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ARTICLE

Kakonein restores hyperglycemia-induced macrophage digestion dysfunction through regulation of cathepsin B-dependent NLRP3 inflammasome activation

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Abstract

In hyperglycemia-induced complications, macrophages play important roles in disease progression, and altered digestion is a key feature that dictates macrophage function. Recent evidence indicates that kakonein (Ka) possesses anti-inflammatory activities for hyperglycemia-induced complication. In this study, we established a mouse model of Nlrp3$^{+/+}$ and Nlrp3$^{-/-}$ hyperglycemia and administering Ka, primary culture macrophages were tested by engulfing and digesting microbes. The role of macrophages in the cathepsin B–NLRP3 pathway involved in the mechanism of Ka in restoring macrophage digestion function was investigated using biochemical analyses, molecular biotechnology, and microbiology. Ka restored the function of macrophage digestion, which were same characterized by Nlrp3$^{-/-}$ mice. Meanwhile, kakonein could decrease NLRP3 inflammasome products expression and NLRP3/ASC or NLRP3/Casp1 colocalization in macrophage. Interestingly, Ka suppressed

Abbreviations: E.coli, Escherichia coli; NLRP3, NOD-like receptor protein 3; ASC, Apoptosis-associated speck-like protein; CASP1, Caspase-1; Ka, Kakonein; ATP, Adenosine triphosphate; LMP, Lysosomal membrane permeabilization; PBS, Phosphate-buffed saline; MOI, Multiplicity of infection; LAMP-1, Lysosomal associated membrane protein 1; BCA, Bicinchoninic acid; PCR, Polymerase chain reaction; CFU, Colony forming unit; HG, High glucose; RILP, Rab interacting lysosomal protein; EEA1, Early endosome antigen 1; NLRP1, NOD-like receptor protein 1; TLR4, Toll-like receptor 4; TNF-R1, Tumour necrosis factor receptor 1; IL-1R1, Interleukin-1 type 1 receptor; PAMP, Pathogen associated molecule patterns; DAMP, Damage associated molecule patterns.

inflammasome response not by reducing NLRP3 and ASC expression but by reducing cathepsin B release and activation. And Ka restored macrophage digestion and inhibited NLRP3 inflammasome activation consistent with cathepsin B inhibitor. It is concluded that Ka reduced the release of lysosomal cathepsin B and consequently inhibited NLRP3 inflammasome activation to prevent macrophage digestion. Hence, Ka may contribute to new targets for treatment of hyperglycemia-associated dysfunction of macrophage digestion and development of innovative drugs.

KEYWORDS
cathepsin B, hyperglycemia, kakonein, macrophage digestion, NLRP3 inflammasome

1 | INTRODUCTION

Hyperglycemia is a chronic metabolic disorder with high incidence rate in the world, and its complications seriously affect the health of patients.[1] Accumulating lines of evidence indicate that hyperglycemia leads to macrophage inflammation and impairs the digestive function of macrophages.[2,3] The digestive function of macrophages is important in maintaining homeostasis,[4] and digestive dysfunction could cause various cardiovascular complications.[5] Therefore, suppressing inflammatory-mediated macrophage digestive dysfunction could be a new approach in preventing hyperglycemia-associated complications.

Natural products from traditional Chinese medicine exert certain effect on preventing hyperglycemia complications. As the major component of Pueraria lobata (Willd.) Ohwi, Kakonein (Ka; 8-(β-d-glucopyranosyl)−4′,7-dihydroxyisoflavone) has the effect of treating hyperglycemia-related diseases.[6] Previous study reported that Ka has shown significant anti-inflammatory effect, especially for the inhibition of NLRP3 inflammasome.[7] NLRP3 inflammasome, an important inflammatory factor, plays an important role in macrophage digestion function.[8] NLRP3 inflammasome belongs to the NOD-like receptor family and combines with adaptor protein.[9] Empirical research indicates that NLRP3 inflammasome assembly and activation can be stimulated by different kind of dangerous factor from hyperglycemia, such as high glucose (HG), Ca^{2+}, and ATP.[10–12] Lysosomal membrane permeabilization (LMP) releases cathepsin B, which is crucial for NLRP3 inflammasome activation.[13] Scholars should explore the mechanism underlying NLRP3 inflammasome-mediated macrophage dysfunction of Ka in the pathogenesis of hyperglycemia.

In this study, we demonstrated a novel role of Ka in hyperglycemia-induced macrophage digestive dysfunction for the first time. Ka attenuated macrophage digestion dysfunction by inhibiting NLRP3 inflammasome activation, which was significantly dependent on the release of cathepsin B. Thus, our findings indicated the clinical potential of Ka for prevention of chronic hyperglycemia complications.

2 | MATERIALS AND METHODS

2.1 | Animals procedures

C57BL/6J ($Nlrp3^{+/+}$ and $Nlrp3^{-/-}$) mice (6 weeks of age, weighing 18–22 g, male), which were bred from breeding pairs, were from The Jackson Laboratory, Bar Harbor, ME, USA. All protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine (20200331042). Control group received normal diet. Model group and treatment group were fed high-fat feed for 4 weeks and then i.p. injected with a freshly prepared STZ (60 mg/kg) (Sigma–Aldrich, St. Louis, MO, USA) dissolved in 0.1 mol/L citrate buffer (pH 4.4) after 12 h fasting; Then injected intraperitoneally three times with a freshly prepared Streptozocin or citrate buffer. After the last injection, we measure fasting blood glucose (FGB) every other day and hyperglycemia model is considered to be established when FGB were above 10.5 mmol/L for 3 consecutive days. Then, mice in treatment group were treated with Ka (10, 20, 40 mg/kg; i.g., daily), metformin (200 mg/kg, i.g., daily), or CA-074 (10 mg/kg, i.p., daily) for 7 days. Similarly, to verify the therapeutic effect of Ka by inhibiting NLRP3 inflammasome, we respectively divided $Nlrp3^{+/+}$ and $Nlrp3^{-/-}$ mice into normal group, model group, and Ka group (40 mg/kg).

2.2 | Cell extraction and identification

After mice were sacrificed by cervical dislocation, we slowly injected 5 ml RPMI1640 medium (Gibco, Waltham, MA) with syringe into mice abdominal cavity and drew out the harvest solution. After 150 g centrifugation for 5 min, the precipitated cells were cultured in RPMI-1640 containing 10% heat-inactivated FBS and 1% penicillin–streptomycin (Gibco) in incubators for 2 h. The suspended cells were discarded and the adherent cells were monolayered macrophages. We identified macrophages extracted from mice in immunofluorescence

and flow cytometry by staining F4/80 (1:200; CST, USA). Meanwhile, neutral red phagocytosis assay was used to verify macrophages. In detail, macrophages were seeded at 1×10^5 cells per well in 96-well microplates. After incubated for 2 h, the supernatant was discarded. Then, neutral red solution was added and incubated at 37°C for 3 h and macrophages were observed.

2.3 | Flow cytometry

The cells were resuspended and added with F4/80 antibody (1:500; #565409; BD, USA), CD11c antibody (1:500; #561241; BD), and CD206 antibody (1:500; #565250; BD). Simultaneously, the cells without an antibody were used as peer control. All cells were incubated for 60 min, collected, and immediately analyzed by flow cytometry.

2.4 | Cell transfection

The cathepsin B shRNA (#sc-29933-SH; Santa Cruz, USA) was used in the experiment. Gene transfection in cells was performed by Lipofectamine 3000 transfection according to manufacturer's instructions (Invitrogen, USA). Briefly, the primary macrophage was extracted and differentiated from bone marrow was spread to more than 80% confluent and replaced serum-free DMEM for 30 min. The plasmid master mix was prepared by dilution in Opti-MEM™ Medium (Gibco, USA). P3000™ and Lipofectamine™ 3000 reagent were then added for 15 min at room temperature. The transfected cells were incubated in medium with 2.5 μg/ml puromycin to screen out the shRNA plasmid-containing cells and the cellular transfection efficiency of cells was analyzed by western blot.

2.5 | Cell culture and treatment

Freshly isolated bone marrow-derived macrophages from mice were grown in α-MEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin (Gibco) and added to M-CSF. Then, primary macrophages were seeded in plates and divided into 6 groups: control, model (30 mM glucose), Ka (12.5, 25, 50 μM Ka + 30 mM glucose, respectively), and metformin (2 mM metformin + 30 mM glucose) for 24 h. Meanwhile, CA-074 (10 μM) or Cathepsin B knock-down were used as inhibitors.

2.6 | Bacterial infection and cytotoxicity assay

Bacterial infection was performed as previously described.[14] The density of *Escherichia coli BL21* (ATCC) was measured by a McFarland Nephelometer, and the macrophages were infected with bacteria (multiplicity of infection MOI = 100) for 3 h in serum-free RPMI-1640. Then, the macrophage was treated with gentamicin (100 μg/ml) in incubators to clear extracellular bacteria for 12 h; the remaining cell viability was assessed by trypan blue assay.

2.7 | Phagocytosis and digestion assays

Phagocytosis and digestion assays were performed as previously described.[15] In brief, after coculture of E.coli and macrophage for 3 h, we collected the supernatant for phagocytosis assay. Then, the suspension was removed, the adhesive cell was washed to eliminate extracellular bacteria, and the cell was washed and lysed in 0.5% saponin at 37°C for phagocytic assay. The rate of phagocytosis was expressed as phagocytic bacteria/total bacteria. Another group in parallel, the macrophage was treated with gentamicin (100 μg/ml) in incubators to clear extra bacteria. Twelve hours later, the cell was washed and lysed in 0.5% saponin at 37°C for digested assay. The digested number of colonies = the phagocytic number of colonies - the number of colonies remaining after digestion, and the rate of digestion/phagocytosis was calculated by digested bacteria/phagocytic bacteria ratio.

2.8 | Immunofluorescence microscopy

In infected assay, fluorescent microscopy of bacterial entry was performed as previously described.[15] In brief, *E. coli* was resuspended in RPMI-1640 medium and stained with CytoTrace™ Red CMTPX (1:800: AAT, US), and primary macrophages had been processed and were stained with CytoTrace™ Green CMTPX (1:800; AAT). Then, the cell was infected by *E. coli* at MOI = 100 in a cell incubator for 3 h. Extracellular bacteria were removed and macrophages were treated with gentamicin (100 μg/ml) in incubators for 12 h. In uninfected assay, the different macrophage groups were incubated with anti-NLRP3 (1:200; #ab4207; Abcam, USA), anti-caspase-1 (1:200; #sc-56036; Santa Cruz), anti-ASC (1:100; #sc-22514-R; Santa Cruz), anti-LAMP-1 (1:200; #sc-20011; Santa Cruz), and anti-cathepsin B (1:200; #31718; CST, USA). All samples were observed through laser scanning confocal microscope (Carl Zeiss, Germany).

2.9 | Assessment of cellular cathepsin B activity

Assessment of cellular cathepsin B activity was performed as previously described.[16] Cathepsin B activities in macrophage were determined using assay kits (BioVision, CA). All operations were carried out as per the manufacturer's instructions.

2.10 | ELISA assay

The samples of cell supernatant and serum were collected. Inflammatory products of IL-1β (R&D System, #MLB00C, MN, USA) and IL-18 (R&D System, #7625) were measured with ELISA kit. All operations were following the manufacturer's instructions.

2.11 | Western blot analysis

Protein samples were collected and prepared. After electrophoresis and membrane transfer, the sample was incubated with primary antibodies. The primary antibodies were NLRP3 (1:1000; #ab91413; Abcam), caspase-1 (1:500; #sc-56036; Santa), ASC (1:1000; #sc-514414; Santa), cathepsin B (1:1000; CST, #31718), β-actin(1:1000; #BM0627; BOSTER, China), and β-tubulin (1:1000; #A05397-1; BOSTER). And then, membranes were treated with anti-rabbit IgG (1:1500; #5127; CST) or anti-mouse IgG (1:1500; #93702; CST). All protein bands were quantitated by Image J software (NIH, Bethesda, USA).

2.12 | RNA isolation and real-time quantitative PCR

Total RNA from cells was extracted with RNAiso Plus (TAKARA, Japan) and then treated as previously described.[17] The primers were synthesized as follows: 5′-ATTRACCRCGCRCCGRAGARAAGRG-3′ (forward primer) and 5′-TCGRCAGRCAARAGARTCCRACARCAG-3′ (reverse primer) for the mouse *Nlrp3* gene; 5′-GGCGAGAGAGGTGAACAAGG-3′ (forward primer) and 5′-GCCAAGGTCTCCAGGAACAC-3′ (reverse primer) for the mouse *Asc* gene; 5′-CCCATCTATGAGGGTTACGC −3′ (forward primer) and 5′- TTTAATGTCACGCACGATTTC-3′ (reverse primer) for the *β-actin* as internal reference control. The results were quantified using the $2^{-\triangle\triangle CT}$ method.

2.13 | Statistical analysis

Two-tailed unpaired Student's *t*-test was performed for comparisons between 2 groups. Two-way ANOVA were used for experiments containing more than 2 groups, followed by Bonferroni's multiple comparisons test. Data are shown as means ± SEM. When $p < 0.05$ or $p < 0.01$, it had statistical significance

3 | RESULTS

3.1 | Therapeutic effect of Ka in hyperglycemia-induced macrophage dysfunction

First, the hyperglycemia model was established and treated with Ka (10, 20, and 40 mg/kg) or metformin (200 mg/kg) for 7 days. After treatment, macrophages were extracted from mice for bacterial phagocytosis and digestion assays (Figure 1(A)). Results showed that the function of macrophage bacterial phagocytosis was not significantly different, but the function of macrophage bacterial digestion evidently recovered under Ka or metformin treatment (Figures 1(B) and 1(C)). In vitro, we extracted macrophages from C57BL/6N mice. Cells were treated with Ka (12.5, 25, and 50 μM) or metformin (2 mM) in different groups. After removing the original culture solution and adding *E. coli* to coculture, the role of Ka in the function of macrophage bacterial phagocytosis and digestion in vitro was detected (Figure 1(D)). *E. coli* and macrophages were stained with CytoTrace™ Red CMTPX and CytoTrace™ Green CMTPX, respectively, to directly monitor the changes in macrophage phagocytosis. As shown in Figure 1(E) (upper panels), the cells of the model group were visualized as red puncta with high fluorescence intensity, whereas Ka or metformin treatment slightly decreased the number and the fluorescence intensity of the red spots. Similar to the in vivo results, the in vitro results showed that Ka or metformin can evidently restore the digestive function of macrophages (Figures 1(E) and 1(F)).

3.2 | Therapeutic effect of Ka on the recovery of macrophage function under hyperglycemia by inhibiting the NLRP3 inflammasome

Our previous studies show that the NLRP3 inflammasome activation induces the digestive dysfunction of macrophages.[16] Therefore, we explored whether the recovery of macrophage digestive function by Ka was related to the NLRP3 inflammasome. We monitored the changes in macrophage digestion through the CytoTrace™ staining and CFU assay in vivo or in vitro, and results showed that the hyperglycemia-induced dysfunction of macrophage bacterial digestion evidently recovered under Ka treatment, but the therapeutic effect disappeared when NLRP3 was deficient (Figure 2).

3.3 | Down-regulation of the activation of NLRP3 inflammasome components by Ka

We detected whether Ka could affect the hyperglycemia-induced macrophage of NLRP3 inflammasome activation in vivo or in vitro. Results (Figures 3(A)–3(F)) showed that Ka could significantly inhibit the cleavage of caspase-1 and IL-1β, which was an important product of NLRP3 inflammasome activation. Furthermore, we analyzed the colocalization of NLRP3 inflammasome components to determine whether Ka affected the NLRP3 inflammasome assembly. We found that the Ka reduced the colocalization between NLRP3/ASC and NLRP3/CASP1 in a concentration-dependent manner, as shown by the decreased yellow staining and colocalization coefficient (Figures 3(G)–3(I)). Thus, these data demonstrated that Ka treatment inhibited the activation of the NLRP3 inflammasome in hyperglycemia-induced macrophage.

3.4 | Effect of Ka on the HG-induced formation of NLRP3 inflammasome in macrophage

To determine the effect of Ka on HG-induced NLRP3 inflammasome production in macrophage, we analyzed the expression of NLRP3

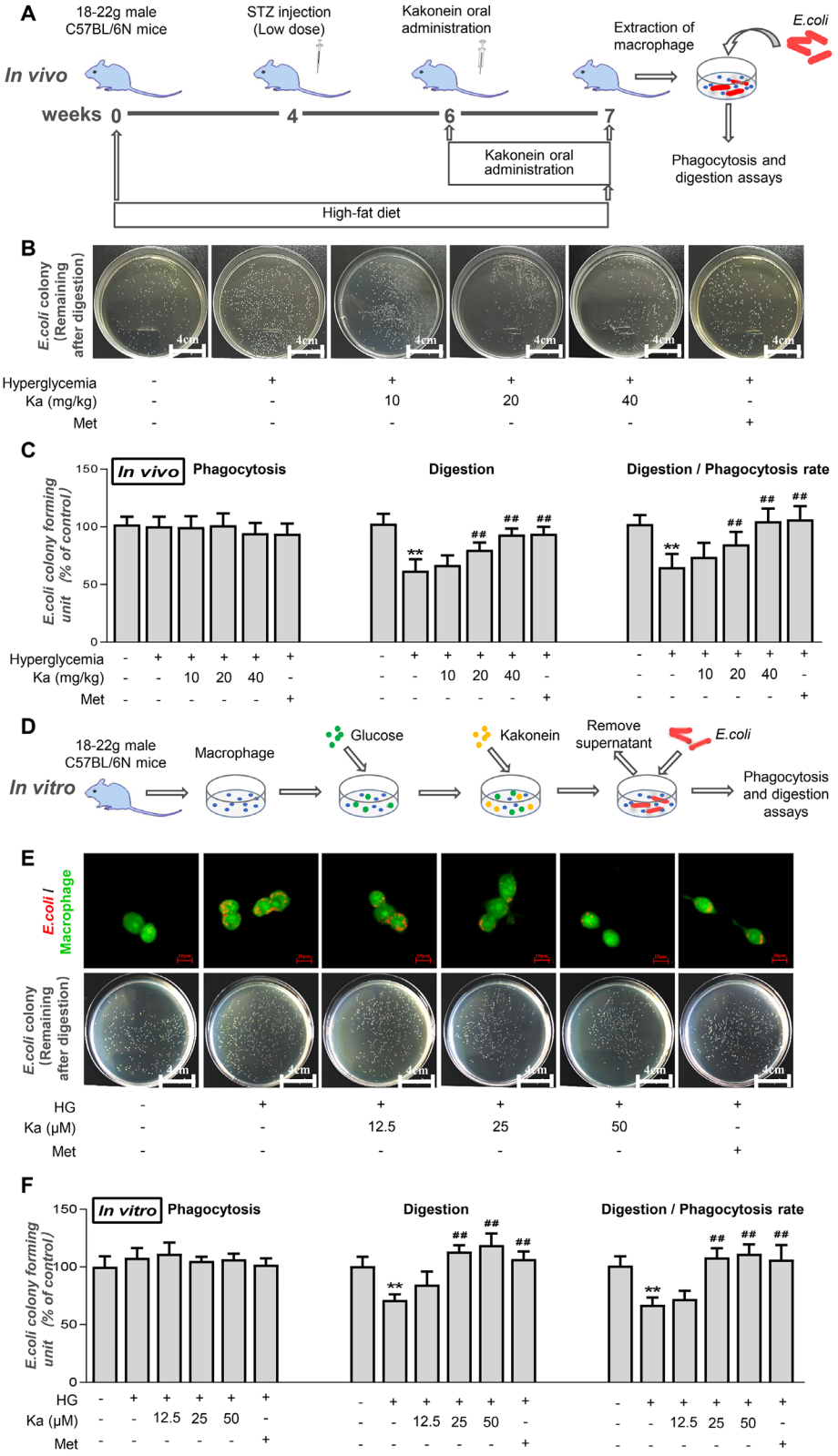

FIGURE 1 Therapeutic effect of Ka in hyperglycemia-induced macrophage dysfunction. (A) Schedule of animal arrangement in vivo. Nlrp3$^{+/+}$ and Nlrp3$^{-/-}$ mice established hyperlipidemia model and treated with Ka (10, 20, 40 mg/kg) or metformin (200 mg/kg) for 7 days and then primary macrophages were extracted from mice for bacterial phagocytosis and digestion assays. (B) and (C) The role of Ka in hyperglycemia-induced macrophage dysfunction of bacterial phagocytosis and digestion was detected by CFU assay ($n = 8$). (D) Schedule of primary macrophages arrangement in vitro. Nlrp3$^{+/+}$ and Nlrp3$^{-/-}$ macrophages established HG model and treated with Ka (12.5, 25, 50 μM) or metformin (2 mM) for bacterial phagocytosis and digestion assays. (E) Confocal microscopy of the cells and bacteria was stained with CytoTrace™ dye. *E. coil* was stained with CytoTrace™ Red CMTPX, and the macrophage was stained with CytoTrace™ Green CMTPX. The role of Ka in high glucose-induced macrophage dysfunction of bacterial digestion was detected by CFU assay. (F) Quantitative data of the CFU assay ($n = 3$). $^{**}p < 0.01$ versus control group. $^{\#\#}p < 0.01$ versus model group

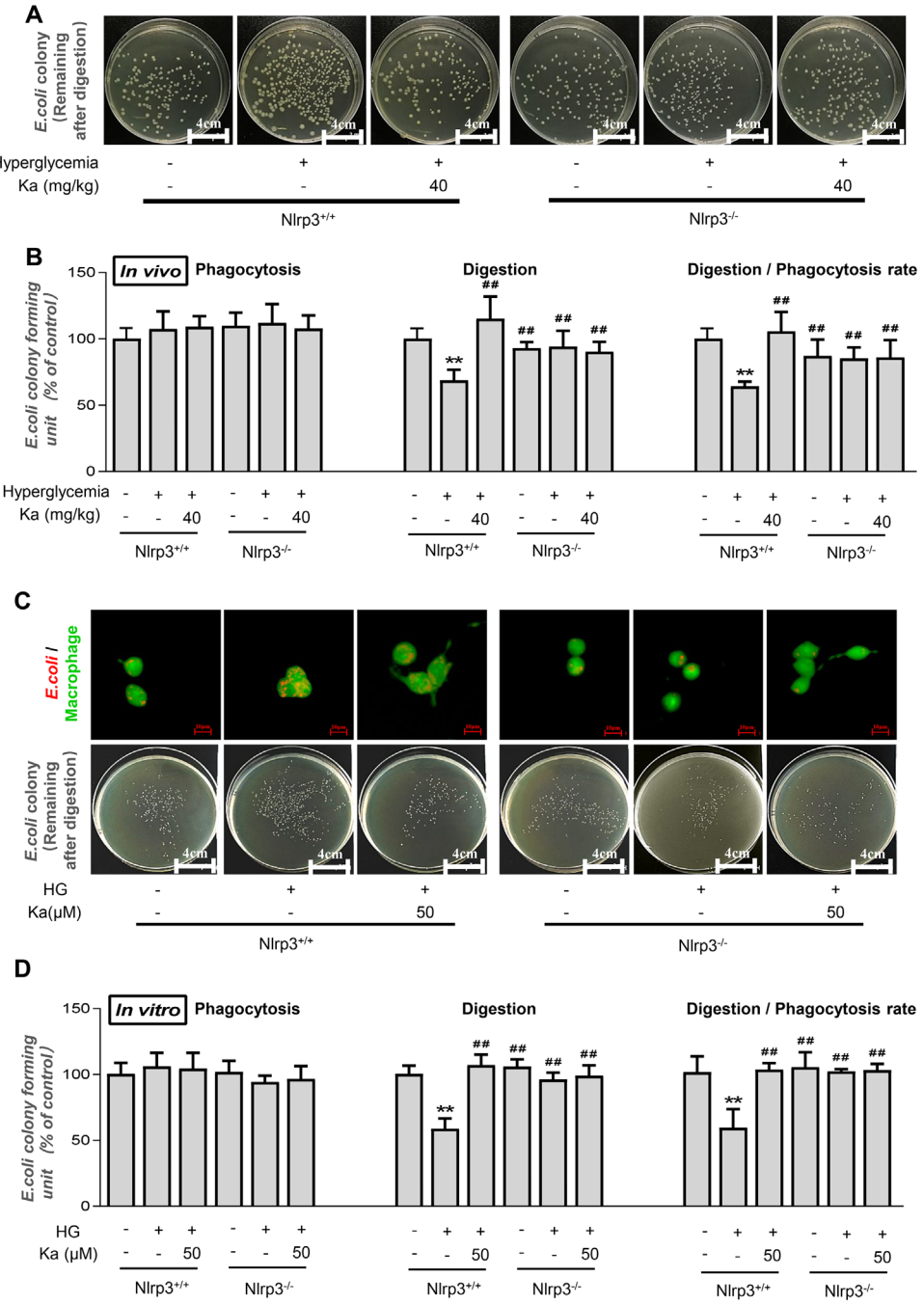

FIGURE 2 Therapeutic effect of Ka on the recovery of macrophage function under hyperglycemia by inhibiting the NLRP3 inflammasome. (A) and (B) The role of Ka in hyperglycemia-induced macrophage dysfunction of bacterial phagocytosis and digestion with or without NLRP3 deficient was detected by CFU assay ($n = 8$). (C) The role of Ka in high glucose-induced macrophage dysfunction of bacterial digestion with $Nlrp3^{+/+}$ or $Nlrp3^{-/-}$ macrophages were showed by CytoTrace™ dye staining and CFU assay. (D) Quantitative data of the CFU assay ($n = 3$). $^{**}p < 0.01$ versus control group. $^{\#\#}p < 0.01$ versus model group

inflammasome components. Results showed that Ka did not significantly decrease the expression of NLRP3 and ASC (Figures 4(A)–4(D)). Similarly, Ka did not significantly decrease the transcription of *Nlrp3* and *Asc* (Figures 4(E) and 4(F)). Thus, Ka inhibited the activation of NLRP3 inflammasome not by inhibiting NLRP3 inflammasome-related protein expression.

3.5 | Down-regulation of the HG-induced LMP and cathepsin B release in macrophage by Ka

The lysosomal release of cathepsin B plays a crucial role in mediating the NLRP3 inflammasome activation induced by LMP. As shown in Figures 5(A) and 5(B), the cathepsin B expression in the cytosol

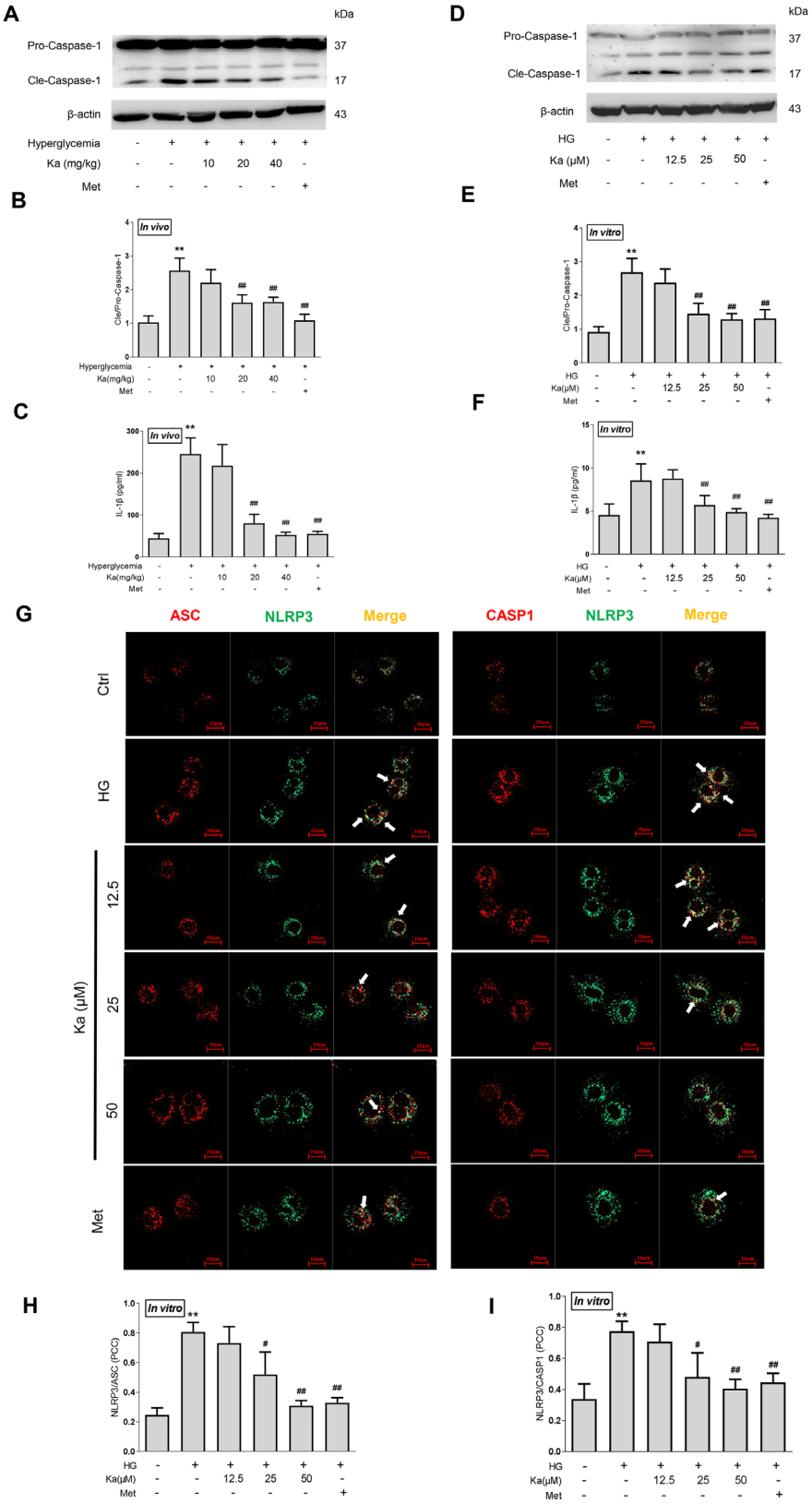

FIGURE 3 Down-regulation of the activation of NLRP3 inflammasome components by Ka. (A) and (B) Western blot analysis and summarized data showed the effect of Ka on macrophage protein expression levels of cle/pro-caspase-1 in hyperglycemic animal models ($n = 6$). (C) The content of IL-1β in serum were detected by ELISA kit ($n = 8$). (D) and (E) Western blot analysis and summarized data showed the effect of Ka on macrophage protein expression levels of cle/pro-caspase-1 in high glucose stimulated ($n = 3$). (F) The content of IL-1β in supernatant were detected by ELISA kit ($n = 3$). (G) Representative confocal fluorescence images indicated the effect of Ka on the colocalization of NLRP3 (green) with ASC (first line, red) or with CASP1 (second line, red) in macrophage. (H) and (I) Quantitative data of the colocalization efficiency of NLRP3 with ASC or NLRP3 with CASP1 ($n = 3$). $^{**}p < 0.01$ versus control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus model group

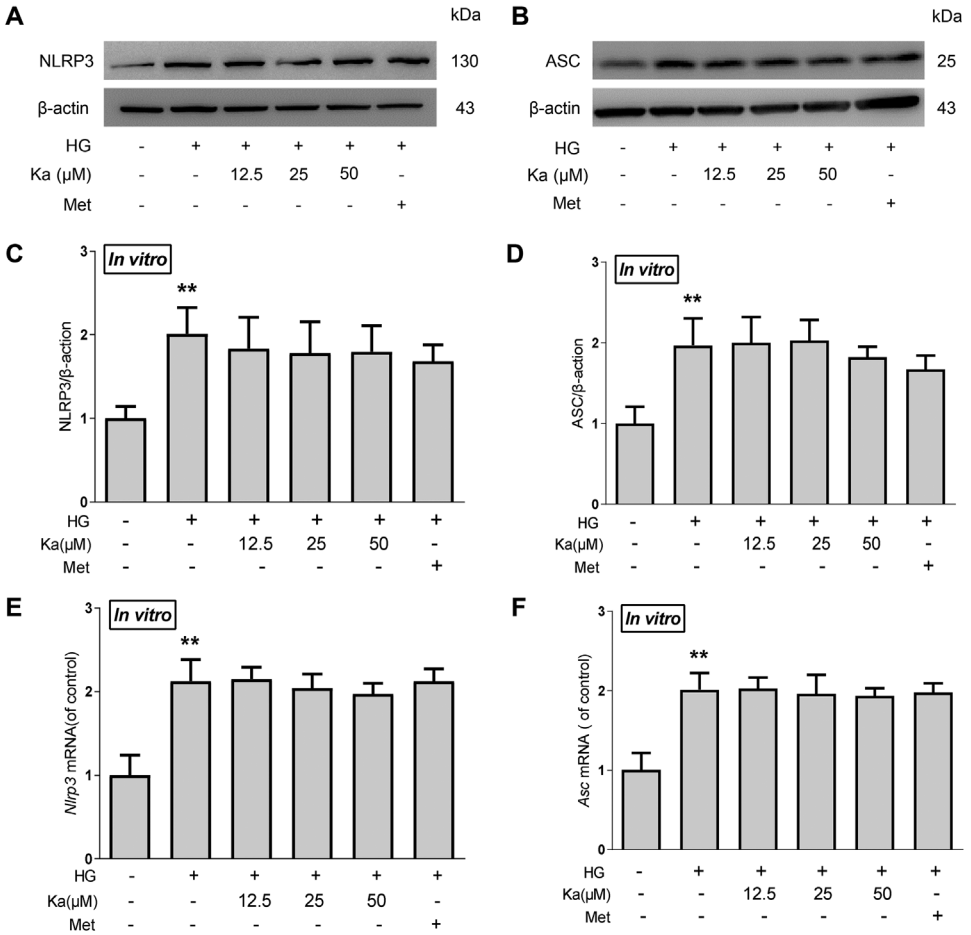

FIGURE 4 Effect of Ka on the high glucose-induced formation of NLRP3 inflammasome in macrophage. (A) and (C) Western blot analysis and summarized data of NLRP3 expression in different Ka concentrations in high glucose stimulated ($n = 3$). (B) and (D) Western blot analysis and summarized data of ASC expression ($n = 4$). (E) and (F) Analysis of *Nlrp3* and *Asc* transcriptional level ($n = 3$). $^{**}p < 0.01$ versus control group

of macrophage markedly decreased upon Ka treatment, suggesting that the down-regulation of cathepsin B release may be the result of reducing the LMP, leading to the inhibition of the NLRP3 inflammasome activation. Similarly, we found that Ka decreased the colocalization between LAMP1 (green) and cathepsin B (red) in a concentration-dependent manner (Figures 5(A)–5(C)). Western blot showed that Ka increased the expression of pro-cathepsin B in lysosome but decreased the expression of mature cathepsin B in the cytosol (Figures 5(D)–5(E)). These findings confirmed that LMP and cathepsin B were involved in the Ka down-regulation of the macrophage NLRP3 inflammasome activation.

3.6 | Validation of the therapeutic effect of Ka in HG-induced dysfunction through the down-regulation of cathepsin B release

We found that the cathepsin B inhibitor (CA-074) abolished the HG-induced NLRP3 protein binding and NLRP3 inflammasome activation in the macrophage. We monitored the changes in macrophage digestion in the presence of vehicle or CA-074 by CytoTrace™ staining and CFU assay. Figures 6(A) and 6(B) showed that the HG-induced dysfunction of macrophage bacterial digestion evidently recovered under Ka treatment, but therapeutic effects disappeared in the presence of CA-074. Our data showed that that CA-074 decreased the cleavage of pro-caspase-1 by Western blot method and IL-1β by ELISA method (Figures 6(C)–6(E)). CA-074 could also reduce the colocalization between NLRP3/ASC and NLRP3/CASP1 (Figures 6(F)–6(H)). To further determine the therapeutic effect of Ka in HG-induced macrophage digestion dysfunction, cathepsin B knock-down cell was established. The result showed that the HG-induced dysfunction of macrophage bacterial digestion evidently recovered under Ka treatment, but therapeutic effects disappeared in cathepsin B knock-down (Figure S4). These data suggested that the inhibition of cathepsin B also blocked the HG-induced macrophage dysfunction.

4 | DISCUSSION

This study demonstrates that inflammation participates in hyperglycemia-induced macrophage dysfunction, resulting in hyperglycemia complications.[18,19] In this regard, scholars must develop

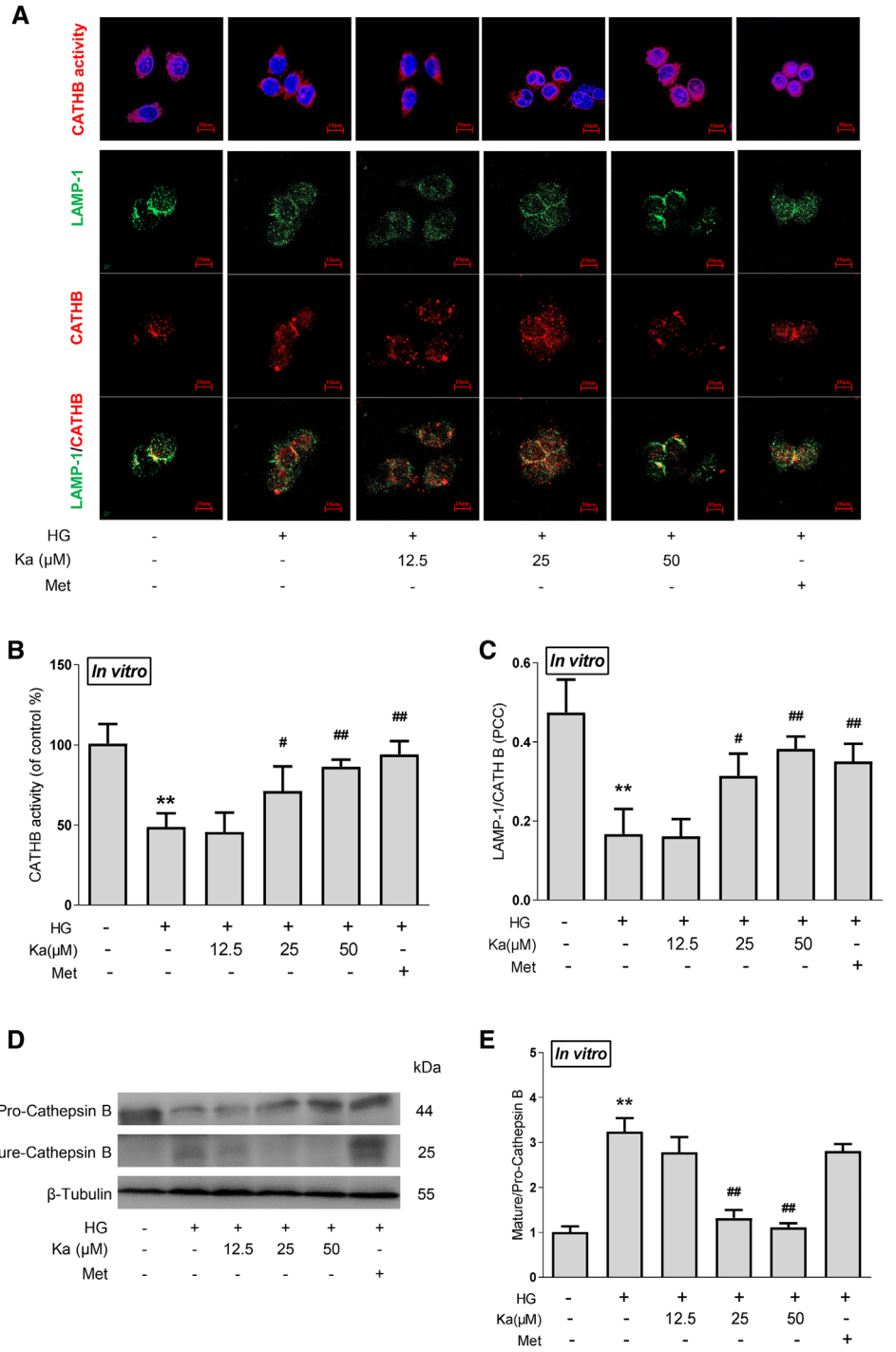

FIGURE 5 Down-regulation of the HG-induced LMP and cathepsin B release in macrophage by Ka. (A) The fluorescent of cathepsin B activity was stained with z-Arg-Arg-cresyl violet (red) and nuclei were stained with Hoechst (first line, blue). LAMP1/CathB was identified by confocal microscopy, the merged images displayed yellow dots or patches indicating the colocalization of LAMP1 (green) with CathB (second line, red). (B) and (C) Quantitative data of the colocalization efficiency of cathepsin B activity or LAMP1/CathB ($n = 3$). (D) and (E) Western blot analysis and summarized data showed the effect of Ka on the protein expression levels of pro-cathepsin B and mature-cathepsin B in macrophage ($n = 3$). $^{**}p < 0.01$ versus control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus model group

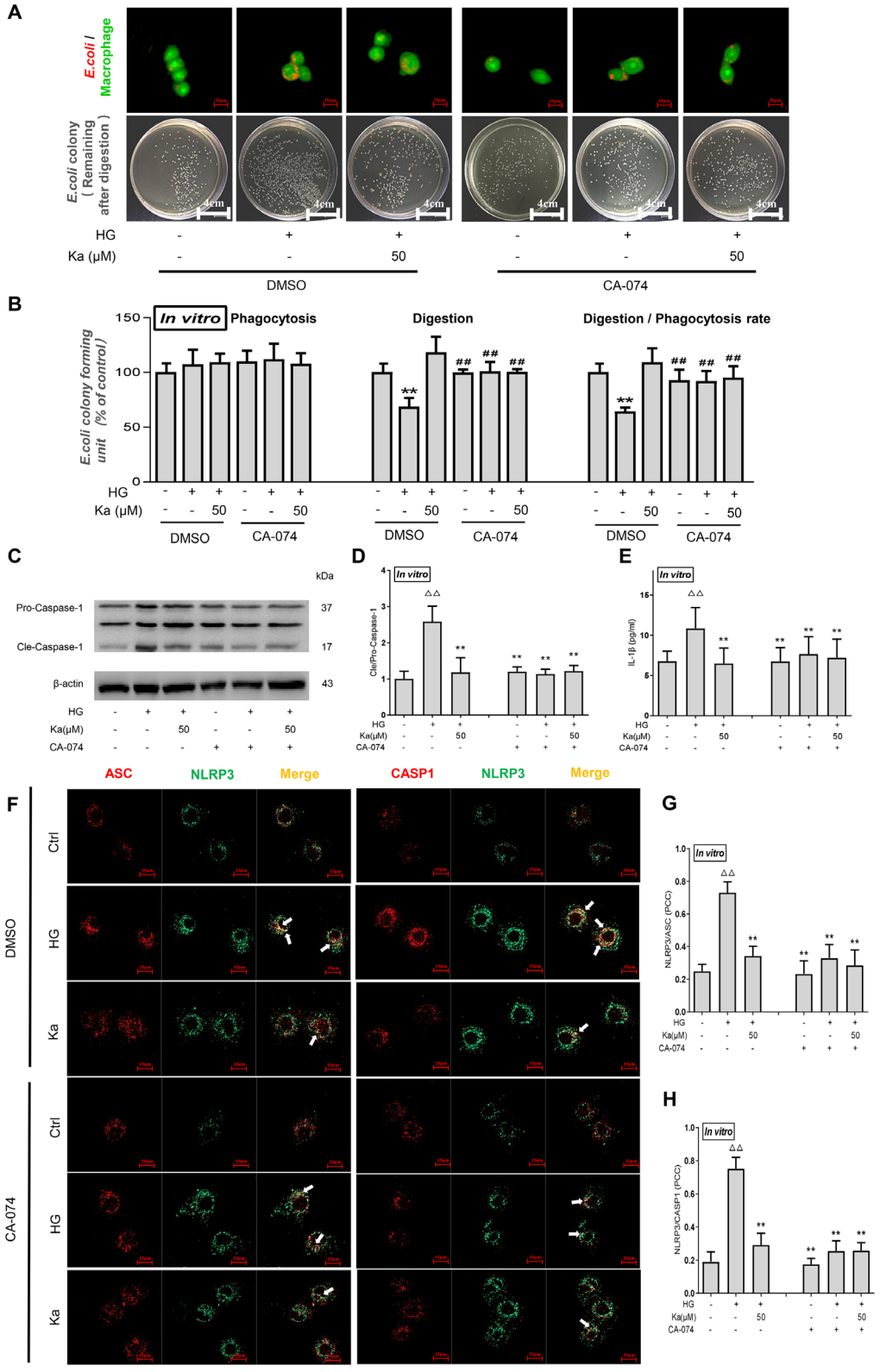

FIGURE 6 Validation of the therapeutic effect of Ka in HG-induced dysfunction through the down-regulation of cathepsin B release. (A) Confocal fluorescence images showed the role of Ka in high glucose-induced macrophage dysfunction of bacterial digestion in the presence of vehicle or cathepsin B inhibitor (CA-074) in vitro. (B) Quantitative data of the CFU assay ($n = 3$). (C) and (D) Western blot analysis and summarized data showed the effect of Ka on the protein expression levels of cle/pro-caspase-1 in macrophage ($n = 3$). (E) The content of IL-1β in supernatant were detected by ELISA kit ($n = 3$). (F) Macrophage was treated with or without Ka for 24 h in the presence of vehicle or CA-074. Confocal fluorescence images indicated the effect of Ka on the colocalization of NLRP3 (green) with ASC (first line, red) or with CASP1 (second line, red) in macrophage. (G) and (H) Quantitative data of the colocalization efficiency of NLRP3 with ASC or NLRP3 with CASP1 ($n = 3$). $^{\Delta\Delta}p < 0.01$ versus control group. $^{**}p < 0.01$ versus model group. (I) The role of Ka in high glucose-induced macrophage dysfunction of bacterial phagocytosis and digestion in scramble or Cathepsin B knock-down by CFU assay ($n = 3$)

drugs for hyperglycemia-associated complications by suppressing the inflammatory-mediated digestive dysfunction of macrophages. *P. lobata* (Willd.) Ohwi is widely used in treatment of hyperglycemia. Ka (daidzein-8-C-glucoside), an isoflavone commonly derived from *P. lobata*, is used for clinical treatment of hyperglycemia-related complications[20] and shows obvious anti-inflammatory effect.[21] Hence, Ka has potential in the clinical treatment of hyperglycemia complications. However, the anti-inflammatory mechanism of Ka in the treatment of hyperglycemia-induced macrophage dysfunction remains unclear. The results demonstrated that Ka could recover macrophage function through NLRP3 inflammasome effects in hyperglycemia, which are related to the cathepsin B–NLRP3 pathway.

The phagocytosis and digestion function of macrophages are important for preventing the damages of risk factors to maintain homeostasis.[4] Therefore, homeostatic imbalances are the common pathologic bases for development of various cardiovascular complication in hyperglycemia.[22] In our experiment, we established animal model of hyperglycemia and treated them with Ka, peritoneal macrophages were extracted and identified, and found that the change of macrophage typing was not significant, most of macrophage did not differentiate into M1 and M2 (Figures S1(B)–S1(D)). Then, the results confirmed the decreased digestion rate of macrophages in hyperglycemia and that Ka dose-dependently restored the digestion function of macrophages in vivo (Figures 1(A)–1(C)). Although it has been reported that Ka has hypoglycemic effect when the administration time or dosage is prolonged, our study found that Ka could improve the digestion function of macrophages even without significantly inducing hypoglycemic effect (Figure S1(A)), which reveals more pharmacodynamic mechanism of Ka. So we used metformin, which has an anti-inflammatory effect for treatment of hyperglycemic complications,[23] as a positive control drug. Furthermore, we identified primary macrophages derived from bone marrow (Figure S2) and established a coculture model of *E. coli* and primary macrophages in HG environment and found that Ka restored the digestion function of macrophages after stimulation with HG in vitro (Figures 1(D) and 1(E)). These data, for the first time, reveal a critical role of Ka in the recovery of macrophage digestion function in hyperglycemia.

As a large intracellular signaling platform in macrophages, inflammasomes play an important role in innate immune response, including in regulating digestive function.[24] The present research showed that cle-caspase-1, the product of inflammasomes, could cleave endosomal early endosome antigen 1 or Rab interacting lysosomal protein,[25,26] wherein both are essential proteins that regulate digestive function by affecting endosome docking and fusion machinery.[27] Although inflammasomes contain many isoforms, such as NLRP1 or NLRC4,[28] our previous data confirmed that the activation of macrophage NLRP3 inflammasomes contributes to the development of the dysfunction of digesting microbes[14] and other studies also supported that NLRP3 inflammasome was related to phagocytosis.[29] Therefore, we used MCC950, which acted as a classical inhibitor of NLRP3 inflammasome activation, to verify the mechanism of NLRP3 inflammasome-mediated macrophage digestion. The results showed that the HG-induced digestive dysfunction evidently recovered under MCC950 treatment (Figure S3(C)). Moreover, many studies showed that the anti-inflammatory effect of Ka is related to NLRP3 inflammasome[30,31] and therapeutic effect is achieved by NLRP3 inflammasome activation.[32–34] Thus, we selected the NLRP3 inflammasome pathway as the critical point to further investigate the mechanism of Ka. In the present study, the decrease in the digestive function of macrophages was inhibited when the *Nlrp3* gene was absent; meanwhile, *Nlrp3* gene deficiency also prevented effect of Ka against hyperglycemia-induced macrophage digestion decreased (Figure 2). In this phase, our data support the view that Ka can restore macrophage digestive function in hyperglycemia by inhibiting the NLRP3 inflammasome pathway.

We found that increasing cle-caspase-1, IL-1β, and IL-18 expression, assembly of NLRP3 inflammasomes was inhibited in macrophages under the treatment of Ka (Figures 3 and S(A) and S(B)). This finding suggested that NLRP3 inflammasome-dependent caspase-1 activity was blocked by Ka. In this regard, we further studied the mechanism of Ka in inhibiting NLRP3 inflammasome activation. The NLRP3 inflammasome pathway needs 2 signal paths to be activated. The signal 1 of NLRP3 inflammasomes was called priming, which was activated by the NF-kB signal pathway through inflammation-related receptors, such as TLR4, TNF-R1, and IL-1R1. NLRP3 inflammation-related genes were induced to be transcribed to increase the protein expression. However, we found that Ka did not alter the gene transcription or protein expression of NLRP3 and ASC, which were both increased induced by HG (Figure 4). This study differs from previous reports[35] on the mechanism of Ka in inhibiting NLRP3 inflammasomes possibly due to differences in the disease model used and dosage of Ka tested. Thus, we decided to focus on other signal pathways. The signal 2 of NLRP3 inflammasome was called activation, which was induced by the assembly of NLRP3 inflammasome complex by pathogen-associated molecule patterns or damage-associated molecule patterns (DAMP), resulting in the activation of caspase-1 maturation. Interestingly, we demonstrated in the present research that Ka reduced the release of cathepsin B from lysosomes and recovery the activity of cathepsin B. Cathepsin B is a cysteine proteolytic enzyme in lysosomes, which is related to the activation of noncanonical inflammasome, especially NLRP3 inflammasome. When stimulated by risk factors, cells increase lysosomal permeability and lead to the leakage of cathepsin B, which interacted with LRR fragment of NLRP3 at endoplasmic reticulum and form transient cathepsin B/NLRP3 complex that activates NLRP3 inflammasome. Ka also inhibited HG-induced cathepsin B maturation to block cathepsin B/NLRP3 binding and NLRP3 inflammasome activation (Figure 5). DAMPs could be a key factor in proinflammatory response as signal 2 in hyperglycemia.[36] Our previous study reported that the release of cathepsin B activates NLRP3 inflammasomes, contributing to injuries on macrophage digesting function.[14] Consequently, our result suggested that the mechanism of Ka may be related to signal 2 rather than signal 1. Metformin also reduced the release of cathepsin B but promoted its maturation, in contrast to Ka. To complement this anti-inflammatory mechanism, we treated macrophages with CA-074, which acted as a selective inhibitor of cathepsin B, as another positive control drug. Similar to Ka, CA-074 both exerted effects such as

inhibiting HG-induced NLRP3 inflammasome activation and protecting the function of macrophage digestion. Moreover, CA-074 prevented the inflammation effect of Ka in hyperglycemia-induced macrophage digestion dysfunction (Figures 6 and S1(E)). Recently, several studies have proved that CA-074 had clear off-target effects on cathepsin B-NLRP3 inflammasome activation,[37–41] thus we further examined that Ka prevented HG-induced dysfunction through cathepsin B by gene-silencing technology. Our result suggested that cathepsin B knock-down also prevented therapeutic effect of Ka in HG-induced digestive dysfunction (Figure S4). Thus, Ka may recover the digestion function of macrophages by inhibiting cathepsin B-NLRP3 inflammasome pathway.

The function of macrophages plays an important role in maintaining homeostasis. The dysfunction of macrophages induced by hyperglycemia may lead to severe clinical complications of diabetes, including wound healing, atherosclerosis, poor cardiac remodeling after myocardial infarction, and increased susceptibility to infection. The change of phagocytic digestive function is the key characteristic to determine the function of macrophages, which is considered to be a marker event of the weakening of antibacterial effect in the process of infection. In our study, we are the first to report that Ka could recover hyperglycemia-associated dysfunction of macrophage digestion. The results exhibit a mechanism of Ka in inhibiting cathepsin B–NLRP3 inflammasome pathway. Overall, our research provides a new target for the treatment of diabetic complications, especially diabetes-related infectious diseases, and the development of innovative drugs.

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AUTHORSHIP

X. W., Y. H., Y. C., and D. L. conceived and designed the studies. D. L. and L. Z. performed the experiments. Y. Y., X. Z., D. C., Y. G., W. X., Y. C., and H. H. analyzed the data. D. L., L. Z., and Y. Y. drafted the manuscript. Y. L., J. L., and R. H. supplemented the experiment. D. X. and C. Z. provided financial support. All authors read and approved the final version of the manuscript. D. L. and L. Z. contributed equally to this work.

DISCLOSURE

The authors have declared that no competing interest exists.

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SUPPORTING INFORMATION

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