of tert and (D-F) percentages of positive cells. (G-J) Representative images of VVG staining, Masson staining and HE staining and summarized data showed the elastin breaks, collagen content and arterial thickness of the aortas, respectively. (K) The responses of isolated aortic rings to Phe, Ach, and SNP were measured and summarized into dose-response curve. (L) The maximum contraction to Phe and the maximum relaxation to Ach and SNP were summarized. Data were analyzed using were analyzed two-way ANOVA followed by Turkey's multiple comparisons. *denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$. n=6-7 per group.

Figure 3. NLRP3$^{-/-}$ ameliorated T2DM-induced vascular senescence and aortic injuries. WT and NLRP3$^{-/-}$ mice were utilized to develop T2DM. (A) Experimental procedure for HFD/STZ-induced type 2 diabetes in 6-weeks old male WT and NLRP3$^{-/-}$ mice. The other age-matched mice with NFD and vehicle were used for control. (B) Representative SA-β-Gal staining images (n=3), immunofluorescence images, immunohistochemistry images and (C-F) summarized data showed the expression of tert, p53, p21, and γ-H2AX. (G-J) Typical images of VVG staining, Masson staining and HE staining and summarized data showed the elastin breaks, collagen content and arterial thickness of the aortas, respectively. (K) The responses of isolated aortic rings to Phe, Ach, and SNP were measured and

summarized into dose-response curve. (L) The maximum contraction to Phe and the maximum relaxation to Ach and SNP were collected. Data were analyzed using two-way ANOVA followed by Turkey's multiple comparisons. *denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$. n=6-7 per group.

Figure 4. Treatment with MCC950 and metformin conferred vascular protection in old diabetic mice. (A) Experimental procedure for HFD/STZ-induced type 2 diabetes in 16-months old male WT mice, then the old diabetic mice were administrated with MCC950 (10mg/kg/2d) and Metformin (200mg/kg/d) for 10 weeks, respectively. (B) SA-β-Gal staining was conducted to measure aortic SA-β Gal activity (n=3). (C) Representative Western blotting gel literature and summarized data showed the expression of p53, p21, and γ-H2AX in the aorta (n=3-4). (D-G) Representative images of aortic sections with VVG staining, Masson staining and HE staining showed the morphology changes. (H-I) Vascular tension recording and summarized into dose-response curve, and the maximum contraction to Phe and the maximum relaxation to Ach and SNP were collected. Data were analyzed using two-way ANOVA followed by Turkey's multiple comparisons. *denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n=6-7 per group.

Figure 5. NLRP3$^{-/-}$ alleviated premature senescence of MAEC induced by HG. MAECs (WT and NLRP3$^{-/-}$) were incubated with or without HG (30mM) for 72 h. (A-B) Representative Western Blot gel documents and summarized data showed the expression of NLRP3, caspase-1 p20/caspase-1, IL-1β/proIL-1β in lysate of MAECs and the expression of caspase-1 p20 and IL-1β in the culture supernatant. (C-G) Representative Western Blot gel documents and summarized data showed protein levels of p-eNOS/eNOS, tert, p53, and p21 in MAECs. (H-I) DNA damage foci were detected by immunofluorescence microscopy with anti-γ-H2AX (red) and cell nuclei were counterstained with DAPI (blue). γ-H2AX foci were quantified as mean fluorescent intensity per cell, and take 20 images per group for analysis. SA-β-Gal positive cells were quantified in MAECs. (J) Representative images of Western Blot and summarized data showed protein levels of integrin β3 and p-p38/p38 in MAECs. (K-M) Typical images and summarized data showed proliferation and migration capacity of MAECs by BrdU assay and wound healing assay. Data were analyzed using two-way ANOVA followed by Turkey's multiple comparisons. *denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$. n=3-6 per group.

Figure 6. The overexpression of NLRP3 exacerbated HG-induced cellular senescence. After MAECs or VSMCs were infected with NLRP3

overexpression plasmids or Vector, both types of cells were incubated with HG (30mM) for 72 h. (A-B) Representative images of Western Blot and summary data showed the protein levels of NLRP3, p-eNOS/eNOS, caspase-1 p20/caspase-1, IL-1β/proIL-1β, p53, p21, and γ-H2AX in MAECs. (C-D) Representative images of Western Blot and summary data showed the protein levels of NLRP3, collagen I, collagen III, caspase-1 p20/caspase-1, IL-1β/proIL-1β, p53, p21, and γ-H2AX in VSMCs. Data were analyzed using one-way ANOVA followed by Turkey's multiple comparisons. *denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$. n=3-4 per group.