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Metformin ameliorates endotoxemia-induced endothelial pro-inflammatory responses via AMPK-dependent mediation of HDAC5 and KLF2

Rui Tian[a,1], Ranran Li[a,*,1], Yiyun Liu[a], Jialin Liu[a], Tingting Pan[a], Ruyuan Zhang[a], Bingya Liu[b], Erzhen Chen[c], Yaoqing Tang[a], Hongping Qu[a,*]

[a] Department of Critical Care Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, PR China
[b] Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, PR China
[c] Department of emergency, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, PR China

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ABSTRACT

Exaggerated endothelial pro-inflammatory response is a hallmark in the early stage of sepsis and contributes to the subsequent tissue injury and organ failure. The anti-inflammatory effects of AMP-activated protein kinase (AMPK) activator metformin in sepsis has been revealed. However, the underlying mechanisms remain not fully understood. In the present study, the potential roles of histone deacetylase 5 (HDAC5) and kruppel-like factor 2 (KLF2) in the effects of metformin on endothelial pro-inflammatory responses were investigated. The results showed that metformin pretreatment increased the phosphorylation of HDAC5 at serine 498, leading to the upregulation of KLF2, and eliminated lipopolysaccharide (LPS) and tumor necrosis factor α (TNFα)-induced upregulation of vascular cell adhesion molecule 1 (VCAM1). Furthermore, the adhesion of HL60 leukocytes to endothelial monolayer was effectively inhibited by metformin. In addition, the in vivo data confirmed that AMPK activation attenuated local and systemic inflammation in endotoxic mice induced by LPS via mediating phosphorylating HDAC5 and restoring KLF2 expression. Our findings revealed that AMPK activation-mediated HDAC5 phosphorylation and KLF2 restoration is, at least partially, responsible to the anti-inflammatory effects of metformin in endotoxemia-induced endothelial cells, which has important implications for the future development of interfering therapies of sepsis.

1. Introduction

Sepsis, characterized by dysregulation of systemic immunity and subsequent multiple organ failure, is the leading cause of death among hospitalized patients [1,2]. Despite substantial efforts in understanding the pathophysiology of sepsis as well as investigation of therapeutic strategies, the effective treatment of sepsis remains a clinical challenge. Endothelial cells lining along the inner surface of the vasculature play a significant role in maintaining vascular homeostasis [3]. In the early stage of sepsis, exaggerated endothelial activation, vascular leakage, disturbance of blood flow, as well as other derangements cause the loss of vascular integrity, which contributes significantly to sepsis-associated organ failure. Therapies interfering endothelial activation are potential to attenuate sepsis-induced organ dysfunction.

Lipopolysaccharide (LPS), also known as endotoxin, is the key structural component of the wall of gram-negative bacteria and is a critical factor that triggers the pathogenesis of sepsis [4]. Circulating LPS activates endothelial cells, leading to the production of inflammatory cytokines, expression of adhesion molecules, leukocyte recruitment, and loss of vascular integrity. In addition, LPS activates monocytes and macrophages in the circulation to release pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), which in turn modulate endothelial pro-inflammatory activation [5,6].

The active molecular responses in inflammation requires intensive metabolic support and modulation of the metabolic pathways might be a novel strategy to restrict inflammatory injury [7]. Metformin, characterized as a metabolic regulator, is widely used as a first-line anti-diabetic drug for type-II diabetes [8]. Metformin activates adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) which plays a pivotal role in maintaining the metabolic homeostasis [9]. Recently, several studies revealed the anti-inflammatory effects of metformin in sepsis [10,11]. However, the underlying mechanisms remains not fully understood.

The regulation of gene expression by histone deacetylases (HDACs)

* Corresponding authors.
E-mail addresses: rebecca1009@hotmail.com (R. Li), hongpingqu0412@hotmail.com (H. Qu).
[1] These authors contribute equally to this work.

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is a kind of post translational modification. HDAC activity is demonstrated to be highly enhanced in sepsis, hemorrhagic shock, and other acute injuries [12–14], leading to the organ dysfunction. The beneficial effects of HDAC inhibition suggests that controlling HDAC activity is a potential therapeutic strategy in these diseases [15,16]. Kruppel-like factor 2 (KLF2) as a vascular-protective transcription factor plays an important role in maintaining endothelial function and vascular integrity [17–19]. KLF2 in endothelial cells is well known to be highly sensitive to fluid shear stress. Recent studies have demonstrated that the regulation of KLF2 by fluid shear stress is controlled by HDAC5, one of the Class IIa HDACs of which the activity is regulated by their phosphorylation-dependent nuclear/cytoplasm shuttling [20]. AMPK has been reported to induce HDAC5 cytoplasm/nuclear translocation in cellular adaptation to hypoxia [21].

Therefore, in this study, we aimed to investigate the potential mechanisms of HDAC5 and KLF2 as the downstream of AMPK in mediating the effects of metformin on endothelial pro-inflammatory responses both in vitro and in a mouse model of endotoxemia.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords. Fresh human umbilical cords were acquired from the Department of Obstetrics in Ruijin Hospital with the concern of donors. Briefly, two fresh umbilical cords were collected and stored in sterile PBS. Before isolation, wash away the residual blood in the lumen of the vein and check the leakage of the whole cord with sterile PBS. Then the lumen of the vein was filled with pre-warmed Collagenase type I (C0130, Sigma Aldrich, St. Louis. MO). The two ends of the cord were clamped and incubated in sterile PBS at 37 °C for 10 min. Gently massage the cord to help the detachment of endothelial cells from the vessel wall. Then drain the collagenase solution with isolated cells into a 50 ml tube. Wash the vein with endothelial cell medium. Collect medium in the 50 ml tube with cell suspension and centrifuge the tube at 1500 rpm for 10 min. Resuspend the cell pellet and seed cells in T25 flask pre-coated with 1% gelatin (V900863, Sigma Aldrich). HUVEC were grown in EGM-2MV medium (CC-3202, Lonza, Walkersville, MD USA) with 10% fetal bovine serum (FBS, CC-4102B, Lonza), suspended with streptomycin and penicillin and maintained at 37 °C under a humidified atmosphere with 5% CO2. HUVEC at passages 1–4 were used for all experiments. Isolated HUVEC were characterized using endothelial-specific markers, CD31(560983, BD Pharmingen, San Jose, CA) and VE-cadherin (561714, BD Pharmingen) (Supplementary Fig. 1). HUVEC were used in accordance with the human subject guidelines of Ruijin Hospital Shanghai Jiao Tong University.

To induce endothelial inflammation, HUVEC were stimulated with LPS (*E. coli* serotype 0111: B4, L2630, Sigma Aldrich) and human recombinant TNFα (300-01A, Pepro tech, USA). For cell pretreatment, AMPK activator metformin from Sigma Aldrich (PHR1084) and AICAR from Selleck (S1802, Houston, TX, USA) were used. HDAC5 specific inhibitor LMK235 was purchased from Selleck (S7569).

2.2. Animals

Male C57BL/6 mice (20–30 g) were randomly divided into experimental groups (n = 6 per group). The animals were housed individually and maintained on a mouse chow diet in a temperature and light-dark cycle-controlled environment (24 °C, 12: 12 h).

To induce endotoxemia, mice were injected intraperitoneally with LPS (5 mg/kg). The dose of LPS has been determined according to the literature [22]. In intervention groups, mice were intraperitoneally injected with metformin (250 mg/kg) 16 h before LPS injection or with AICAR (500 mg/kg) 1 h before LPS injection. The pre-treatment time and doses of metformin and AICAR have been selected according to the literatures [23–27]. As vehicle control, 200 ul 0.9% NaCl were i.p. injected 1 h before LPS injection. 6 h after LPS injection, mice were anesthetized and blood was collected. Organs were snap frozen or fixed with formalin. Frozen organs were stored at −80 °C. All animal experiments were conducted according to the principles of laboratory animal care.

2.3. Western blot

The protein samples from total cell or tissue lysates were subjected to 12% SDS-PAGE and transferred to PVDF membrane (162-0177, Bio-Rad, USA). Membranes were blocked with 5% non-fat dried milk and then incubated with appropriate primary and secondary antibodies. Blots were detected with HRP Substrate (WBLUF0500, Millipore, USA). Antibodies against VCAM1 (sc-1504), KLF2 (sc-28675), HDAC5 antibody (sc-133225), and phospho-HDAC5 (Ser498, sc-101692) were purchased from Santa Cruz (USA). Antibody against acetyl-Histone H3 (06-599) were purchased from Sigma Aldrich. Antibodies against AMPK alpha (2532) and phospho-AMPK-alpha (Thr172, 4188) were purchased from Cell Signaling Technologies (Danvers, MA). Images were taken using a Tanon 5500 Imaging System (Tanon, Shanghai, China).

2.4. Immunofluorescence staining of HDAC5

HUVEC were grown on Millicell 8-well glass (PEZGS0416, Millipore, USA). After indicated treatments, cells were fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.25% Triton X-100 (Sigma Aldrich) for 5 min on ice. Cells were blocked with 3% BSA (Sigma Aldrich), and then incubated with HDAC5 antibody (sc-133225, Santa Cruz) overnight at 4 °C. After washing, cells were incubated with the secondary antibody conjugated with Alexa flour 488 (A11029, Thermo Fisher Scientific). Nuclei was stained with DAPI (D9542, Sigma Aldrich) and slides were mounted with microscope cover glass (80340-0130, CITOGLAS, Jiangsu, China). Images were taken with Olympus microscope (DP73, Japan).

2.5. Endothelial leukocyte adhesion assay

HUVEC monolayer was seeded in 6-well plate. HL60 were grown in RPMI-1640 media (R-1383, Sigma) supplemented with 10% FBS (10091148, Gibco, USA). HL60 were stained with Hochest 33342 (H1399, Thermo Fisher Scientific) for 10 min, washed, and resuspended in RPMI-1640 containing 1% FBS at a concentration of 500,000 cells/ml. 1 ml of the labeled cells were allowed to attach to HUVEC monolayers for 1 h at 37 °C. Unattached HL60 were washed off with PBS. 6–8 random images per well were taken with Fluorescence microscope (DP73, Olympus).

To quantify the number of adhered HL60 leukocytes on endothelial monolayer, HL60-adhered HUVEC were trypsinized with trypsin after washing properly. Trypsinized cells were collected with 5% FBS in PBS. HL60 in cell suspension were incubated with FITC-conjugated CD45 antibody (560976, BD Bioscience, San Diego, CA, USA) for 1 h in dark on ice, and resuspended with FACS buffer. The percentage of adhered HL60 was determined by flow cytometry (FACSCalibur, BD Bioscience).

2.6. Small interfering RNA–mediated gene silencing

AMPKα was knocked down using small interfering RNA (siRNA) sequence for global AMPKα (AMPKα1 and AMPKα2) [28]. Negative control siRNA was used as control. Global AMPKα siRNA and negative control siRNA were purchased from GenenPharma (Shanghai, China). HiPerFect (301704, QIAGEN, Germany) was used as the transfection reagent according to the manufacturer's instructions.

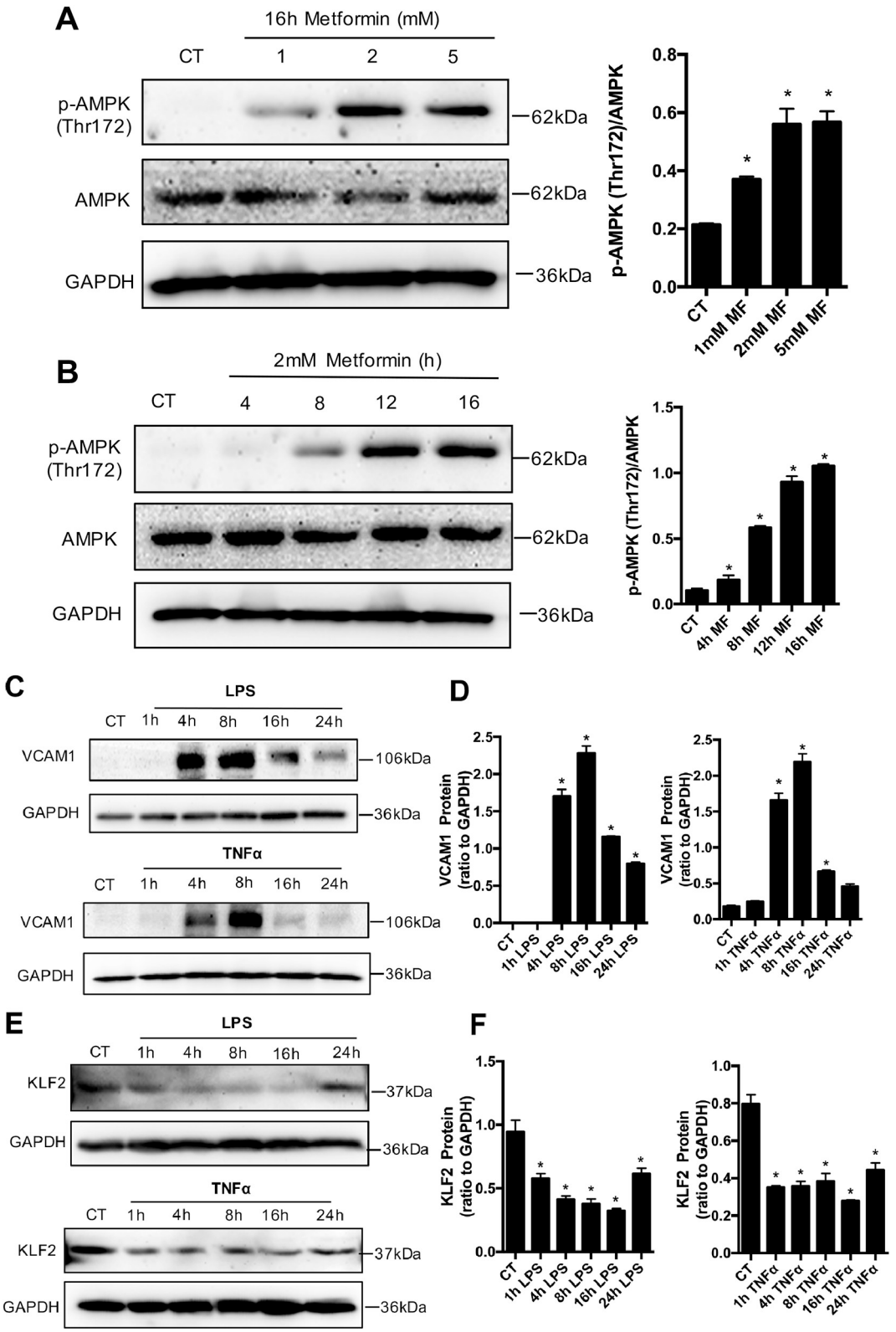

Fig. 1. The responses of HUVEC to metformin treatment and LPS/TNFα stimulation in vitro. (A, B) Human umbilical vein endothelial cells (HUVEC) were incubated with metformin at different doses (1 mM, 2 mM, and 5 mM) and for different time points (4 h, 8 h, 12 h, and 16 h). The protein levels of phosphorylated AMPK (Thr172) were determined by western blot. (C–F) HUVEC were stimulated with LPS (1 μg/ml) and TNFα (10 ng/ml) respectively for different time points. The protein levels of VCAM1 and KLF2 were determined by western blot. Images were representative of three independent experiments. The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3).

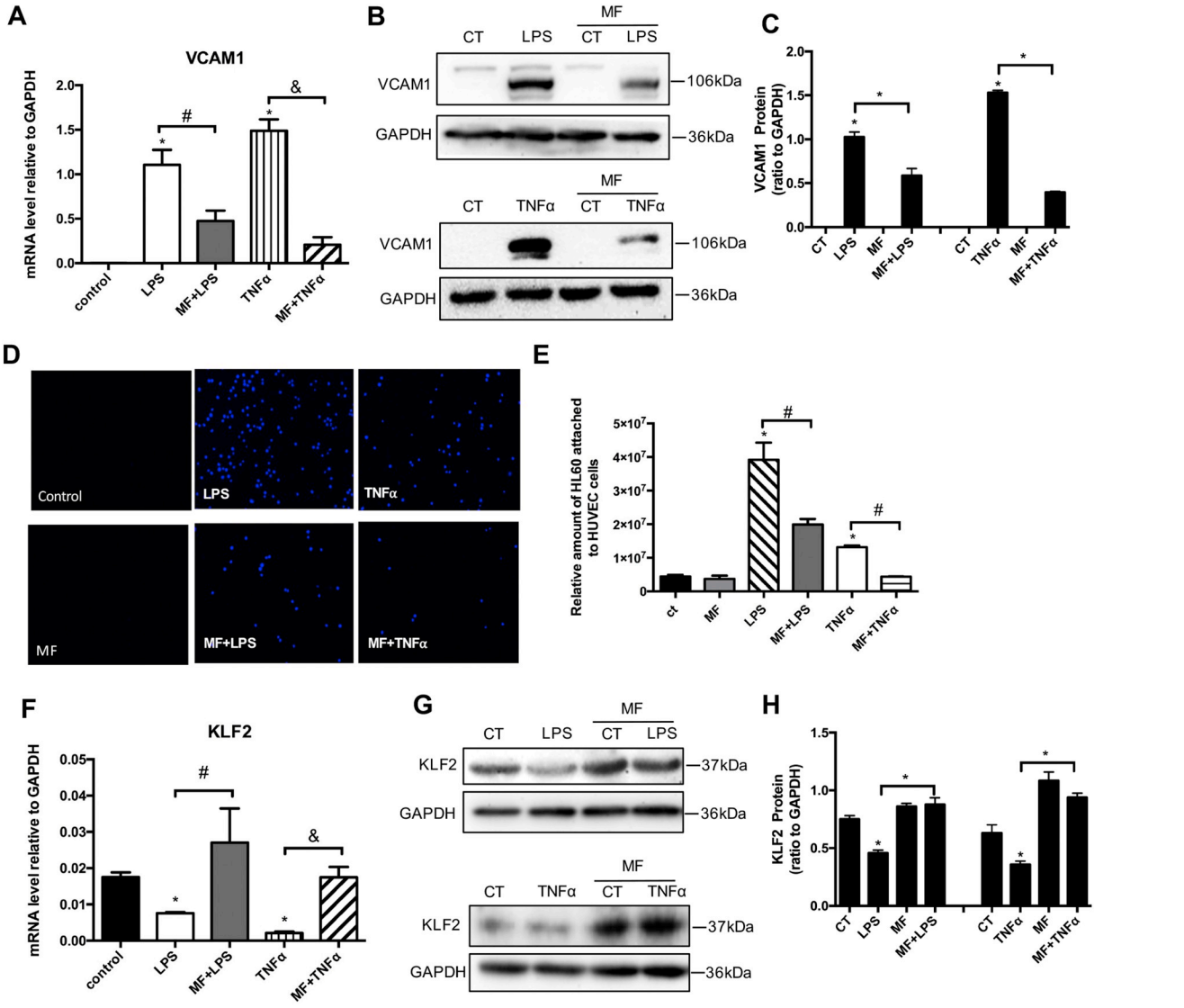

Fig. 2. Metformin inhibited the pro-inflammatory activation of endothelial cells induced by LPS and TNFα. HUVEC were pre-incubated with metformin (2 mM) for 16 h before exposed to LPS (1 μg/ml) and TNFα (10 ng/ml) for another 4 h. (A, F) VCAM1 and KLF2 mRNA level expression was determined by quantitative RT-PCR using GAPDH as the housekeeping gene. Values represented mRNA level relative to GAPDH. Data were expressed as mean ± SD, n = 3 per group. *, $P < 0.05$, LPS/TNFα vs. control; #, $P < 0.05$, MF + LPS vs. LPS; &, $P < 0.05$, MF + TNFα vs. TNFα. (B, C and G, H) The protein expression of VCAM1 and KLF2 was determined by western blot. Images were representative of three independent experiments. The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3). (D) HL60 stained with Hoechst-33342 were co-cultured with HUVEC for 1 h. Images were taken to show HL60 leukocytes attached to HUVEC. Images were representative of three independent experiments. (E) HL60 attached to HUVEC were stained for CD45 and quantified by flow cytometry. Data were expressed as mean ± SD, n = 3. *, $P < 0.05$, LPS/TNFα vs. control; #, $P < 0.05$, metformin + LPS/TNFα vs. LPS/TNFα.

2.7. Cytokine quantification by enzyme-linked immunosorbent assay

The concentrations of TNFα and IL-6 in mice plasma were measured by enzyme-linked immunosorbent assay (TNFα and IL-6: R&D Systems, Minneapolis, MN).

2.8. Quantitative real-time PCR

Total RNA was extracted from cell and tissue lysates using TRIzol® Reagent (15596-026, Thermo Fisher Scientific) following the protocol of the manufacturer. RNA concentration and purity were measured with Gen5 Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Reverse transcription of RNA was carried out using the RevertAid First Stand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific). SYBR Green reagent (A25777, Thermo Fisher Scientific) was used for qRT-PCR to analysis mRNA expression. Sequences of mouse primers used were as follows:

human VCAM1 (5′-ACCACATCTACGCTGACAATGAATCC-3′ and 5′-AACACTTGACTGTGATCGGCTTCC-3′); mouse VCAM1 (5′-CCTCACT TGCAGCACTACGG-3′ and 5′-CATGGTCAGAACGGACTTGG-3′); human KLF2 (5′-GGTGAGAAGCCCTACCACTG-3′ and 5′-GCACAGATGGCACT GGAAT-3′); mouse KLF2 (5′-CGGCAAGACCTACACCAAGA-3′ and 5′-AACTTCCAGCCGCATCCTT-3′); human GAPDH (5′-GGTGAAGGTCG GAGTCAACG-3′ and 5′-CAAAGTTGTCATGGATGGACC-3′); mouse GAPDH (5′-AAGCCCATCACCATCTTCCA-3′ and 5′-CCTGCCTCACCACC TTCTTG-3′).

2.9. Immunohistochemistry (IHC) staining

IHC staining was conducted using streptavidin–biotin–peroxidase complex method. Briefly, mouse kidney and lung tissue samples were fixed and paraffin-embedded. Tissue sections were dewaxed,

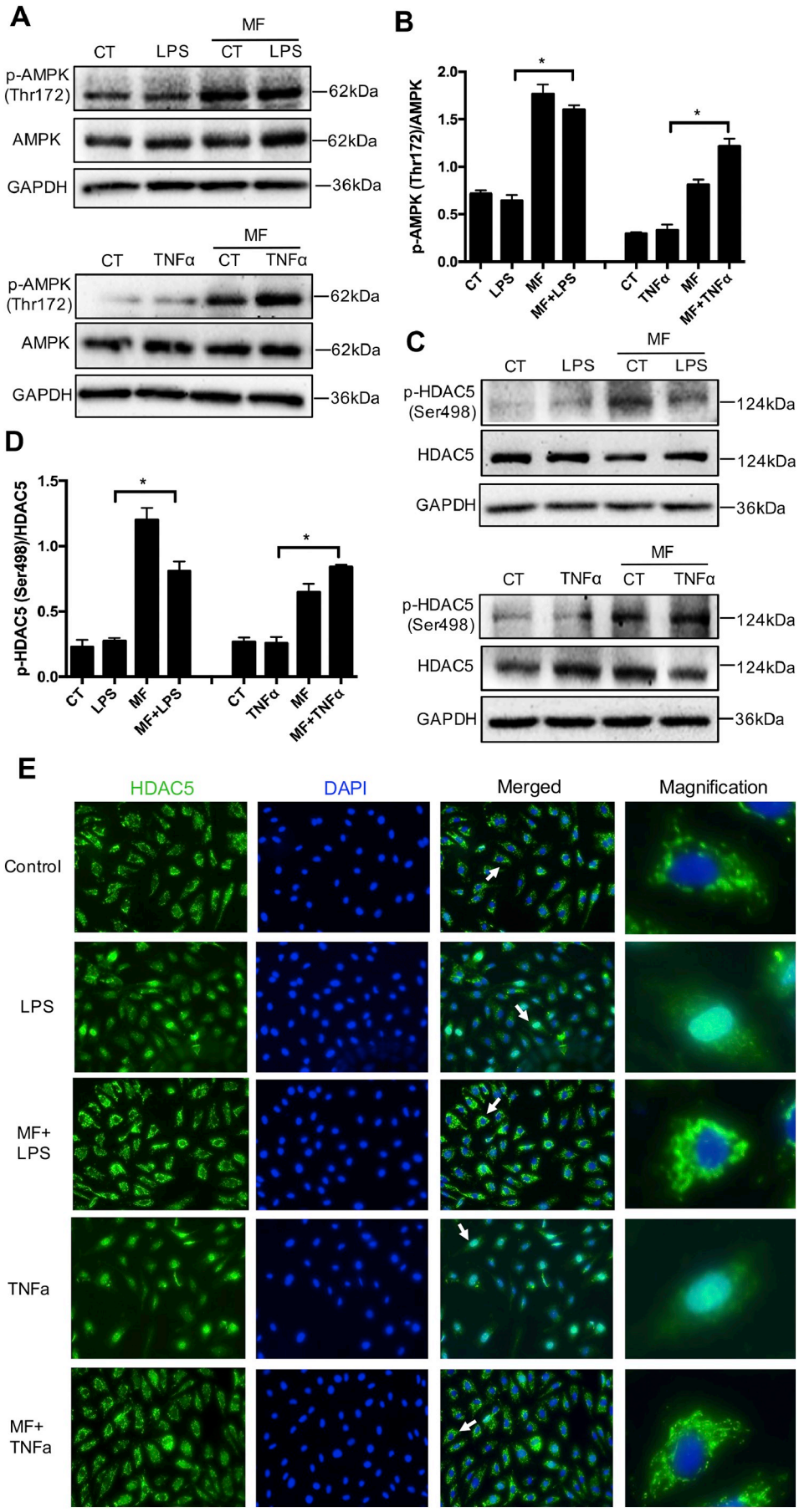

Fig. 3. The effects of metformin on AMPK and HDAC5 phosphorylation in HUVEC. (A-D) HUVEC were pre-incubated with metformin (2 mM) for 16 h before stimulated with LPS (1 μg/ml) and TNFα (10 ng/ml) for another 4 h. Phosphorylated AMPKα (Thr172) and phosphorylated HDAC5 (Ser498) were analyzed by western blot. The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3). (E) Immunofluorescence staining of HDAC5 in HUVEC. HDAC5 was stained in red and nucleus was stained with DAPI (blue). The image magnification is 400×. Images were representative of three independent experiments.

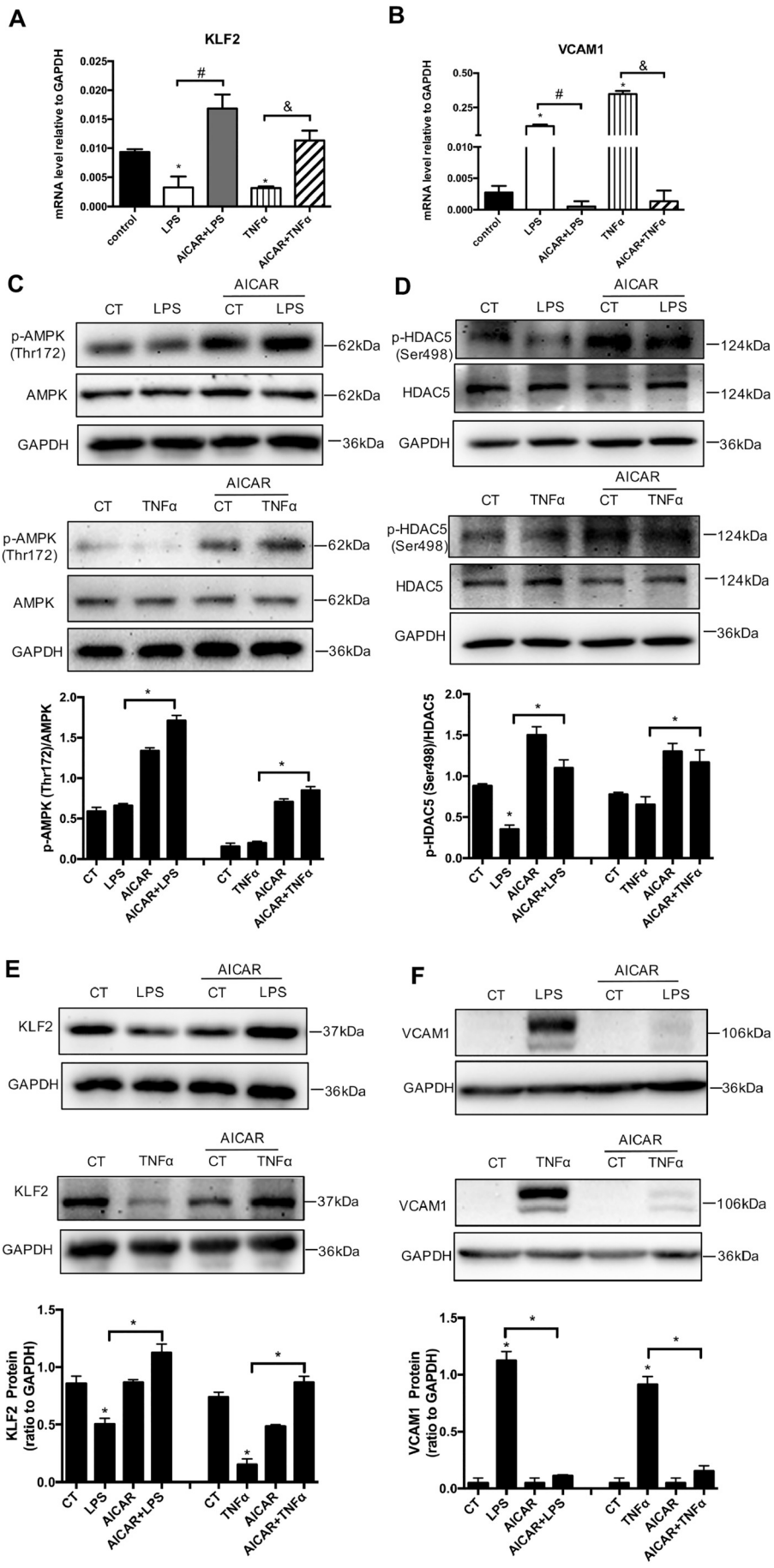

Fig. 4. AICAR abolished endothelial pro-inflammatory responses via mediating AMPK-HDAC5-KLF2 signaling. HUVEC were pre-incubated with AICAR (1 mM) for 1 h before stimulated with LPS (1 μg/ml) and TNFα (10 ng/ml) for another 4 h. (A, B) VCAM1 and KLF2 mRNA level expression was determined by quantitative RT-PCR using GAPDH as the housekeeping gene. Values represented mRNA level relative to GAPDH. Data were expressed as mean ± SD, n = 3 per group. *, $P < 0.05$, LPS/TNFα vs. control; #, $P < 0.05$, AICAR + LPS vs. LPS; &, $P < 0.05$, AICAR + TNFα vs. TNFα. (C–F) The protein levels of phosphorylated AMPK (Thr172), phosphorylated HDAC5 (Ser498), KLF2, and VCAM1 were determined by western blot. Images are representative of three independent experiments. The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3).

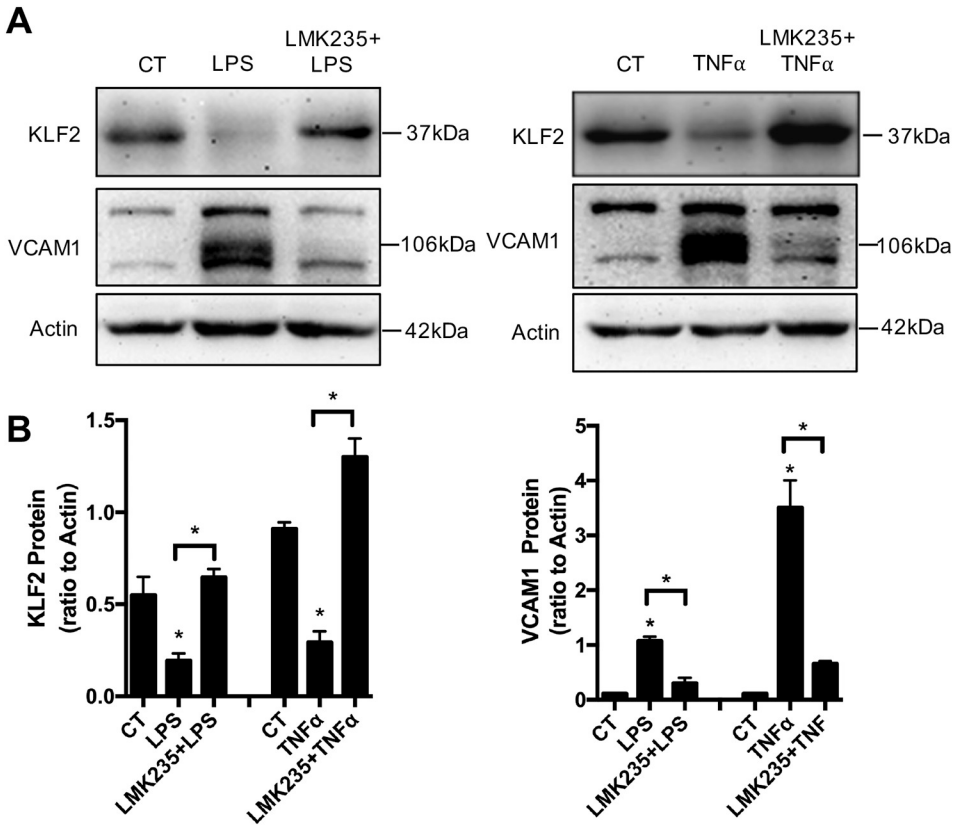

Fig. 5. The effects of specific inhibition of HDAC5 on the expression of KLF2 and VCAM1. (A, B) HUVEC were pre-incubated with HDAC5 specific inhibitor LMK235 (50 nM) 0.5 h before LPS (1 μg/ml) and TNFα (10 ng/ml) stimulation for another 4 h. The protein level of KLF2 and VCAM1 was determined by western blot. The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3).

rehydrated, and antigen retrieved. Then sections were stained with VCAM1 antibody (sc-1504, Santa Cruz) at 4 °C overnight, followed by incubation in biotinylated secondary antibody for 30 min at 37 °C, and finally visualized with DAB solution and counterstained with hematoxylin. Images were taken under a light microscope (BX50, Olympus).

2.10. Statistics

All data are expressed as the mean ± standard deviations (SD) of at least three independent experiments. All multiple groups were compared with ordinary one-way analysis of variance (ANOVA) on Ranks followed by Bonferroni's multiple comparisons test. Statistical significance was set at $P < 0.05$. Figures were prepared using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Metformin inhibited endothelial pro-inflammatory responses induced by LPS and TNFα in vitro

Metformin exhibited its pharmacological effects in endothelial cells in a dose- and time-dependent manner (Fig. 1A, B; Supplementary Figs. 2 and 3), showing the highest induction of phosphorylated AMPK at threonine 172 at the dose of 2 mM at 16 h. To investigate endothelial activation by LPS and TNFα, we stimulated HUVEC with LPS and TNFα for different time points. The results showed that the expression of adhesion molecule VCAM1 was highly induced, showing the peak at both 4 h and 8 h (Fig. 1C, D). In addition, LPS and TNFα stimuli resulted in the downregulation of the transcription factor KLF2 compared to control (Fig. 1E, F).

To investigate the effects of metformin on endothelial pro-inflammatory responses, we pre-treated HUVEC with metformin before LPS and TNFα stimulation. The results showed that the upregulation of VCAM1 upon 4 h LPS and TNFα stimulation was inhibited by the pre-treatment of metformin both at mRNA and protein level (Fig. 2A, B and C). As confirmed in Fig. 2D and E, the number of HL60 leukocytes adhered to the endothelial monolayer was also significantly reduced by metformin pre-treatment. Furthermore, metformin effectively restored KLF2 expression which was downregulated by LPS and TNFα both at mRNA and protein level (Fig. 2F, G and H). These data suggested that metformin may exhibit its anti-inflammatory effects in endothelial cells by restoring KLF2 expression.

3.2. AMPK activation induced the phosphorylation-dependent nuclear export of HDAC5 in endothelial cells

Considering that KLF2 is regulated by HDAC5 in endothelial cells, and HDAC5 is the downstream phosphorylation target of AMPK, we investigated whether HDAC5 is involved in metformin-mediated KLF2 restoration. We found that metformin increased the phosphorylation of AMPKα at threonine 172 (Fig. 3A, B). Meanwhile, the level of phosphorylated HDAC5 at serine 498 was also increased by metformin pre-treatment (Fig. 3C, D). Furthermore, the immunofluorescence results showed that LPS and TNFα stimulation induced nuclear accumulation of HDAC5 in HUVEC, which was effectively diminished by metformin pre-treatment (Fig. 3E).

To confirm the effects of metformin on HDAC5 and KLF2 as well as on endothelial pro-inflammatory responses, we pre-incubated HUVEC with another AMPK activator, AICAR, before LPS and TNFα stimulation. As shown in Fig. 4A and B, AICAR significantly inhibited the upregulation of VCAM1 and the downregulation of KLF2 induced by LPS and TNFα at mRNA level. At protein level, AICAR significantly increased the levels of phosphorylated AMPKα at threonine 172 (Fig. 4C) as well as phosphorylated HDAC5 at serine 498 (Fig. 4D). The protein level of KLF2 was also effectively increased by AICAR pre-treatment (Fig. 4E). Furthermore, LPS and TNFα-induced upregulation of VCAM1 was dramatically abolished by AICAR (Fig. 4F).

To investigate the regulatory effects of HDAC5 on KLF2 expression

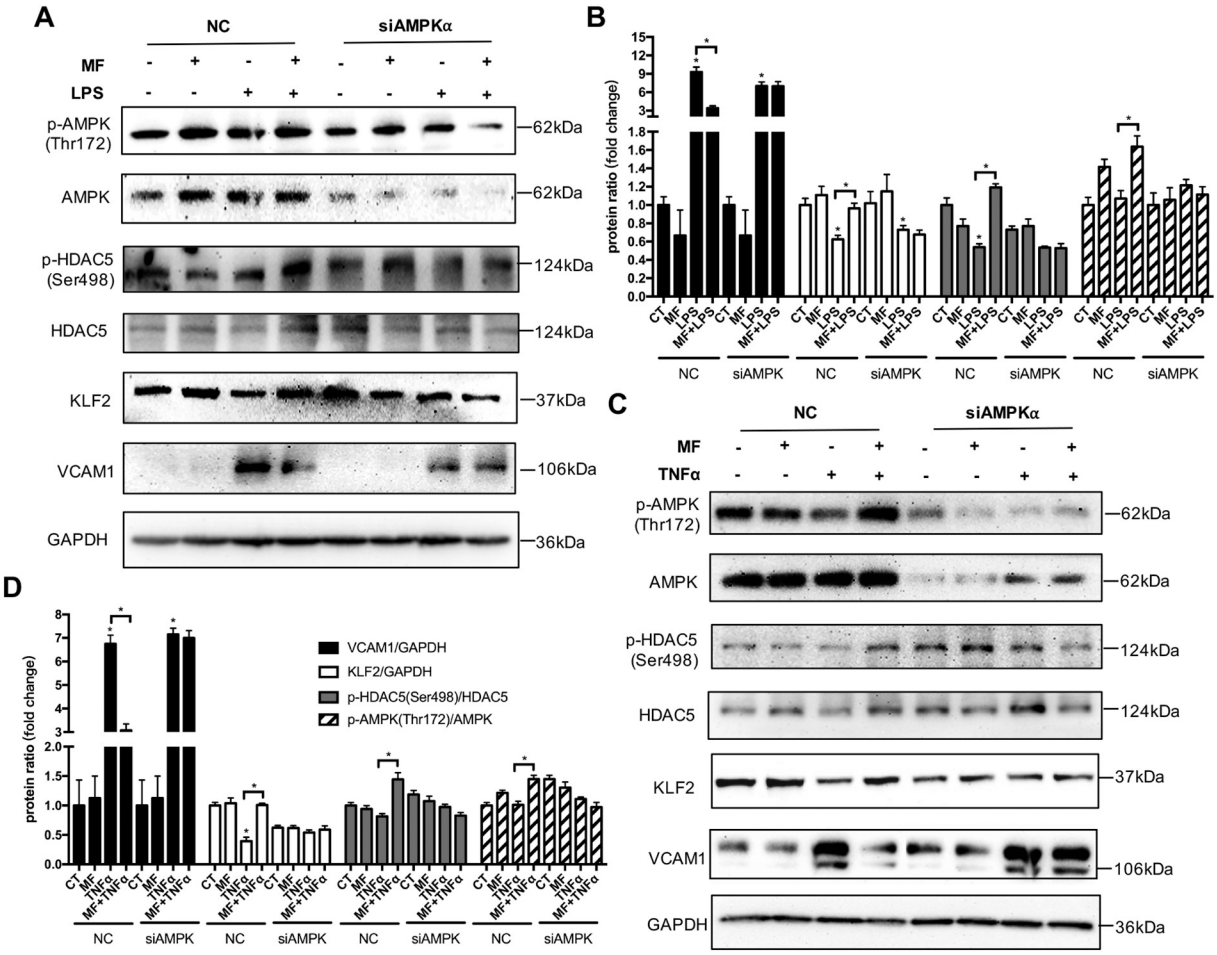

Fig. 6. Tian et al. The effects of metformin on endothelial pro-inflammatory responses were dependent on AMPK activation. (A, C) AMPK was genetically knocked down with siRNA before cells were treated with metformin (2 mM) for 16 h. After stimulated with LPS(1 μg/ml) and TNFα (10 ng/ml) for another 4 h, cells were harvested for protein analysis. Phosphorylated AMPK (Thr172), phosphorylated HDAC5 (Ser498), KLF2, and VCAM1 were determined by western blot. Images were representative of three independent experiments. (B, D) The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3).

in endothelial cells, we pre-incubated HUVEC with LMK235, the HDAC5 specific inhibitor, before LPS/TNFα stimulation. As shown in Fig. 5, LMK235 pre-treatment effectively restored the reduction of KLF2 induced by LPS/TNFα stimuli. In the meantime, the induction of VCAM1 by LPS/TNFα was significantly inhibited by LMK235 pre-incubation. This demonstrated that HDAC5 plays an important role in rescuing KLF2 expression in endothelial pro-inflammatory activation.

These data revealed that in the presence of LPS and TNFα, metformin-mediated AMPK activation induced the phosphorylation-dependent nuclear export of HDAC5, which in turn regulated the upregulation of KLF2, eventually exhibiting its anti-inflammatory effects on endothelial cells.

3.3. The effects of metformin on HDAC5 and KLF2 was AMPK-dependent

To investigate whether the anti-inflammatory effects of metformin via mediating HDAC5 and KLF2 is associated with AMPK, we knocked down AMPKα in HUVEC prior to metformin pre-treatment. As shown in Fig. 6A–D, when AMPKα was knocked down, metformin-mediated upregulation of phosphorylated AMPKα at threonine 172 was abolished. In the meantime, AMPK knock-down diminished metformin-induced HDAC5 phosphorylation at serine 498 as well as KLF2 upregulation. Furthermore, when AMPKα was knocked down, metformin had no effects on LPS/TNFα-induced VCAM1 expression. Taken together, these data indicated that the metformin mediated HDAC5 phosphorylation and KLF2 restoration in endothelial cells in an AMPK-dependent manner.

3.4. The administration of AMPK activators attenuated the systemic and local pro-inflammatory responses in mice subjected to endotoxemia

To further investigate the anti-inflammatory effects of AMPK activation in vivo, AMPK activator metformin and AICAR was administered respectively to mice prior to LPS injection. As shown in Fig. 7A, LPS injection significantly increased the level of VCAM1 in the arterioles, peritubular capillaries, postcapillary venules, and glomerulus in the kidneys as well as in the arterioles, alveolar capillaries, and venules in the lungs. Metformin and AICAR administration effectively reduced VCAM1 expression in the microvascular beds both in the kidneys and lungs. Furthermore, LPS-induced increase of the serum levels of pro-inflammatory cytokines TNFα (Fig. 7B) and IL-6 (Fig. 7C) were both significantly decreased by metformin and AICAR.

In addition, Metformin and AICAR administration effectively reversed LPS-induced decline of phosphorylated AMPK and HDAC5 in mouse kidneys and lungs (Fig. 7D–G). The upregulation of VCAM1 as well as the downregulation of KLF2 were reversed by metformin and AICAR both at mRNA level (Fig. 7H, I) and at protein level (Fig. 7D–G). These data revealed that metformin and AICAR exhibited their

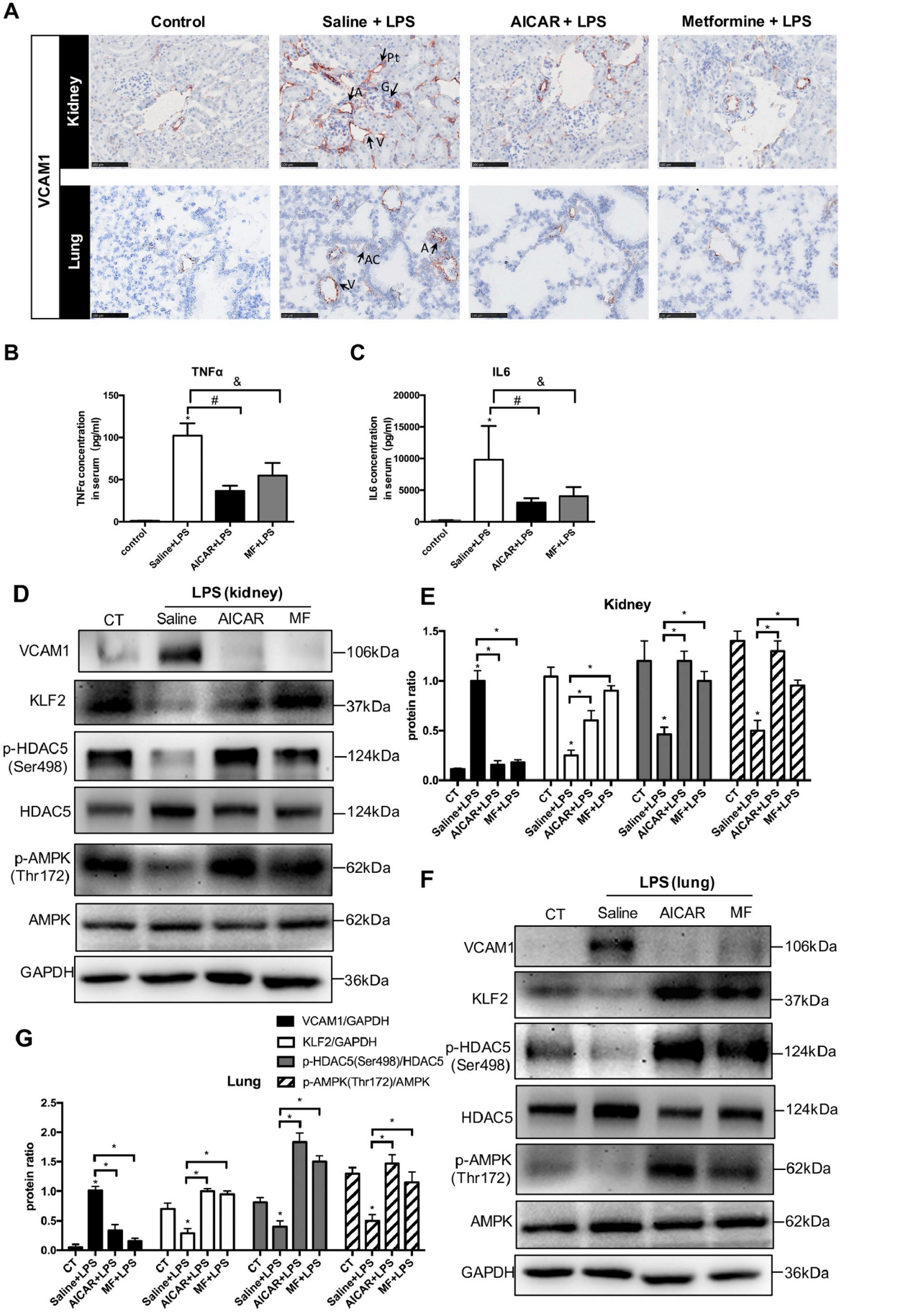

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Fig. 7. The administration of AMPK activators effectively ameliorated systemic and local pro-inflammatory responses in mice subjected to endotoxemia. (A) Immunohistochemical staining of vascular cell adhesion molecule (VCAM)-1 in the kidneys and lungs of control mice, mice subjected to 6 h LPS with or without AICAR or metformin pre-administration. VCAM1 was stained in red. Specific vascular beds are indicated by arrows. A = arteriole, G = glomerulus, Pt = peritubular capillary, V = venule, AC = alveolar capillary. The original magnification is 200×. (B, C) Systemic concentrations of TNFα and IL6 in the serum of different groups of mice were measured by ELISA. Data were expressed as mean ± SD, n = 6 animals per group. *, $P < 0.05$, saline + LPS vs. control; #, $P < 0.05$, AICAR + LPS vs. saline + LPS; &, $P < 0.05$, MF + LPS vs. saline + LPS. (D, F) The protein levels of phosphorylated AMPK (Thr172), phosphorylated HDAC5 (Ser498), KLF2, and VCAM1 in mouse kidneys and lungs were analyzed by western blot. (E, G) The blots were semi-quantitatively analyzed and the data were presented as mean ± SD (n = 3). (H, I) The mRNA levels of KLF2 and VCAM1 in kidneys and lungs of mice from control group, and mice subjected to LPS with or without metformin/AICAR were determined by quantitative RT-PCR using GAPDH as the housekeeping gene. Values represented mRNA level relative to GAPDH. Data were expressed as mean ± SD, n = 6 animals per group. *, $P < 0.05$, saline + LPS vs. control; #, $P < 0.05$, AICAR + LPS vs. saline + LPS; &, $P < 0.05$, MF + LPS vs. saline + LPS.

pharmacological effects on endotoxemia-induced endothelial pro-inflammatory responses via AMPK-mediated HDAC5 phosphorylation and KLF2 restoration.

4. Discussion

In the early stage of sepsis, endothelial cells are crucial in the onset of pro-inflammatory responses and the inhibition of endothelial inflammation is expected to attenuate sepsis-associated multiple organ failure. In the present study, we explored the regulatory mechanism of AMPK activators in endotoxemia-induced endothelial inflammation. We demonstrated that by activating AMPK, metformin and AICAR increased HDAC5 phosphorylation and led to the upregulation of KLF2, resulting in the inhibition of endotoxemia-induced VCAM1 expression both in vitro and in vivo (as summarized in Fig. 8). Our results indicated that AMPK-mediated HDAC5 phosphorylation and KLF2 restoration is, at least partially, responsible to the anti-inflammatory effects of metformin in endotoxemia-induced endothelial activation, which has important implications for interfering therapies of sepsis.

AMPK is a cellular energy sensor and plays a crucial role in the maintenance of metabolic homeostasis. AMPK activator metformin modulates cellular metabolism and limit cell growth under energy stress [29]. In addition to the well-known metabolic regulatory activities of metformin, increasing evidences revealed its anti-inflammatory effects. However, the underlying mechanisms remains not fully understood yet. In the current study, we demonstrated that AMPK activation by metformin abolished endotoxemia-induced endothelial inflammation via inducing phosphorylation-dependent nuclear export of HDAC5 and the upregulation of KLF2, suggesting the important role of HDAC5 and KLF2 in the anti-inflammatory properties of metformin. It is worthy to note that AMPK as a protein kinase may also phosphorylate other class IIa HDACs, such as HDAC4, HDAC7, and HDAC9, which might be of interest to be investigated in the future.

In addition, it has been reported recently that AMPK-dependent

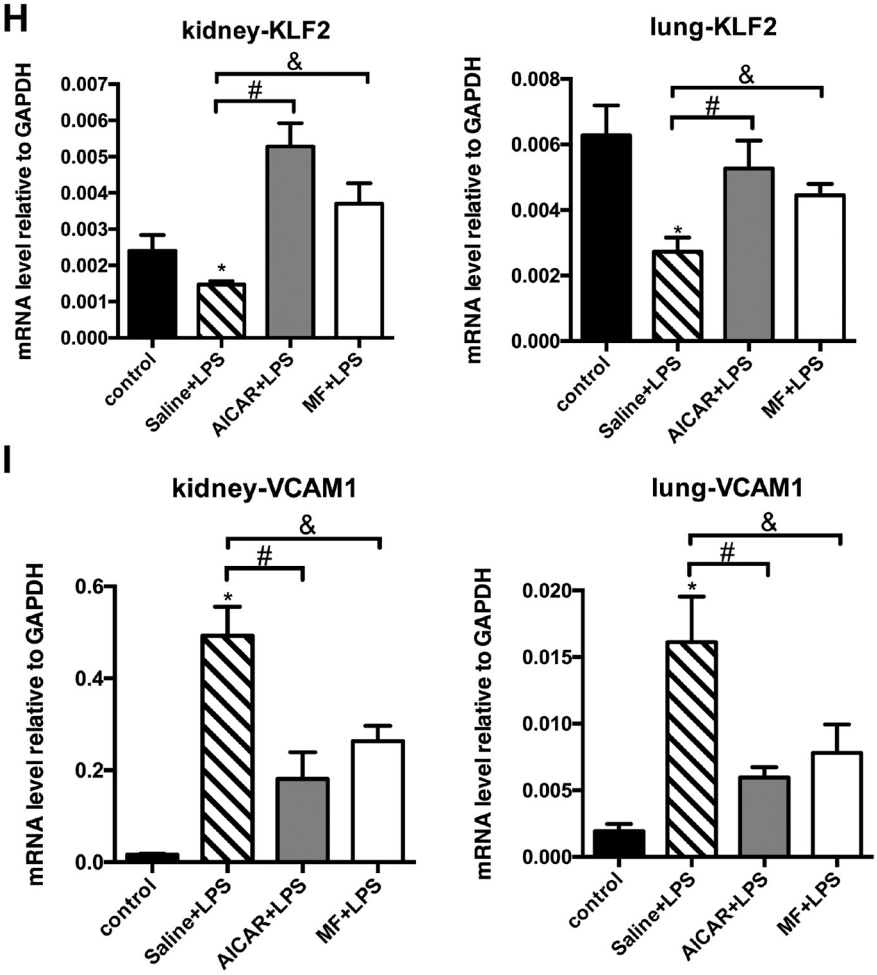

Fig. 7. (*continued*)

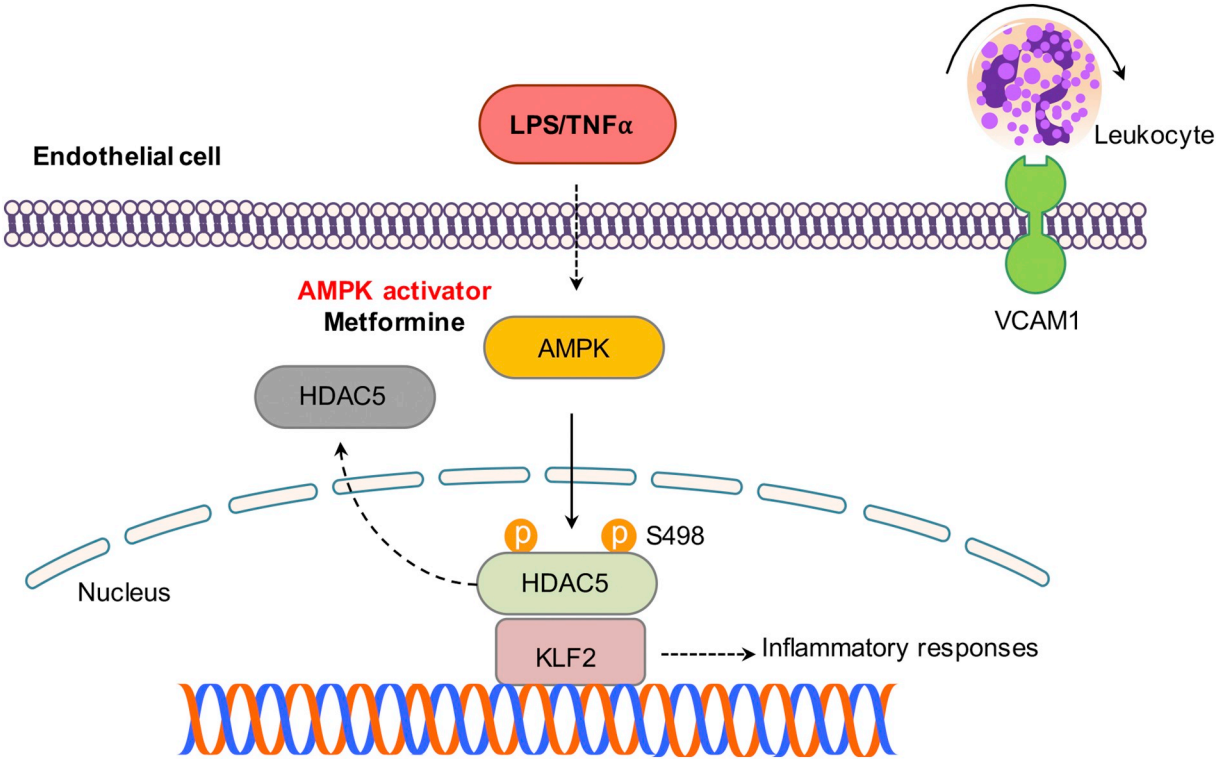

Fig. 8. Schematic description of the role of AMPK-HDAC5-KLF2 in endothelial pro-inflammatory activation in endotoxemia. KLF2 as a transcription factor negatively regulates endothelial pro-inflammatory activation by inhibiting the expression of adhesion molecule VCAM1. In the nucleus, HDAC5 binds directly to KLF2 and represses its transcriptional activity. As a phosphokinase of HDAC5, activated AMPK can phosphorylate HDAC5 at serine 498, leading to the nuclear export of HDAC5, which in turn increases KLF2 expression. Upon the stimulation of pro-inflammatory factors (e.g., LPS and TNFα) during endotoxemia, AMPK activity is reduced, leading to the accumulation of HDAC5 in the nucleus. Thereafter, KLF2 is inhibited and the downstream VCAM1 expression is upregulated, mediating the adhesion of leukocytes to endothelial cells, and then impairs the integrity of endothelial barrier. In this study, by using AMPK activator metformin and AICAR, we examined the role of AMPK-HDAC5-KLF2 axis in endotoxemia-induced endothelial pro-inflammatory responses both in vitro and in vivo. Full-line arrow indicates activation while dash-line arrow indicates inhibition.

suppression of mTOR might be responsible to the anti-inflammatory effects of metformin [30]. On the other hand, metformin may also exhibit its anti-inflammatory effects via AMPK-independent pathways. For instance, it has been demonstrated that metformin suppressed the activity of phospholipase C which plays crucial roles in the expression of inflammatory genes as well as neutrophil infiltration [31–33]. Our study, together with others', by elucidating the potentially anti-inflammatory mechanisms, provided theoretical support for the application of metformin in clinic.

HDACs play important roles in post-transcriptional modification. The existing HDAC inhibitors are lack of specificity, which may lead to complicated pharmacological effects and even toxicities. Therefore, the exploration of potential drugs that have regulatory effects on specific HDAC or specific class of HDACs is of importance. The regulatory effects of HDAC5 in endothelial cells were firstly reported under fluid shear stress condition where HDAC5 modulates the transcriptional activity of KLF2 and the downstream eNOS expression, suggesting that HDAC5 may modulate vascular functions in health and disease [20]. We found here that metformin effectively increased HDAC5 phosphorylation (Ser498) by AMPK activation, exhibiting its anti-inflammatory benefits. In addition, class IIa HDACs also exert regulatory effects on inflammation in other cell types. Shakespear et al. found that HDAC7 promoted the expression of a subset of Toll-like receptor 4 (TLR4)-dependent pro-inflammatory genes in macrophages via a HIF-1α-dependent mechanism [34]. Therefore, class IIa HDACs might be viable targets in the development of novel anti-inflammatory interventions in future research.

Clinical data shows that the level of KLF2 in renal tissue of patients with sepsis is significantly lower than that of non-septic patients [35]. KLF2-knockout mice exacerbate inflammatory responses in multi-bacterial infections and sepsis [36]. We found in this study that metformin showed its anti-inflammatory effect by restoring the expression of KLF2, revealing the role of KLF2 in mediating metformin-induced anti-inflammatory effects. KLF2 also shows its regulatory effects on inflammation in other cell types. Das et al. showed that KLF2 negatively regulated the transcriptional activity of both NF-κB and AP-1, thereby inhibiting inflammatory activation of monocytes [37]. In the study by Manoharan et al., KLF2 suppressed the expression of chemokines Ccl2 and Ccl1 by modulating miR-124a and miR-150 in myeloid cells [38]. Therefore, the restoration of KLF2 in interventions of inflammatory diseases is of importance.

Collectively, our data for the first time revealed that metformin treatment ameliorated endotoxemia-induced endothelial pro-inflammatory responses both in vitro and in vivo via suppressing HDAC5 activity and restoring KLF2 expression. Although the detailed mechanisms still need more intensive investigation, the present study suggests that the anti-inflammatory effects of metformin on endothelial cells in endotoxemia is, at least partially, via mediating AMPK-HDAC5-KLF2 signaling.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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