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Krill oil activates NRF2 to prevent diabetic endothelial injury in part through astaxanthin-induced inhibition of KEAP1

Huali Meng[a,b], Xiaoli Huang[c], Jian Zhou[d], Yan Zheng[b], Lei Du[a,b], Hui Li[e], Zhiyue Zhang[e], Linlin Xu[f,*], Hao Wu[a,b,*]

[a] Department of Nutrition and Food Hygiene, School of Public Health, Cheeloo College of Medicine, Shandong University, Jinan 250012, China
[b] Research Center of Translational Medicine, Jinan Central Hospital, Shandong University, Jinan 250012, China
[c] Department of Nutrition, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, China
[d] Medical Center of Gastrointestinal Surgery, Weifang People's Hospital, Weifang 261041, China
[e] NMPA Key Laboratory for Technology Research and Evaluation of Drug Products, Key Laboratory of Chemical Biology (Ministry of Education), Department of Pharmaceutics, School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan 250012, China
[f] Department of Neurology, The Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, China

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ABSTRACT

Diabetes mellitus (DM) is a severe chronic disease that results in high morbidity and mortality. DM causes endothelial injury (DEI) as a basis for cardiovascular complications of DM with few effective approaches developed for its intervention. Krill oil (KO) possesses anti-inflammatory and anti-oxidative activities, but its effect on DEI is unknown. Hence, the aims of this study were to investigate the effect and molecular mechanism of KO on DEI. To investigated the preventive effect of KO on DEI, streptozotocin and high-fat diet-induced type 2 diabetic mice were fed with KO for 6 months. RNA sequencing for endothelial cells (ECs) was used to explore the mechanism of KO's protective function. To clarify the role of nuclear factor erythroid 2-related factor 2 (*Nfe2l2* or NRF2) signaling in KO's protection against DEI, *Nfe2l2* gene-silenced ECs or knockout mice were treated with KO. Molecular docking assay and surface plasmon resonance assay were carried out to reveal binding between Kelch like ECH associated protein 1 (KEAP1) and major components of KO. KO significantly alleviated DEI and aortic pathological injury in the wild-type diabetic mice. RNA sequencing revealed that KO dramatically activated NRF2 antioxidant signaling in high glucose-challenged ECs, the effect of which was further confirmed in the diabetic aortas. *Nfe2l2* gene deletion or silencing completely abolished KO's protection against DEI *in vivo* and *in vitro*, demonstrating that NRF2 was required for KO's action. Further, molecular docking assay and surface plasmon resonance assay identified that KO's functional component astaxanthin (AST), but not docosahexaenoic acid and eicosapentaenoic acid, was able to bind the Kelch domain of KEAP1, promoting nuclear translocation of NRF2 which activated antioxidant gene expression. The comparison of the effects of KO and AST on endothelial NRF2 nuclear translocation suggested that KO might activate NRF2 at least partially through AST-KEAP1 interaction. KO activates NRF2 to prevent diabetic endothelial injury in part through AST-induced inhibition of KEAP1.

1. Introduction

Diabetes mellitus (DM) is a chronic disease, posing a severe threat to global public health and social economy. In 2021, the diabetic population of the world was 537.7 million. It is estimated by International Diabetes Federation that this number will increase up to 783 million by 2045[1].

* Corresponding authors.
Email address: hwu@sdu.edu.cn (H. Wu); linlinxu@sdu.edu.cn (L.L. Xu)
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DM results in high morbidity and mortality predominantly through complications, such as cardiovascular disease, nephropathy, retinopathy and neuropathy. Among these, cardiovascular disease is a major cause for the mortality of the diabetic population[2]. As compared with non-diabetics, the diabetic individuals develop higher risk of cardiovascular disease[3]. Under diabetic condition, the vascular endothelium is injured, characterized by chronic inflammation, oxidative stress and endothelial dysfunction[4]. The DM-induced endothelial injury (DEI) is a critical first step toward atherosclerosis (AS) as a pathological basis for cardiovascular disease[4]. Therefore, blockage of DEI is a viable strategy to prevent cardiovascular complications of DM[5]. However, to date, there have still been in short of effective approaches for DEI intervention.

Dietary supplementation with *n*-3 long-chain polyunsaturated fatty acids (LCPUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has yielded beneficial outcomes in diabetic patients with cardiovascular complications[6-8]. Krill oil (KO), extracted from the *Euphausia superba* (Antarctic krill), has emerged as an alternative source of *n*-3 LCPUFAs. KO contains EPA and DHA in forms of phospholipids which have higher bioavailability compared with the triacylglycerol forms found in fish oil (FO)[9]. Moreover, KO contains astaxanthin (AST) as one of the most potent natural antioxidants[10].

To date, the understanding of the effect and molecular action of KO on DM and complications has still been very limited. We previously found that KO could prevent diabetic nephropathy and cardiomyopathy, and accelerated wound healing in a mouse model of type 2 DM (T2DM)[11-13]. Beyond that, KO was reported to improve metabolic syndrome, cardiovascular disease, overweight, hypertriglyceridemia, cardiovascular disease and non-alcoholic fatty liver disease[14-19]. Notably, KO was found in a clinical trial to attenuate endothelial dysfunction in patients with T2DM[19]. However, this study did not standardize the diets of the participants, especially fatty fish, downgrading the hierarchy of evidence. Moreover, the molecular action of KO was not known. The investigation of this mechanism will facilitate a deeper understanding of KO's action, and provide an experimental basis for further application of KO in larger clinical trials.

KO combats inflammation and oxidative stress as hallmarks of DEI[20]. The key functional components, EPA, DHA and AST, alleviate inflammation and oxidative stress through several mechanisms, such as inhibiting NLR family pyrin domain containing 3 (NLRP3) inflammasome activation, activating PPAR-γ signaling pathway, and regulating the competence of GRP120, etc.[8,21-22]. Among these, nuclear factor erythroid 2-related factor 2 (*Nfe2l2* or NRF2) is a key factor that can be activated by EPA, DHA and AST. NRF2 is a transcription factor governing the transcription of a series of antioxidant genes, forming an endogenous antioxidant defense system that scavenges excessive free radicals under DM condition. We thus hypothesized that KO might attenuate DEI through activation of NRF2, a phenomenon preliminarily identified by RNA sequencing using KO or vehicle-treated endothelial cells (ECs) upon high glucose (HG) stimulation. To this end, KO was studied for its effect on DEI in C57BL/6J wild-type and *Nfe2l2* gene knockout (*Nfe2l2*$^{-/-}$) diabetic mice.

Kelch like ECH associated protein 1 (KEAP1) is a negative regulator of NRF2. By sequestering NRF2 with the Kelch domain, KEAP1 restricts NRF2 from nuclear translocation, thereby hampering the transcription of NRF2 downstream antioxidant genes[23]. Based on the prediction of binding affinity using molecular docking assay, AST was culled as a positive control for the study of KO's molecular action on KEAP1/NRF2 in HG-challenged ECs.

In summary, the purpose of this study was to explore the preventive effect and mechanism of KO on DEI.

2. Materials and methods

2.1 Animal experiments

C57BL/6J wild-type and *Nfe2l2*$^{-/-}$ male mice were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA). All the animals were housed in the Animal Center of Shandong University at 22 °C, on a 12/12 h light–dark cycle, with free access to food and tap water. The experimental procedure was approved by the Preventive Medicine Ethics Committee of Shandong University (Permission number: SYKX20200022). To establish DM, after 1 week of adaption, the 8-week-old mice were intraperitoneally injected with streptozotocin (STZ, Sigma-Aldrich, Shanghai, China; dissolved in 0.1 mol/L sodium citrate, pH 4.5, 50 mg/kg·body weight, once per day for 5 consecutive days). The non-diabetic control mice received the same volume of sodium citrate solution. One week after the last injection, fasting (6-h fast) blood glucose levels were measured, with a value above 13.89 mmol/L diagnosed as DM[24]. Once DM was confirmed, the diabetic mice were immediately fed a high-fat diet (HFD) or a KO-containing HFD. The nondiabetic mice were continuously fed a standard AIN-93G diet. Composition of the experimental diets is described in Table S1. The KO used in the present study was provided by Zhongguan Biotechnology (Hunchun) Co., Ltd. (Hunchun, Jilin, China). The average food intake of KO-treated mice was approximately 2.79 g/day. Based on body surface area normalization, the KO supplementation was equivalent to 7 g/day for a 60 kg adult, forming a dose of *n*-3 PUFAs (the KO used contained 23% EPA, 14% DHA and an AST content of 232 mg/kg) at approximately 2.59 g/day. The detailed ingredients of KO are presented in Table S1. The detailed diet formulas are presented in Table S2. To prevent the oxidation of KO, the feed containing KO was stored in a sealed container at −80 ℃, and the mice's feed was changed daily.

The mice were treated with KO for a total period of 6 months. During the experimental procedure, body weight and fasting blood glucose levels were monitored every week and every 4 weeks, respectively. Glucose tolerance test (GTT) was performed 24 weeks post DM onset. At the end of the experiment, the mice were euthanized under anesthesia with isoflurane (3% isoflurane/air) under a gas anesthesia machine (RWD, Shenzhen, Guangdong, China), with the aortas harvested for analysis.

2.1.1 Endothelial function analysis

After harvesting, the thoracic aortas were cut into 4 mm segments and mounted on 2 L-shaped metal prongs. Vascular contraction in response to phenylephrine (PE) and relaxation in response to acetylcholine (ACh) were recorded to reflect endothelium-dependent vascular function, as described in our previous study[25].

2.1.2 Histopathological staining

To assess aortic histology and pathology, the aortas were fixed in 4% neutral buffered formalin for 48 h, and processed into 4 μm paraffin sections, followed by hematoxylin and eosin (H&E) and Masson's trichrome staining. ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used for quantitative analysis.

2.1.3 Immunohistochemical staining

Immunohistochemical (IHC) staining was performed using a 3-step IHC kit following the manufacturer's instructions (BOSTER Biotechnology, Wuhan, Hubei, China). Primary antibodies against VCAM-1 (1:200, sc-13160, Santa Cruz Biotechnology, Santa Cruz, California, USA) and ICAM1 (1:500, sc-8439, Santa Cruz Biotechnology) were used for incubation overnight at 4 ℃.

2.1.4 Analysis of reactive oxygen species

The 4-μm paraffin sections were treated with 0.1% Triton X-100 PBS for 15 min and incubated with dihydroethylenediamine (DHE, 1 μmol/L, Beyotime, Shanghai, China) solution for 30 min at 37 °C in the dark. Images were taken with a fluorescence microscope at 580 nm (EVOS FL, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.2 Cell experiments

2.2.1 Cell culture and treatments

To investigate the protective effect of KO on HG-induced endothelial cell injury, human aortic ECs (American Type Culture Collection, Manassas, Virginia, USA) were treated with normal glucose (NG, 5 mmol/L), mannitol (M, 25 mmol/L) or HG (30 mmol/L), in the presence of either glycerol (GLY, 1‰) or KO (250 mg/mL), for 48 h. The cells underwent RNA sequencing, and analyses of qRT-PCR, Western blot, immunofluorescence and DHE, as well as *Nfe2l2* gene silencing. To compare the effects of KO and AST, HG-challenged ECs were treated with KO (250 mg/mL) or equal amount of AST to that in KO (58 μg/mL).

In order to assess monocyte adhesion onto ECs, THP-1, a human acute monocytic leukemia cell line (Shanghai Cell Bank of the Chinese Academy of Sciences, Shanghai, China), were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 0.05 mmol/L *β*-mercaptoethanol (Thermo Fisher Scientific).

2.2.2 Monocyte adhesion assay

ECs were seeded and treated in a 24-well plate. THP-1 cells were labeled by 5 μmol/L calcei (Thermo Fisher Scientific) for 30 min. After washing twice with pre-warmed RPMI 1640, the cells were re-suspended in a complete medium, and were then added (5×10^4 cells) onto the 48-h HG pre-stimulated ECs for 1 h at 37 °C. The uncaptured THP1 cells were carefully removed by washing with PBS. The remaining cells were washed 3 times with cold PBS, and the fluorescence was detected under a microscope (EVOS FL, Thermo Fisher Scientific).

2.2.3 RNA sequencing

To screen out potential molecular pathways or genes regulated by KO, RNA was extracted from the NG-, HG/GLY- and HG/KO-treated ECs, followed by RNA sequencing. Briefly, the mRNA collection was processed according to the standardized mRNA library construction sequencing method of Beijing Genomics Institute (Beijing, China), using SOAPnuke (v1.5.6) to filter out data with a poor quality. Dr. Tom Multiomics Data Mining system (https://biosys.bgi.com) was used for data analysis, mapping and mining. Clean data was compared to the reference genome using HISAT2 (v2.1.0) software. RSEM (v1.3.1) was used for quantification of gene expression, and GraphPad Prism 9.0 was used to map gene expression calorimetry in different samples. Differential gene tests were performed using DESeq2 (v1.4.5) with Q value ≤ 0.05 or false discovery rate ≤ 0.001.

2.2.4 Nfe2l2 gene silencing

To evaluate whether NRF2 mediated KO' protective effect, HG-stimulated ECs were transfected with a negative control small interfering RNA (si-NC) or *Nfe2l2* small interfering RNA (si-*Nfe2l2*), using a transfection reagent (Lipofectamine 2000, #11668019I, invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The sequence (antisense) targeting *Nfe2l2* gene was 5′-UCCCGUUUGUAGAUGACAA-3′. Transfection efficacy was tested 24 h post-transfection.

2.2.5 Separation of nucleus and cytoplasm

Protein lysates of the nuclei and cytoplasm were separated using the nuclear and cytoplasmic protein extraction kit (Beyotime), as described in our previous work[26].

2.2.6 Western blot

Protein lysate was obtained from ECs using RIPA (Beyotime), with concentration determined using bicinchoninic acid method. Western blot was carried out as previously described, using primary antibodies against alpha tubulin (Tubulin, 1:1 000, 66031-1-Ig, Proteintech, Wuhan, Hubei, China), selectin E (SELE, 1:1 000, sc-137054, Santa Cruz Biotechnology), heme oxygenase 1 (HMOX1, 1:1 000, 66743-1-Ig, Proteintech), histone H3 (H3, 1:1 000, 4499S, Cell Signaling Technology, Danvers, Massachusetts, USA), KEAP1 (1:1 000, 60027-1-Ig, Proteintech), NAD(P)H quinone dehydrogenase 1 (NQO1, sc-376023, Santa Cruz Biotechnology), NRF2 (1:1 000, GTX103322, GeneTex, Shenzhen, Guangdong, China), and vascular cell adhesion molecule 1 (VCAM-1, 1:1 000, sc-13160, Santa Cruz Biotechnology)[27]. Image Studio™ Lite software (LI-COR, Lincoln, Nebraska, USA) was used for quantification of grey value.

2.3 Immunofluorescent staining

After snap freezing in optimum cutting temperature with dry ice, the thoracic aortas were cut into 4 μm cryo-sections, and fixed with pre-cold acetone for 20 min. The sections were blocked with 5% normal donkey serum for 1 h at room temperature. After washing with PBS, the sections were incubated with primary antibodies against

α-smooth muscle skeleton (α-SMA, 1:200, A10111, Abcam, Shanghai, China), and NRF2 (1:50, 12721S, Cell Signaling Technology) at 4 °C overnight, followed by incubation with secondary antibodies (1:500, Jackson ImmunoResearch, Shanghai, China) at room temperature for 1 h. The nuclei were stained with DAPI (Beyotime) for 10 min at room temperature. Images were acquired on a Keyence Fluorescent Microscope, and were assessed using ImageJ.

For cellular immunofluorescence, ECs were fixed with 4% formaldehyde at room temperature for 20 min, followed by washing 3 times with PBS. The cells were subjected to 0.4% Triton-X-100 (Solarbio, Beijing, China) at room temperature for 20 min, and blocked with 5% BSA-containing PBS at 37 °C for 1 h. The cells were then incubated with a primary antibody against NRF2 (1:100, 12721S, Cell Signaling Technology) at 4 °C overnight, followed by incubation with a secondary antibody (1:500, Jackson ImmunoResearch, Shanghai, China) at room for 1 h. After washing 3 times with PBS, the cells were stained with DAPI (Beyotime) at room temperature for 5 min. The fluorescence was observed by a fluorescence microscope (EVOS FL).

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total aortic and cellular RNA was extracted using an RNA extraction kit (Omega Bio-Tek, Guangzhou, Guangdong, China), and treated with RNase (Sigma-Aldrich, R6148). A small amount of RNA (1 μg) was then reverse transcribed into cDNA using a cDNA synthesis kit (Vazyme, Nanjing, Jiangsu, China). cDNA was analyzed by quantitative PCR using SYBR® Green (Vazyme), using specific forward and reverse primers. The primer sequences are shown in Table S3.

2.5 Molecular docking assay

Molecular-docking assay was performed using AutoDock Vina. After constructing structural formulas of EPA, DHA and AST in Chem3D, the structures were optimized by the Minimise program in Sybyl. The structure of KEAP1 protein was acquired from Protein Data Bank. All hetero molecules in KEAP1, including water, were removed prior to docking. The optimal binding pose for the EPA, DHA and AST with KEAP1 was predicted based on binding affinity. The 3D-structure diagrams showing the interaction between the AST and KEAP1 were drawn with PyMol 1.7 software.

2.6 Surface plasmon resonance (SPR)

SPR assay was performed using the Biacore T200 (Lund, Skane, Sweden) following the manufacture's operating manual, using recombinant KEAP1 protein and AST provided by MedChem Express (Shanghai, China).

2.7 Statistical analysis

A total of 8 mice per group were studied. The experiments were performed in triplicate. The measurements for each group were summarized as means ± standard deviation. One-way ANOVA was used for the comparisons among the groups, Tukey's test was used for post-hoc testing. $P < 0.05$ is considered as statistically significant.

3. Results

3.1 KO prevented the DM-induced aortic endothelial dysfunction

The diabetic mice developed high fasting blood glucose levels which were not improved by KO (Fig. 1A). During GTT assay, although blood glucose levels were not affected by KO statistically (Fig. 1B), the area under the curve was significantly decreased by KO in the diabetic mice (Fig. 1C), suggesting that KO improved the DM-induced glucose intolerance (Figs. 1B, C). Notably, KO dramatically improved the DM-impaired aortic endothelial function as reflected by the decreased vascular contractility in response to PE and enhanced vascular relaxation in response to ACh (Figs. 1D, E). The diabetic mice developed thickened tunica media and enhanced aortic fibrosis as revealed by H&E (Figs. 1F, G) and Masson's trichrome staining (Figs. 1H, I). These pathological injuries were prevented by KO (Figs. 1F-I).

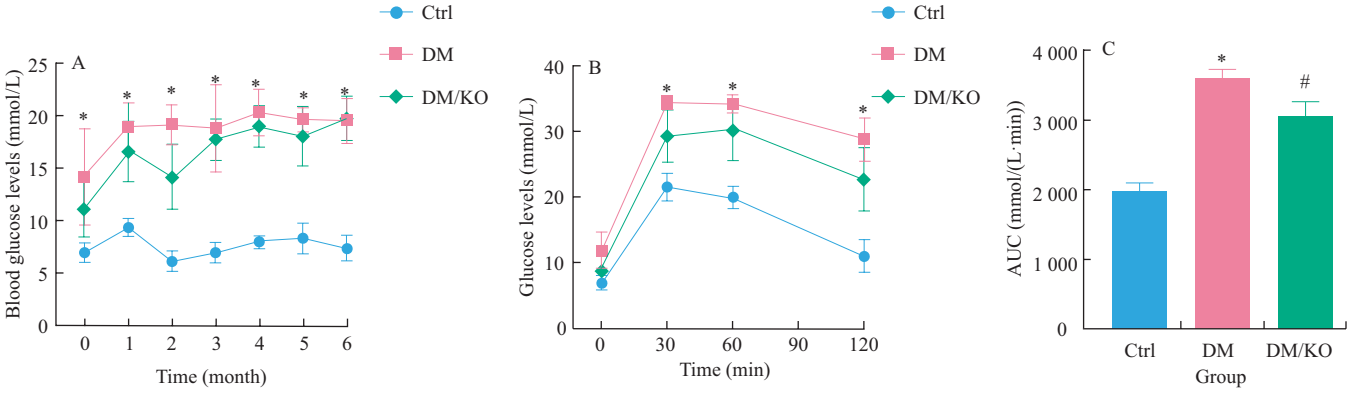


Fig. 1 KO prevented the DM-induced aortic endothelial dysfunction. A mouse model of T2DM was established in 8-week-old C57BL/6J wild-type male mice. (A) Fasting blood glucose levels were monitored every month, for a total period of 6 months. (B) GTT was performed 24 weeks post DM onset, with blood glucose levels measured every 30 min post the intraperitoneal injection of glucose. (C) The area under the curve of panel B was quantified. Aortic (D) contraction and (E) relaxation were measured using thoracic aorta rings in response to PE and ACh, respectively. To assess aortic histopathology, (F) H&E and (G) Masson's trichrome staining was performed, with (H) tunica media thickness and (I) Masson's positive area quantified from H&E staining and Masson's trichrome staining, respectively. The data were summarized as means ± SD ($n = 10$). *$P < 0.05$, DM *vs.* Ctrl; #$P < 0.05$, DM/KO *vs.* DM. Ctrl, control; Masson, Masson's trichrome staining.

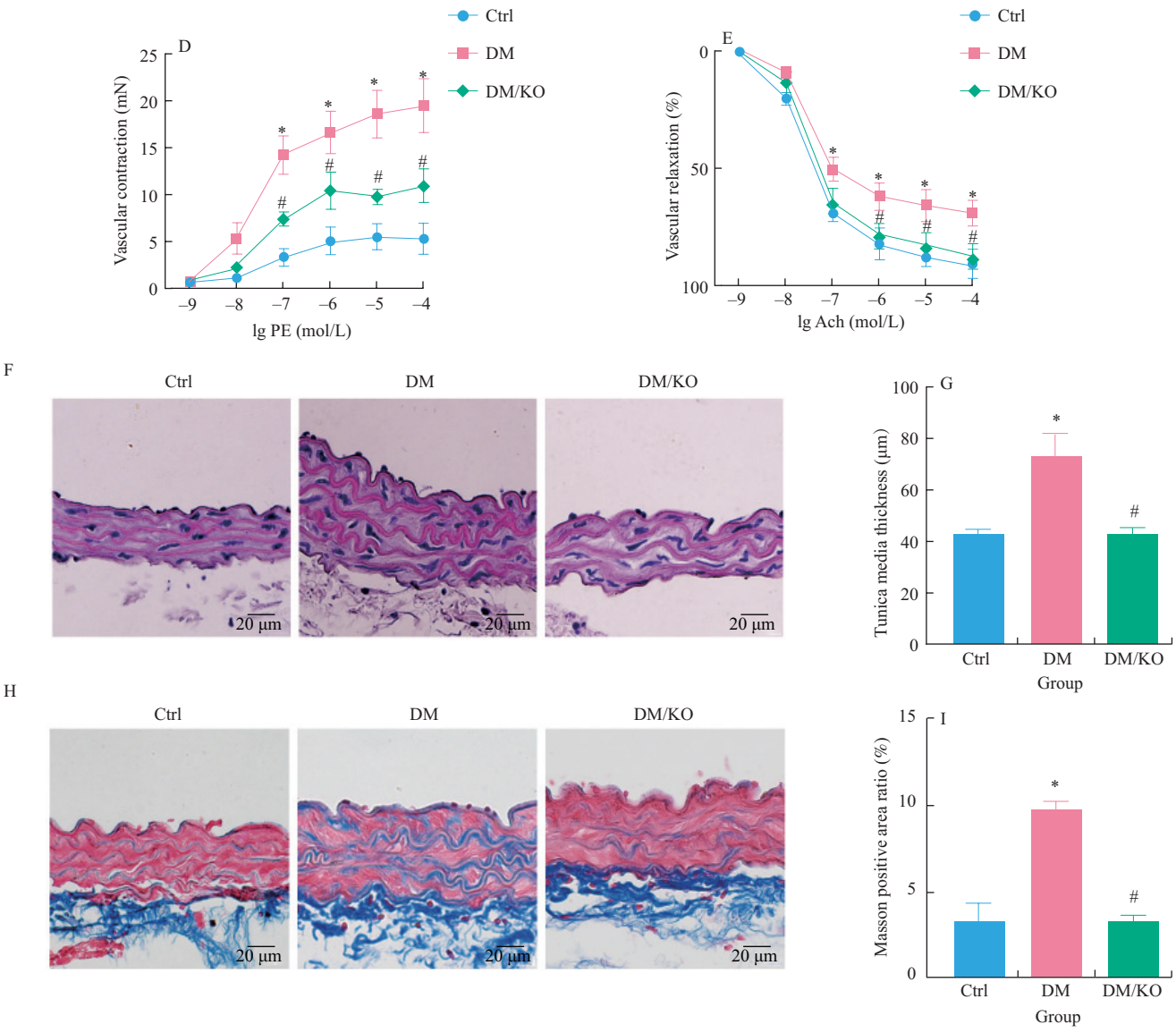


Fig. 1 (*Continued*)

3.2 KO inhibited the aortic expression of inflammatory and oxidative molecules in diabetic mice

Inflammation and oxidative stress contribute to DM-induced endothelial dysfunction and pathological injury. The diabetic mice had increased RNA expression of the inflammatory genes *Vcam1* and *Sele*, and oxidative gene *Nos2* (nitric oxide synthase 2) in the aortas (Fig. 2A). Moreover, VCAM1 and SELE proteins, as well as −OH were accumulated in the diabetic aortas (Figs. 2B-D). These effects were significantly alleviated by KO (Fig. 2).

3.3 KO decreased the expression of pro-inflammatory and oxidative genes in HG-challenged ECs

To explore the molecular mechanism by which KO prevented the DM-induced aortic endothelial injuries, ECs were challenged with HG, and were co-treated with GLY or KO. HG and KO globally altered mRNA expression of ECs as revealed by the heat map (Fig. S1A). Go enrichment analysis identified the KO-modulated key biological processes, all of which represented inflammation (Fig. S1B). Further heat map summarization showed that KO drastically inhibited the HG-provoked mRNA expression of *TNF-α* downstream inflammatory genes (Fig. S1C).

The inhibitory effect of KO on the HG-induced endothelial inflammation was further verified by determining the mRNA and protein levels of the adhesion molecule VCAM1 and chemokine SELE, which were significantly decreased by KO (Figs. 3A-D). Moreover, KO reversed the HG-induced monocyte adhesion onto ECs, and blocked the HG-stimulated formation of endothelial superoxide anion (Figs. 3E-G).

3.4 KO activated the expression of antioxidant genes in HG-stimulated ECs

To investigate the mechanism by which KO alleviated the HG-induced endothelial inflammation and oxidative stress, a scatter

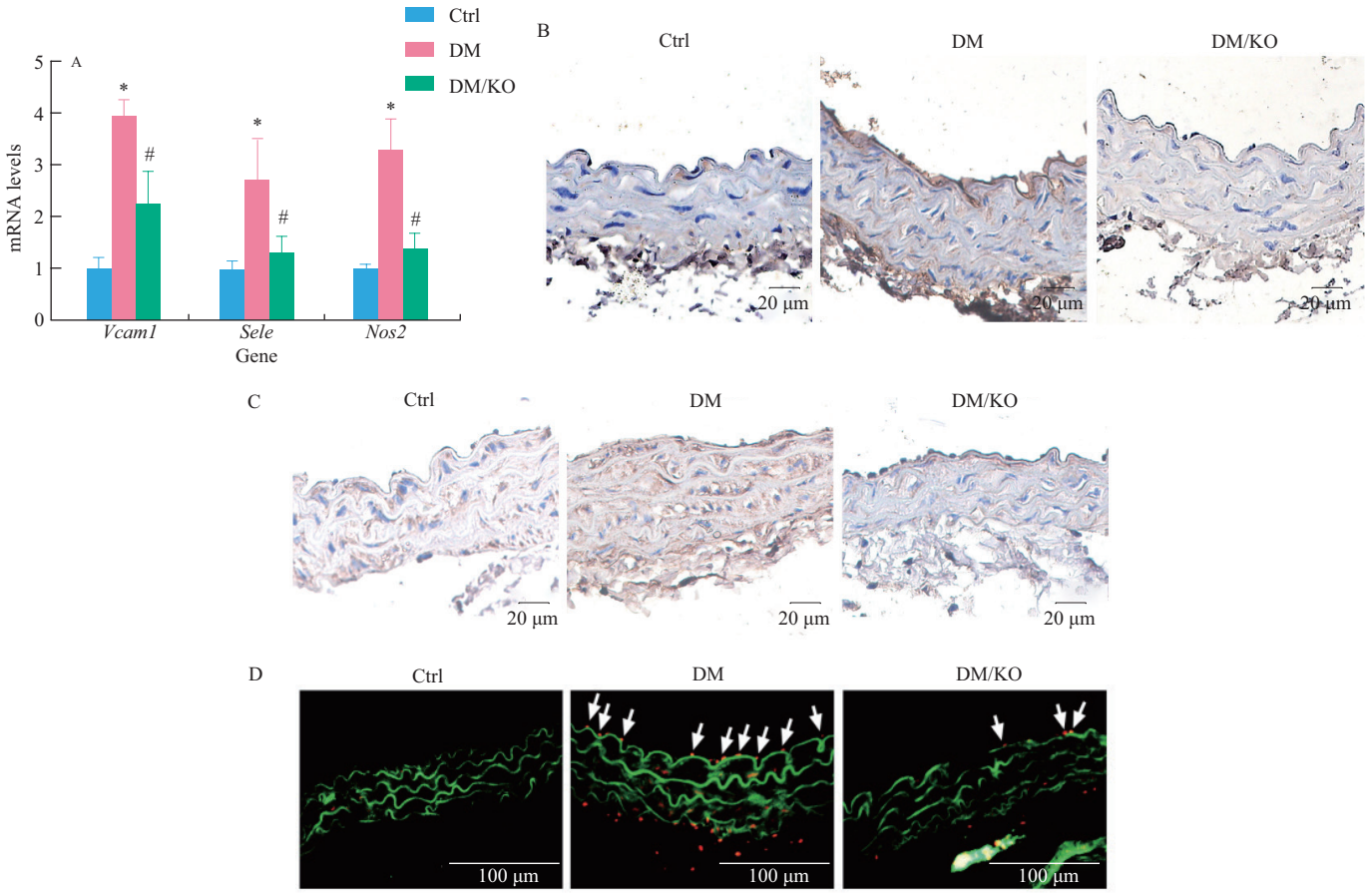


Fig. 2 KO inhibited the aortic expression of inflammatory and oxidative molecules in diabetic mice. To evaluate aortic inflammation and oxidative stress, (A) mRNA levels of *Vcam1*, *Sele* and *Nos2* were determined by qRT-PCR, proteins of (B) VCAM1 and (C) SELE were detected by IHC staining, and (D) reactive oxygen species were displayed by DHE staining (arrows mean positive areas). For qRT-PCR, *Rplp0* (also known as *36b4*) was used as an endogenous control. The data were normalized to Ctrl and summarized as means ± SD ($n = 6$). *$P < 0.05$, DM *vs*. Ctrl; $^{\#}P < 0.05$, DM/KO *vs*. DM.

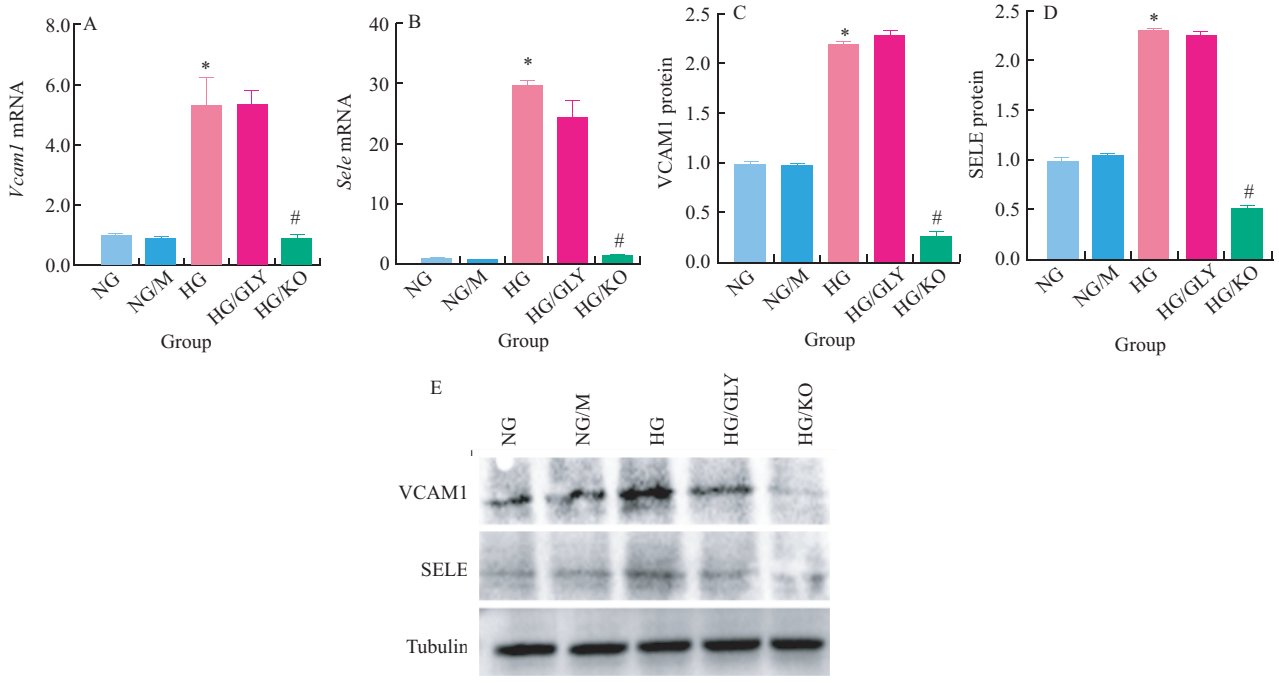


Fig. 3 KO inhibited the expression of inflammatory and oxidative genes in HG-stimulated ECs. To verify the effect of KO on HG-induced inflammation in ECs, VCAM1 and SELE mRNA (A, B) and protein (C, D, E) levels were determined. (F) Monocyte adhesion onto ECs was carried out. (G) DHE staining was performed to detect−OHin ECs. *Rplp0* and Tubulin were used as endogenous controls for qRT-PCR and Western blot, respectively. The data were normalized to NG and summarized as means ± SD ($n = 3$). *$P < 0.05$, HG *vs*. NG/M; $^{\#}P < 0.05$, HG/KO *vs*. HG/GLY.

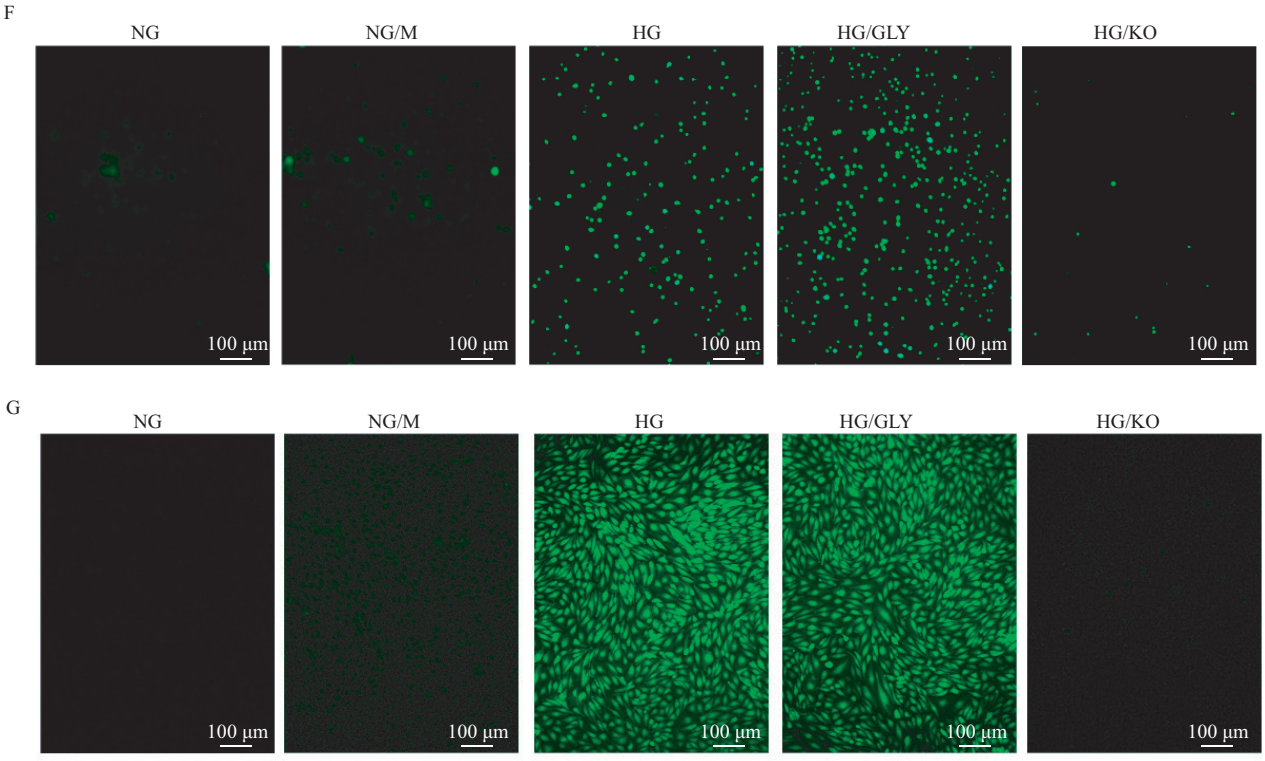


Fig. 3 (*Continued*)

chart was plotted, showing that the mRNA levels of the antioxidant genes *Hmox1* and *Nqo1* were significantly increased by KO (Fig. 4A). qRT-PCR and Western blot further confirmed that KO significantly activated *Hmox1* and *Nqo1* gene expression at both mRNA and protein levels (Figs. 4B-D). As *Hmox1* and *Nqo1* are target genes of NRF2, a heat map was constructed, showing that KO evoked the RNA expression of a series of NRF2 downstream genes, including *Hmox1*, *Nqo1*, glutamate-cysteine ligase catalytic subunit (*Gclc*) and glutathione-disulfide reductase (*Gsr*) (Fig. 4F).

3.5 KO promoted nuclear translocation of NRF2 as a master regulator of antioxidant genes

The following study researched the effect of KO on *Nfe2l2* gene expression and activation in HG-stimulated ECs. KO did not increase *Nfe2l2* mRNA level, but significantly elevated whole cellular NRF2 protein level (Figs. 5A, B and S1D). Cytosolic NRF2 protein was reduced, whereas nuclear NRF2 protein was increased, by KO (Figs. 5C, D and S1D). This resulted in a dramatic elevation of nuclear to cytosolic NRF2 ratio (Figs. 5E and S1D). The KO-induced nuclear translocation of NRF2 was further verified by immunofluorescence assay (Fig. 5F).

In order to validate KO's effect on NRF2 nuclear translocation *in vivo*, NRF2 protein was probed by immunofluorescence, showing that KO enhanced NRF2 expression in the diabetic aorta, especially in the nuclei of the endothelium (Fig. 5G, white arrows). KO reversed the DM-induced inhibition of aortic *Hmox1* and *Nqo1* gene transcription, as an outcome of NRF2 nuclear accumulation (Figs. 5H, I).

3.6 NRF2 was required for KO's protective effects on DM or HG-induced endothelial injuries

To verify whether KO's protective effects were mediated by NRF2, *Nfe2l2* gene knockout diabetic mice and *Nfe2l2* gene silenced ECs were treated with KO. *In vivo*, in the absence of NRF2, KO completely lost the effects on the protection against endothelial dysfunction (Figs. 6A, B), the induction of *Hmox1* and *Nqo1* gene transcription (Fig. 6C), and the decrease of *Vcam1*, *Sele* and *Nos2* mRNA levels (Fig. 6D).

In *Nfe2l2* gene silenced ECs (Figs. 7A, B), KO failed to increase the levels of NRF2 protein and expression of HMOX1 and NQO1 (Figs. 7C-E), and lost the inhibitory effect on the expression of *Vcam1* and *Sele*, monocyte adhesion and −OH production under HG condition (Figs. 7F-J).

3.7 KO activation of NRF2 signaling was at least in part mediated by AST-induced KEAP1 inhibition

To further elucidate the molecular action of KO on the activation of NRF2 antioxidant signaling, KO was studied for its effect on KEAP1, a canonical negative regulator that restricts NRF2 nuclear translocation. Both KEAP1 mRNA and protein levels were not affected by KO (Figs. S2A, B). We therefore speculated that KO might exert a structural inhibitory effect on KEAP1 protein, and therefore performed molecular docking assay predicting the binding affinities between KEAP1 and AST, EPA or DHA which are major functional components in KO. The molecular docking assay suggested that AST, but not EPA and DHA, had potential

strong structural binding affinity with KEAP1 protein (Figs. 8A, B). SPR assay further confirmed the direct binding between AST and KEAP1 with a strong response (Fig. 8C).

The following study compared the effects of AST and KO with equivalent AST content on the promotion of NRF2 nuclear translocation. Both AST and KO increased whole cellular NRF2, but had no impact on cytosolic NRF2 (Figs. 8D, E). Notably, both AST and KO increased nuclear NRF2 levels and the ratio of nuclear to cytosolic NRF2, with KO having a more prominent effect (Figs. 8F, G).

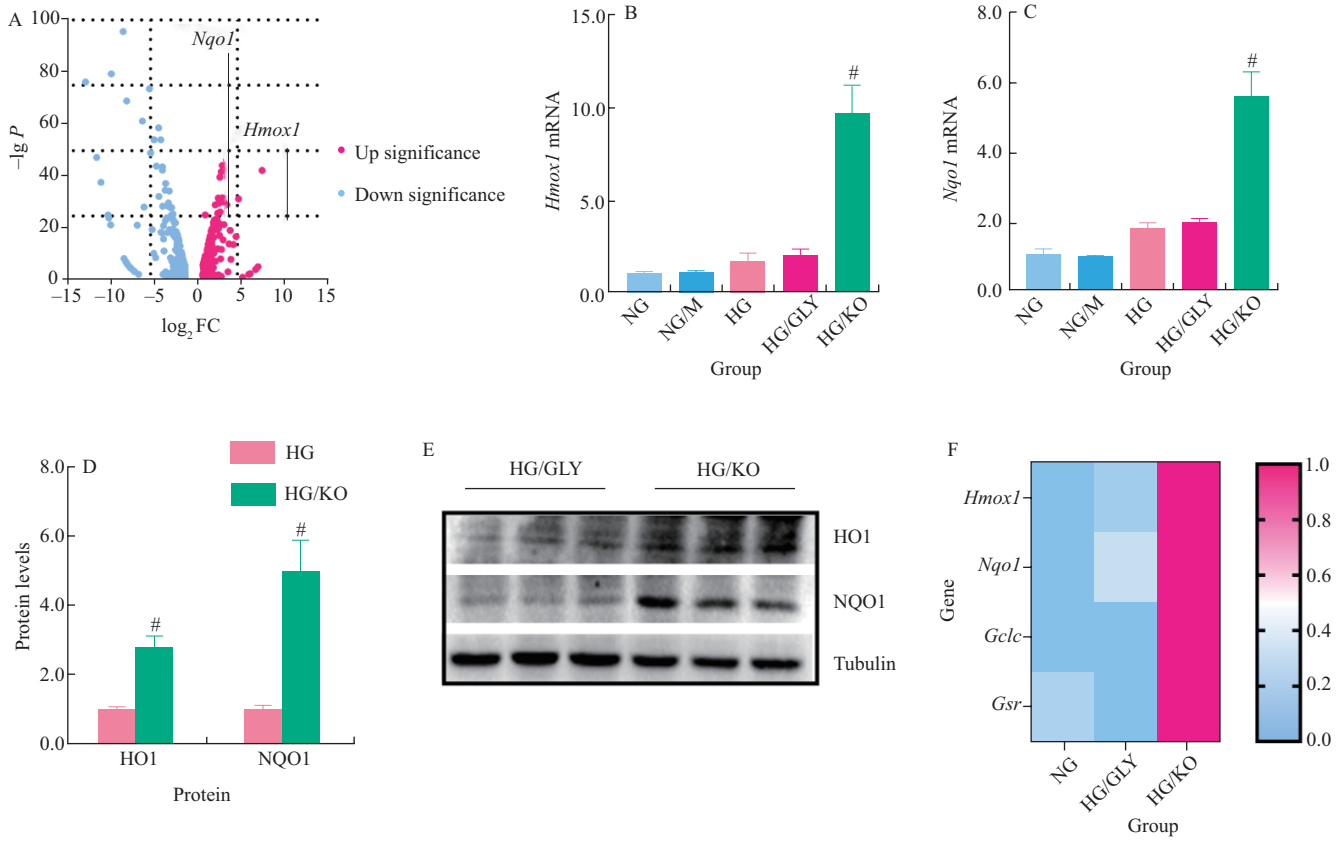


Fig. 4 KO activated the expression of antioxidant genes in HG-stimulated ECs. To further investigate the effect of KO on gene expression in HG-stimulated ECs. (A) A volcano map displaying differentially expressed mRNAs was constructed, showing KO-provoked expression of *Hmox1* and *Nqo1*. (B, C) mRNA levels of *Hmox1* and *Nqo1* determined by qRT-PCR. (D, E) Protein levels of HO1 and NQO1 measured by Western blot. (F) A heat map showing the expression of antioxidant genes *Hmox1*, *Nqo1*, *Gclc* and *Gsr*. *Rplp0* and Tubulin were used as endogenous controls for qRT-PCR and Western blot, respectively. (B-D) The data were normalized to NG and summarized as means ± SD (n = 3). *P < 0.05, HG *vs*. NG/M; $^{\#}P$ < 0.05, HG/KO *vs*. HG/GLY.

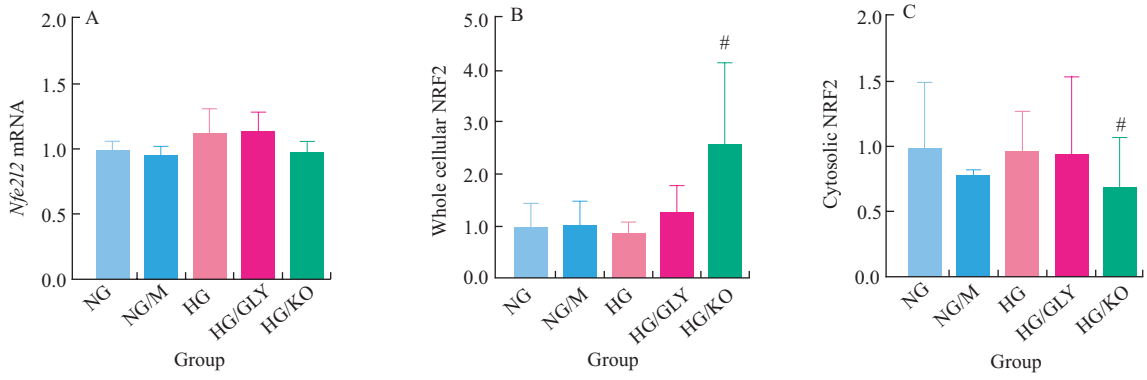


Fig. 5 KO promoted nuclear translocation of NRF2 in ECs and the aortas of the diabetic mice. To explore the molecular action of KO on NRF2, the master regulator of antioxidants, (A) *Nfe2l2* mRNA, as well as (B) whole cellular, (C) cytosolic and (D) nuclear NRF2 protein were determined, with (E) the ratio of nuclear to cytoplasmic NRF2 protein levels calculated. (F) NRF2 immunofluorescence was detected in HG-treated ECs (scale bar = 20 μm). *Rplp0* was used as an endogenous control for qRT-PCR. For Western blot, Tubulin was used as an endogenous reference for whole cellular and cytosolic NRF2, and H3 for nuclear NRF2. To verify the effect of KO on NRF2 antioxidant signaling *in vivo*, (G) NRF2 immunofluorescence and mRNA levels of (H) *Hmox1* and (I) *Nqo1* were determined in the aortas of the C57BL/6J wild-type diabetic mice. *Rplp0* was used as an endogenous control for qRT-PCR. (B-E) The data were normalized to NG and summarized as means ± SD (n = 3), $^{\#}P$ < 0.05, HG/KO *vs*. HG/GLY. (G-I) The data were normalized to Ctrl and summarized as means ± SD (n = 12), *P < 0.05, DM *vs*. Ctrl; $^{\#}P$ < 0.05, DM/KO *vs*. DM.

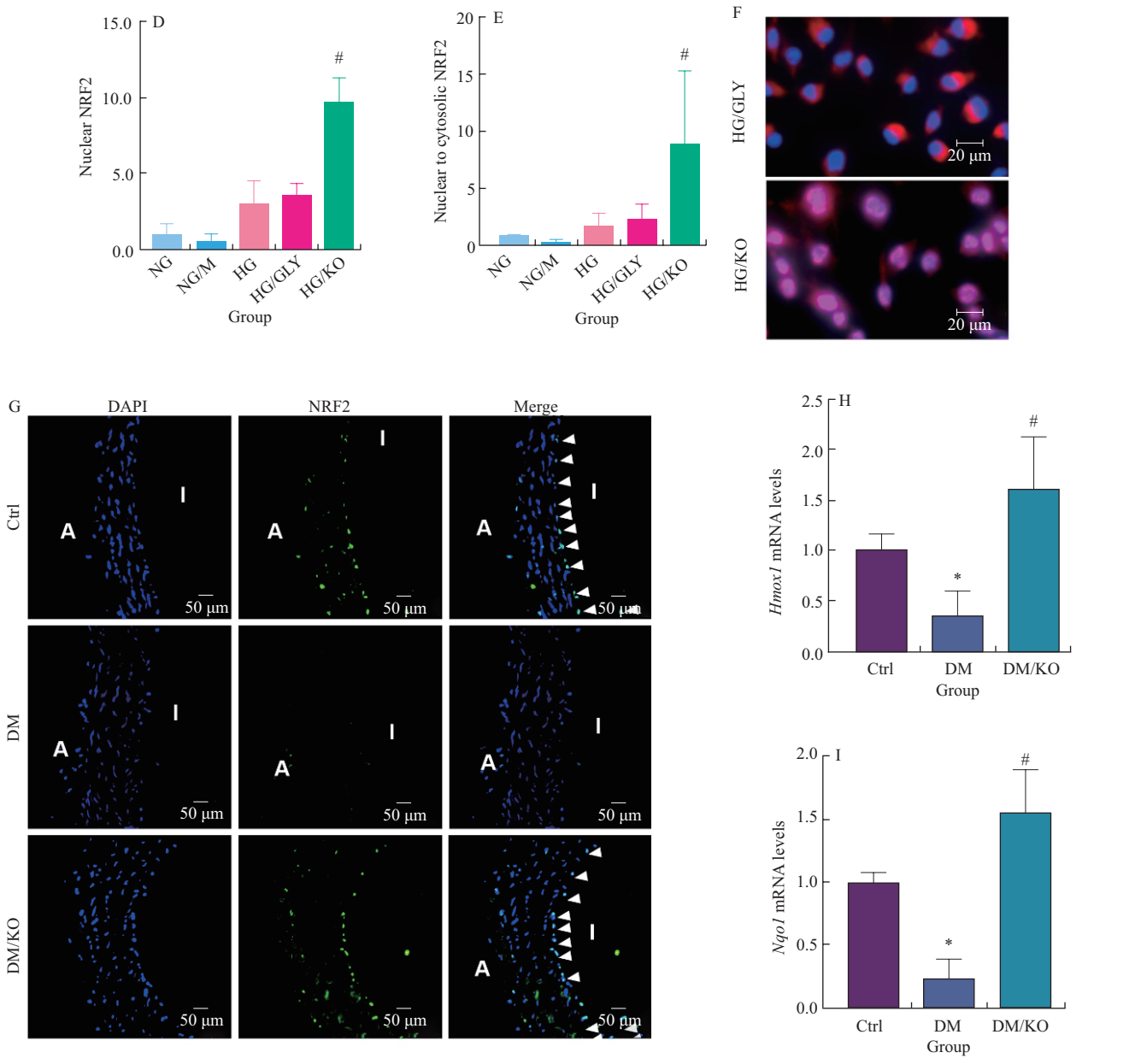


Fig. 5 (*Continued*)

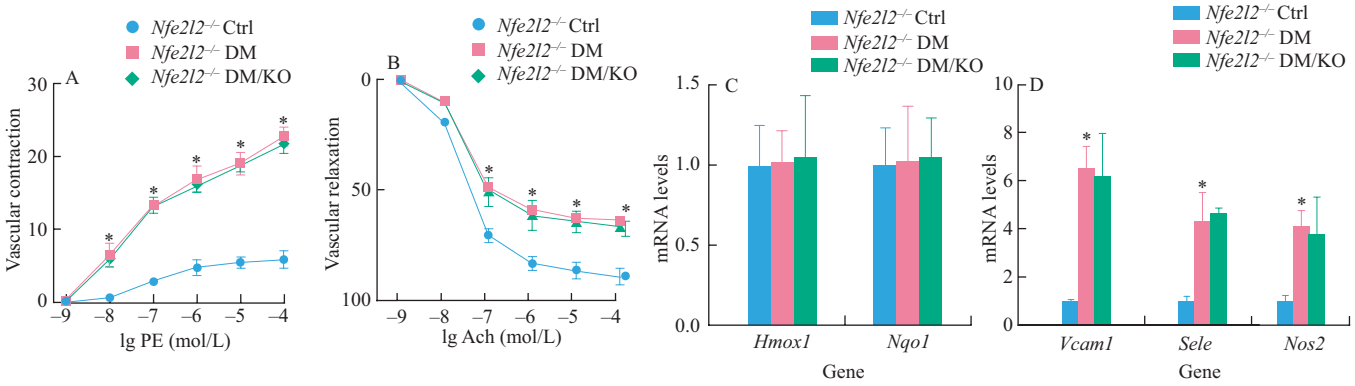


Fig. 6 *Nfe2l2* gene deletion completely abolished KO's protection against DM-induced aortic endothelial injury. To study whether NRF2 was required for KO's protection against DM-induced aortic endothelial injury, C57BL/6J *Nfe2l2* gene knockout mice were induced to T2DM for 6 months, with aortic (A) vascular contraction in response to PE and (B) relaxation in response to ACh determined. In addition, mRNA levels of the antioxidant genes (C) *Hmox1* and *Nqo1*, (D) the inflammatory genes *Vcam1* and *Sele*, and the oxidative gene *Nos2* were measured by qRT-PCR. For qRT-PCR, *Rplp0* was used as an endogenous control. (C, D) The data were normalized to Ctrl and summarized as means ± SD ($n = 10$). *$P < 0.05$, *Nfe2l2*$^{-/-}$ DM *vs*. *Nfe2l2*$^{-/-}$ Ctrl.

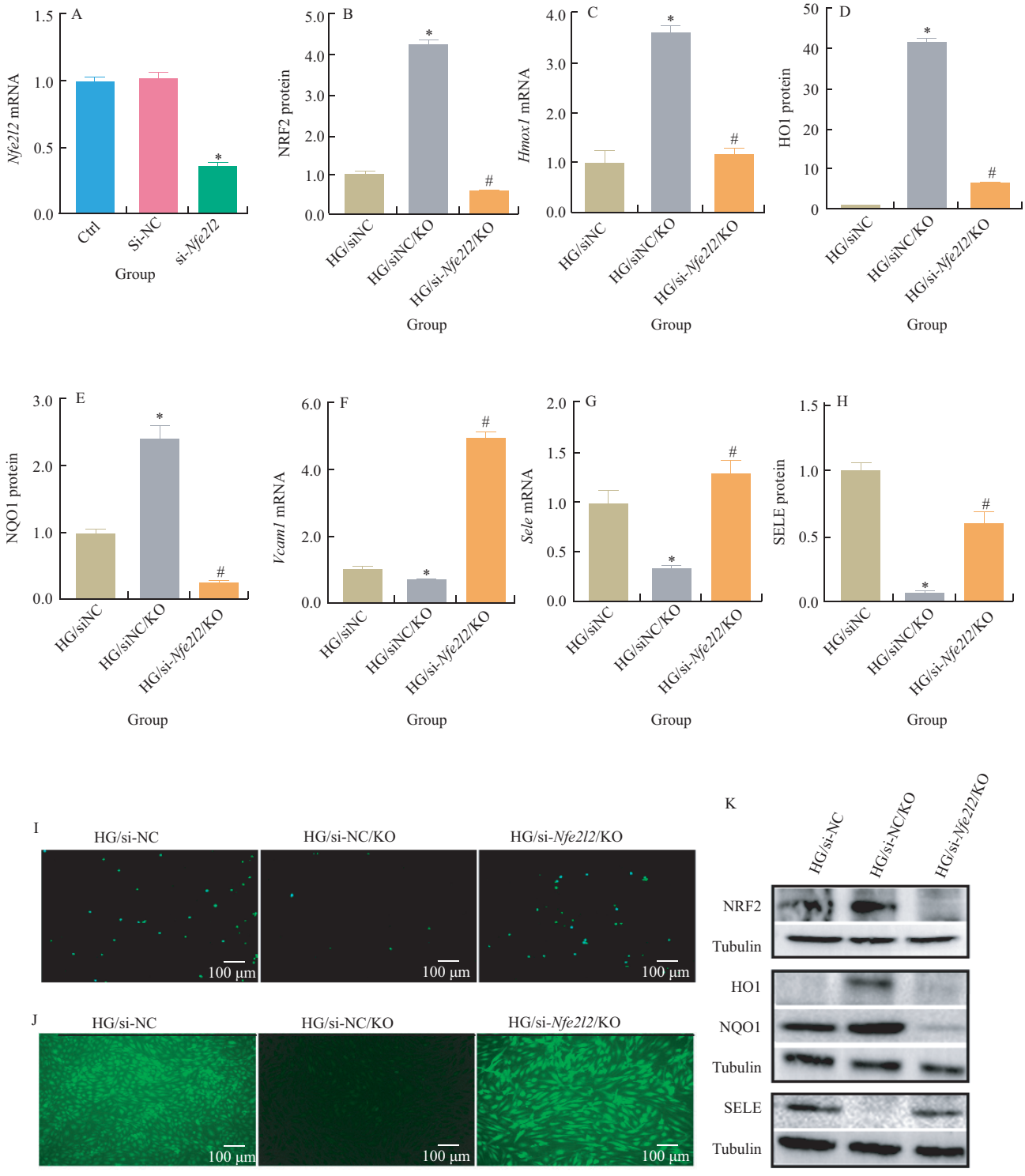
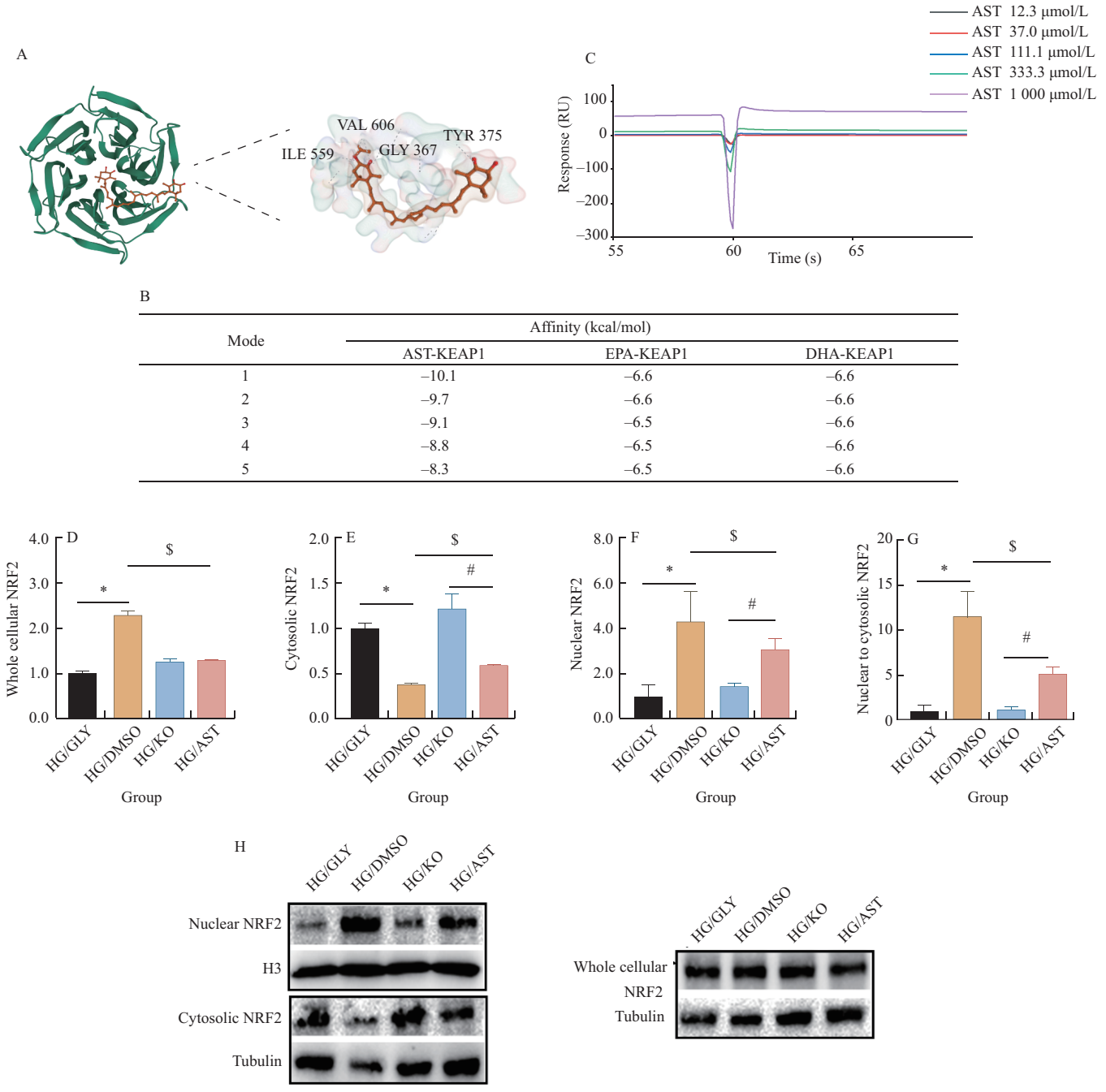


Fig. 7 *Nfe2l2* gene silencing resulted in the abolishment of KO's protective effects *in vitro*. To further verify the role of NRF2 in mediating KO's action *in vitro*, (A) the *Nfe2l2* gene was silenced in ECs, and KO was investigated for its effect on NRF2 antioxidant signaling expression, including (B) NRF2 protein levels, (C) *HMOX1* mRNA level sand (D, E) HO1 and NQO1 protein levels, in the presence of *NFE2L2* small interfering RNA or its negative control. *Nfe2l2* gene silencing was further investigated for its effecton the expression of (F) *VCAM1* mRNA, and (G, H) SELE mRNA and protein, (I) the adhesion of monocytesonto ECs, and (J) the formation of −OH. (K) Western blotband of NRF2, HO1, NQO1 and SELE. *Rplp0* and Tubulin were used as endogenous controls for qRT-PCR and Western blot, respectively. (A) The data were normalized to Ctrland summarized as means ± SD (n = 3). (B-I) The data were normalized to HG/si-NC and summarized as means ± SD (n = 3). *P < 0.05, si-*Nfe2l2* vssi-NC or HG/si-*Nfe2l2*/KO *vs*. HG/si-NC; $^{\#}P$ < 0.05, HG/si-NC/KO *vs*. HG/si-*Nfe2l2*/KO.



Mode	Affinity (kcal/mol)		
	AST-KEAP1	EPA-KEAP1	DHA-KEAP1
1	–10.1	–6.6	–6.6
2	–9.7	–6.6	–6.6
3	–9.1	–6.5	–6.6
4	–8.8	–6.5	–6.6
5	–8.3	–6.5	–6.6

Fig. 8 KO activation of NRF2 signaling was at least in part mediated by AST-induced KEAP1 inhibition. (A, B) Molecular docking assay was performed to predict binding affinities between KEAP1 and major functional components of KO, including AST, EPA and DHA. (C) SPR assay was carried out to verify the binding between AST and KEAP1. *Rplp0* and Tubulin were used as endogenous controls for qRT-PCR sand Western blot, respectively. In order to confirm the contribution of AST to KO's activation of NRF2, ECs were treated with HG, in the presence of GLY, KO or AST, with (D) whole cellular, (E) cytosolic and (F) nuclear NRF2 protein levels determined by Western blot, and (G) the nuclear to cytosolic NRF2 ratios calculated. (H) Western blot band of NRF2. For Western blot, Tubulin was used as an endogenous reference for whole cellular and cytosolic NRF2, and histone H3 for nuclear NRF2. (D-G) The data were normalized to HG/GLY and summarized as means ± SD ($n = 3$). *$P < 0.05$, HG/GLY *vs*. HG/KO; $^{\#}P < 0.05$, HG/AST *vs*. HG/DMSO; $^{\$}P < 0.05$, HG/AST *vs*. HG/KO.

4. Discussion

The present work researched the preventive effect and molecular action of KO on DEI. KO was found to protect against endothelial injury in both wild-type diabetic mice and HG-stimulated ECs. RNA sequencing identified NRF2 antioxidant signaling to be a major target of KO. By employing *Nfe2l2*$^{-/-}$ mice and *Nfe2l2* gene-silenced ECs, NRF2 was found to predominantly mediate KO's protective effects. Mechanistically, KO promoted NRF2 nuclear translocation, triggering the activation of antioxidants genes. Through molecular docking prediction, AST was culled for the comparison with KO, contributing at least partially to KO's activation of NRF2 *via* inhibiting KEAP1. To the best of our knowledge, this is the first study reporting the molecular action of KO on DEI.

AS is a foundation for cardiovascular disease. AS develops more rapidly and occurs at an earlier age in diabetic individuals compared with non-diabetics[28]. An early and effective intervention is crucial for the prevention of cardiovascular complications of DM. However, few effective approaches have been developed. A successful control of hyperglycemia, hypertension and hyperlipidemia was not satisfactory for the prevention of cardiovascular complications of DM-a phenomenon named metabolic memory or glycemic memory, suggesting that other key mechanisms are involved in the development of DM complications[29]. Endothelial oxidative stress and inflammation are provoked upon DM, mutually promoting each other, forming a vicious circle that induces endothelial dysfunction and accelerates atherogenesis[30]. This could be one explanation for metabolic/glycemic memory. Therefore, the intervention of DM-induced endothelial oxidative stress, inflammation and dysfunction, namely DEI, is a viable approach. Hyperglycemia is an important cause for complications of DM[31]. A previous study showed that KO lowered fasting blood glucose in diabetic mice[16]. Similarly, KO improved glucose intolerance in the present work. Thus, we speculate that the improvement of DM might contribute, at least in part, to KO's protection against DEI. However, KO should have exerted a direct impact on the endothelium, as evidenced by the KO-attenuated endothelial injury in the cell experiments. Hence, KO might alleviate DEI through both direct and indirect mechanisms.

A growing body of clinical evidence has demonstrated the effectiveness of *n*-3 LCPUFAs supplementation on DEI. An observational study found that a diet rich in fish, bilberries and wholegrains improved endothelial dysfunction and inflammation in individuals with impaired glucose metabolism[32]. Subsequently, a series of studies confirmed the benefits gained from FO supplementation in patients with T2DM[33-37]. Among these, Rizza et al.[38] found that FO improved endothelial function and reduced inflammatory markers in normal offspring of T2DM patients. Although it is believed that KO has advantage over FO due to the better bioavailability of *n*-3 LCPUFAs and the antioxidant AST, a comparison of the effects of KO and FO on DEI has not been performed. This comparison warrants to be studied in future experiments and clinical trials, the results of which may direct the choice of a better *n*-3 LCPUFAs source for diabetic patients. AST is known as the most potent naturally existing antioxidant. However, natural AST is difficult to be acquired due to the complex extracting process[39]. This results in the prevalence of synthetic AST which has a lower biological activity[40]. KO contains natural AST, and phospholipid forms of EPA and DHA. This has granted KO unique advantages in future application in health promotion and disease prevention.

Our RNA sequencing revealed NF-κB as a top pathway that was regulated by KO. The inhibitory effect of KO on NF-κB pathway was further confirmed by the KO-decreased expression of *Vcam1* and *Sele* which are NF-κB downstream inflammatory genes. Under DM, chronic inflammation and oxidative stress boost mutually, forming a vicious circle that accelerates endothelial injury and other vascular complications[41]. Therefore, inhibition of either diabetes-induced inflammation or oxidative stress should yield beneficial outcomes. NRF2 is a master regulator of cellular antioxidants that combat oxidative stress, leading to inhibition of inflammation[42]. NRF2 activation-induced NF-κB inhibition has often been observed. Therefore, NRF2 could be considered as an upstream negative regulator of NF-κB. This notion was evidenced by the enhancement of inflammation when NRF2 was deleted or silenced[43]. The reason why NRF2 signaling was culled for the study was basically due to the identification of the KO-provoked expression of NRF2 downstream antioxidant genes, especially *Hmox1*, by volcano plot. The upregulation of these genes led to the exploration of NRF2 as their transcription factor. The GO analysis did not highly rank NRF2 pathway. This was possibly because the number of genes in this pathway was less. However, despite the less number of genes, NRF2 pathway played a key role in KO's effect, as shown by the prominent effect of gene silencing or deletion.

Most of AST in KO are in the form of AST esters, which have been studied for their beneficial effects in several studies. For example, DHA-AST was recently reported to improve cognitive function in mice by reducing brain oxidative stress and inflammation[44-45]. In addition, AST esters' protective effects on renal dysfunction induced by vancomycin have been linked to its potent antioxidant properties[46]. As AST esters are important components of KO, it would be interesting to further investigate to what extent these esters contribute to KO's protective effect on DEI.

Different from EPA and DHA which are more easily absorbed in the small intestine, AST esters are able to go further into the colon[47-48]. Therefore, AST esters should have a direct impact on gut microbiota and intestinal barrier function. This speculation is supported by the study showing that AST esters improved intestinal health by modulating gut microbiota and enhancing the intestinal barrier function[49-50]. This nature of AST esters suggests a great potential of their future application in intestinal diseases, such as inflammatory bowel disease. However, for organs/tissues other than the intestine, AST esters might exert an indirect effect through improving intestinal health, including gut microbiota and metabolites, as well as intestinal barrier function, etc. In summary, the awareness of AST esters not only helps understand KO's action, but also improve their application in the food/health industry as functional food components.

In accordance with our findings, KO was reported to activate NRF2, alleviating oxidative stress in patients with liver iron overload and coronary heart disease[51-52]. However, in our previous studies, KO was shown to inhibit TGF-β1 signaling pathway in diabetic nephropathy, block NLRP3 inflammasome in diabetic cardiomyopathy, and promote neovascularization and collagen production during diabetic wound healing[11-13]. One reason for the discrepancies could be that *Nfe2l2* gene knockdown or deletion was not carried out in those work. It is thus not known whether NRF2 was activated and played a key role in KO's protection against those complications of DM. Another reason might be the difference in cell identities with distinct predominantly expressed genes in the different cells and organs, even upon the same diabetic condition. It is therefore not surprising that KO might function through different advantageous signaling pathways in these studies.

A number of studies have confirmed the beneficial effects gained from NRF2 activation on DEI and AS[53-56]. The present work further confirmed that NRF2 was a crucial protective factor in DEI, since *Nfe2l2* gene deletion aggravated DEI in the diabetic mice (Figs. 6A, B). However, global deletion of *Nfe2l2* was found by Sussan et al.[57] to retard, but not accelerate, AS progression in $Apoe^{-/-}$ mice, perplexing the understanding of the role of NRF2 in AS. Several cell types, such as ECs, vascular smooth muscle cells and immune cells,

involve in the pathogenesis of AS. NRF2 may play differential roles in the different cells during AS. This is supported by the findings that *Nfe2l2* gene deletion in ECs exacerbated AS, whereas its deletion in vascular smooth muscle cells halted AS progression[58]. The effect of global *Nfe2l2* gene deletion on AS was the consequence of all, including ECs, vascular smooth muscle cells, immune cells and cells of other organs and tissues. Cell specific gene knockout or over expression should help dissect the exact roles of NRF2 in AS. Different from AS, DEI is a model of endothelial injury under the condition of DM, with ECs to be the only research object. It is understandable that the global *Nfe2l2* gene deletion aggravated DEI in the present work.

In this study, AST was found to have a similar, but weaker, effect on NRF2 activation compared with KO in ECs (Figs. 8D-G). This result suggests that AST partially contributed to KO's activation of NRF2, and that other components of KO, such as EPA and DHA, might also mediate this effect. In line with this speculation, EPA and DHA have been reported to dissociate KEAP1 from Cullin3. This deprived KEAP1's facilitation of NRF2 ubiquitination, and further promoted NRF2 nuclear translocation[23]. Our molecular docking assay showed that EPA and DHA had no binding affinities with KEAP1 protein, implicating indirect actions of EPA and DHA on KEAP1 inactivation. To date, the exact molecular mechanism of EPA and DHA on the inhibition of KEAP1 has not yet been elucidated, which needs to be explored in future studies. Nevertheless, NRF2 mediated the protective effects of AST, EPA and DHA-the 3 major functional components in KO, because KO completely lost its protection against DEI in the absence of NRF2 (Figs. 6A, B). Theoretically, KO might also activate NRF2 through mechanisms other than KEAP1. Since KO did not increase *Nfe2l2* mRNA, but increased whole cellular NRF2 protein level, KO might enhance either the efficiency of NRF2 protein production or its stabilization. For example, microRNA-induced inhibition of NRF2 translation might play a role in KO's elevation of NRF2 protein level.

One innovation of the current study is the identification of AST as an inhibitor of KEAP1. Although a few studies indicated that AST could activate NRF2 *via* inactivation of KEAP1[59-60], to date, the present work has been the first to demonstrate a direct molecular binding between AST and KEAP1. Molecular interaction may or may not decrease the level of a target protein. In the livers of aging rats, AST reduced KEAP1 protein level, suggesting that AST might decrease the stability of KEAP1[61]. In our study, KEAP1 protein level was not decreased by KO which contained AST (Fig. S2B). The discrepancy might result from various reasons, including dose, cell types, experimental conditions, and the influence from other functional components in KO.

In summary, KO was found in the present work to prevent DEI through activation of NRF2. This action was at least in part mediated by AST through a molecular binding with KEAP1. This work could provide a theoretical support and an experimental basis for the application of KO in DEI and cardiovascular complications of DM.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250386.

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