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RESEARCH ARTICLE

Acetylcholine suppresses LPS-induced endothelial cell activation by inhibiting the MAPK and NF-κB pathways

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ABSTRACT. *Background and Objective:* Endothelial cell activation plays a critical role in leukocyte recruitment during inflammation and infection. We previously found that cholinergic stimulation (via vagus nerve stimulation) attenuates vascular endothelial impairment and reduces the inflammatory profile in ovariectomized rats. However, the specific molecular mechanism is unclear. This study was designed to explore the effects and molecular mechanisms of cholinergic agonists (acetylcholine [ACh]) on lipopolysaccharide (LPS)-induced endothelial cell activation *in vitro*. *Methods:* Human umbilical vein endothelial cells (HUVECs) were treated with different concentrations of LPS (10/100/1000 ng/mL) to activate endothelial cells. HUVECs were untreated, treated with ACh (10^{-5} M) alone, treated with 100 ng/mL LPS alone, or treated with different concentrations of ACh (10^{-9}/10^{-8}/10^{-7}/10^{-6}/10^{-5} M) before LPS stimulation. HUVECs were also pre-treated with 10^{-6} M ACh with or without mecamylamine (an nAChR blocker) (10 μM) and methyllycaconitine (a specific α7 nAChR blocker) (10 μM) and incubated with or without LPS. ELISA, western blotting, cell immunofluorescence, and cell adhesion assays were used to examine inflammatory cytokine production, adhesion molecule expression, monocyte-endothelial cell adhesion and activation of the MAPK/NF-κB pathways. *Results:* LPS (at 10 ng/mL, 100 ng/mL and 1,000 ng/mL) increased VCAM-1 expression in HUVECs in a dose-dependent manner (with no significant difference between LPS at 100 ng/mL and 1,000 ng/mL). ACh (10^{-9} M–10^{-5} M) blocked adhesion molecule expression (VCAM-1, ICAM-1, and E-selectin) and inflammatory cytokine production (TNF-α, IL-6, MCP-1, IL-8) in response to LPS in a dose-dependent manner (with no significant difference between 10^{-5} and 10^{-6} M Ach). LPS was also shown to significantly enhance monocyte-endothelial cell adhesion, which was largely abrogated by treatment with ACh (10^{-6}M). VCAM-1 expression was blocked by mecamylamine rather than methyllycaconitine. Lastly, ACh (10^{-6} M) significantly reduced LPS-induced phosphorylation of NF-κB/p65, IκBα, ERK, JNK and p38 MAPK in HUVECs, which was blocked by mecamylamine. *Conclusions:* ACh protects against LPS-induced endothelial cell activation by inhibiting the MAPK and NF-κB pathways, which are mediated by nAChR, rather than α7 nAChR. Our results may provide novel insight into the anti-inflammatory effects and mechanisms of ACh.

Key words: acetylcholine (ACh), human umbilical vein endothelial cells (HUVECs), endothelial cell activation, nAChR

Increasing evidence suggest that the endothelium plays a critical role in the initiation, occurrence and development of inflammation. Vascular endothelial cells are the targets of inflammation and are also involved in the further expansion of inflammation [1, 2]. On the one hand, proinflammatory cytokines produced by infection, sepsis, oxidative stress, atherosclerosis, hypertension, and other inflammatory diseases can activate endothelial cells and cause dysfunction of endothelial cells. On the other hand, the activated endothelium can further release various inflammatory cytokines (*e.g.*, TNF-α, IL-6, MCP-1, IL-8, CINC/KC, *etc.*) and express multiple adhesion molecules (*e.g.*, vascular cell adhesion molecule 1 [VCAM-1], intercellular adhesion molecule 1 [ICAM-1], and E-selectin, *etc.* [1, 3, 4]). A critical function of the activated endothelium during inflammation is to coordinate the migration of peripheral blood leukocytes to culprit sites of infection/injury. Cell-associated adhesion molecules, such as E-selectin, ICAM-1, VCAM-1 and chemokines, expressed by the endothelium facilitate the rolling, adhesion, activation, and migration of circulating leukocytes across the endothelial cell barrier to the site of infection or inflammation [5, 6]. However, excessive leukocyte accumulation during infection and inflammation mediated by the overexpression (or sustained expression) of adhesion molecules by the endothelium can lead to tissue damage. In contrast, insufficient endothelial cell

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activation and subsequent impaired immune cell trafficking can result in host immunosuppression. Thus, the regulation of endothelial cell activation must be precisely controlled.

The recently described cholinergic anti-inflammatory pathway is a physiological mechanism that modulates host inflammatory responses via cholinergic mediators or electrical stimulation of the vagus nerve [7]. A previous study by our laboratory showed that modification of vagal activity contributes to the beneficial effects of the cholinergic anti-inflammatory pathway in inflammatory bowel disease (IBD)-related inflamed colonic mucosa [8]. Some studies of the cholinergic anti-inflammatory pathway revealed that acetylcholine (ACh), the primary neurotransmitter released by the vagus nerve, and nicotinic acetylcholine receptor (nAChR) agonists block TNF production by lipopolysaccharide (LPS)-stimulated macrophages [9] via α7 nAChR [10]. Stimulation of the vagus nerve releases ACh, leading to suppressed TNF production *in vivo* [9] via α7 nAChR [10]. Nicotine, a nicotinic cholinergic agonist, binds and activates nAChRs [11, 12] and exerts anti-inflammatory effects *in vitro* and *in vivo* [9, 12, 13]. Nicotine has also been shown to be an effective treatment in ischaemic rats [14], for experimental sepsis [15], and endotoxin rats [12]. Studies by Wang and co-workers [10] demonstrated that the anti-inflammatory activity of nicotine after LPS treatment is mediated via α7 nAChR expressed by macrophages. Unfortunately, the use of nicotine as a therapeutic agent is limited by its toxicity [16].

Endothelial cells express nAChRs, including α7 nAChR [17-21]. It has been reported that cholinergic receptor agonists, nicotine, CAP55, and ACh are closely related to endothelial cell dysfunction, but the underlying molecular mechanisms are still unclear [22]. This study aims to explore the potential effects and molecular mechanisms of cholinergic agonists (ACh) on endothelial cell activation. We analysed the impact of ACh on adhesion molecule (E-selectin, ICAM-1 and VCAM-1) expression, inflammatory cytokine release and monocyte-endothelial cell adhesion induced by LPS. In addition, we investigated receptor-mediated ACh action and the signalling pathways involved.

Materials and methods

Materials and reagents

ACh, LPS (from *Escherichia coli* 0111:B4), mecamylamine (an nAChR blocker), and methyllycaconitine (MLA) (a specific α7 nAChR blocker) were purchased from Sigma-Aldrich (St. Louis. MO, USA). Antibodies against VCAM-1, ICAM-1, and CD62E (E-selectin) were purchased from Abcam (Cambridge, UK). Antibodies against NF-κB/p65, p-NF-κB/p65, IκBα, and p-IκBα using the MAPK Family Antibody Sampler Kit and the Phospho-MAPK Family Antibody Sampler Kit were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-rabbit IgG, mouse anti-goat IgG, and 4,6-diamidino-2-phenylindole (DAPI) were also purchased from Cell Signaling Technology. ELISA kits for human TNF-α, MCP-1 (CCL2), IL-6, and IL-8 were purchased from RayBiotech Company (Georgia, USA). All other chemicals of analytical grade were purchased from Guangzhou Chemical Reagents (Guangzhou, China).

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh anonymous human umbilical cords using standard techniques, collected from the Third Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). Primary HUVECs were obtained by using collagenase I to digest the subendothelial basement layer and then cultured in DMEM supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37˚C in a humidified 7.5% CO_2 atmosphere. The HUVECs were grown in gelatin-coated flasks (0.1%) in DMEM containing essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, *etc*. When the endothelial cells of the second to third generation were confluent, they were cultured for eight hours in a DMEM-free medium at 37˚C and 0.5% CO_2. Finally, 4% DMEM was used. Then, the drug was added after grouping. In all experiments, HUVECs of passages 2-3 were used.

Western blotting analysis

Total proteins were extracted as previously described [23]. Briefly, the cells were lysed by homogenization in lysis buffer (containing 1 M pH 6.8 Tris-HCl, 20% glycerol, 5% SDS, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM PMS) and incubated for 5 minutes on ice, boiled for 10 minutes, centrifuged (4°C, 13,500 rpm) for 7 minutes and stored at -20°C as whole-cell protein extracts. Cellular lysates containing equal amounts of total protein (30 μg of protein/lane) were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were then washed with TBST, blocked with 5% BSA for 30 minutes at room temperature and probed with primary antibodies against VCAM-1 (no. ab134047, 1:2000), ICAM-1 (no. ab171123, 1:2000), CD62E (E-selectin, ab18981, 1:1000), NF-κB/p65 (#8242, 1:1000), p-NF-κB/p65 (#3033, 1:1000, Ser536), IκBα (#4814, 1:1000), p-IκBα (#9246, 1:1000, Ser32/36), ERK1/2 (#4695, 1:1000), p-ERK1/2 (#4370, 1:1000, Thr202/Tyr204), JNK (#9252, 1:1000), p-JNK (#9251, 1:1000, Thr183/Tyr185), p38 MAPK (#8690, 1:1000), p-p38 MAPK (#4511, 1:1000, Thr180/Tyr182), and β-actin (sc-81178, 1:1000) at 4°C overnight. The membrane was then blocked with 5% BSA solution at 37°C and incubated for one hour in PBS containing goat anti-rabbit (#7074, 1:2000) or mouse anti-goat (#7076, 1:2,000) IgG conjugated with horseradish peroxidase. These proteins were visualized using the enhanced chemiluminescence plus substrate-chromogen system (ChemiDoc XRS+ System, Bio-Rad, Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

HUVECs were divided into several groups as following: control, treated with ACh (10^{-5} M) alone for 24 hours, treated with 100 ng/mL LPS alone for 8 hours, or treated

with different concentrations of ACh (10^{-9}-10^{-5} M) for 24 hours before incubation with LPS (100 ng/mL, 8 hours). The cell culture supernatant was centrifuged and collected. The TNF-α (code: ELH-TNF-α1), MCP-1 (CCL2, code: ELH-MCP1-1), IL-6 (code: ELH-IL6-1), and IL-8 (code: ELH-IL8-1) levels in these groups were analysed using ELISA kits according to the manufacturer's instructions (Raybiotech, Georgia, USA).

Cell immunofluorescence

Cell immunofluorescence was performed as previously described [23]. HUVECs were placed on coverslips and allowed to grow to 50-70% confluence. Following treatment for the indicated times, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, and then permeabilized with 0.5% Triton for 15 minutes at room temperature. After non-specific protein was blocked with 5% BSA-PBS for one hour at room temperature, the fixed cells were incubated with NF-κB/p65 (D14E12) XP Rabbit mAb (#8242, 1:400) overnight at 4°C. The cells were washed three times with PBS and incubated with the secondary antibody, Alexa Fluor 488-labelled goat anti-rabbit IgG (#7074, 1:1000) at room temperature for one hour. After the cells were washed using PBS for three times, they were mounted with 4,6-diamidino-2-phenylindole (DAPI) for detection of cell nuclei (#4083). Immunofluorescence was visualized using a confocal microscope (LSM710, ZEISS) at 512 × 512-pixel resolution. Images were collected using a 40× apochromatic objective.

Cell adhesion assay

HUVECs were placed in 24-well plates and cultured until confluence as a single layer. After cells were starved without serum overnight, the cells were treated with ACh (10^{-6}M) for 24 hours, and subsequently LPS (100 ng/ml) was added for another 8 hours. Afterwards, 3×10^4 THP-1 monocytes labelled with CellTracker™ Red CMTPX (Life Technologies, 5 μM) were co-cultured with HUVECs for one hour. The cells were then washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes, and photographed under a fluorescence microscope (Leica DMi8, German) with five random fields per well.

Data analysis and statistics

The data are presented as mean ± standard error of mean (SEM). Statistical analysis was performed with SPSS statistical software (version 19.0). SigmaPlot (version 10.0) and Graphpad Prism (version 7.0) software were used to illustrate the results. Comparisons among different groups were performed by one-way ANOVA with post hoc testing using Tukey's method. $P < 0.05$ was considered significantly.

Results

ACh suppresses adhesion molecule expression in LPS-treated HUVECs

The high expression of adhesion molecules, such as VCAM-1, ICAM-1, or E-selectin, is a typical feature of endothelial cell activation. To determine the role of ACh in endothelial cell activation, HUVECs were treated with vehicle, ACh, or ACh + LPS, and the expression level of adhesion molecules was detected by western blotting. The results showed that LPS (100 ng/mL) significantly increased the level of VCAM-1, ICAM-1 and E-selectin. As expected, treatment with ACh substantially decreased the LPS-induced expression of VCAM-1, ICAM-1 and E-selectin in a dose-dependent manner (10^{-9} – 10^{-5} M) (*figure 1A-C*). ACh (10^{-5} M) alone did not affect adhesion molecule expression level in the absence of LPS.

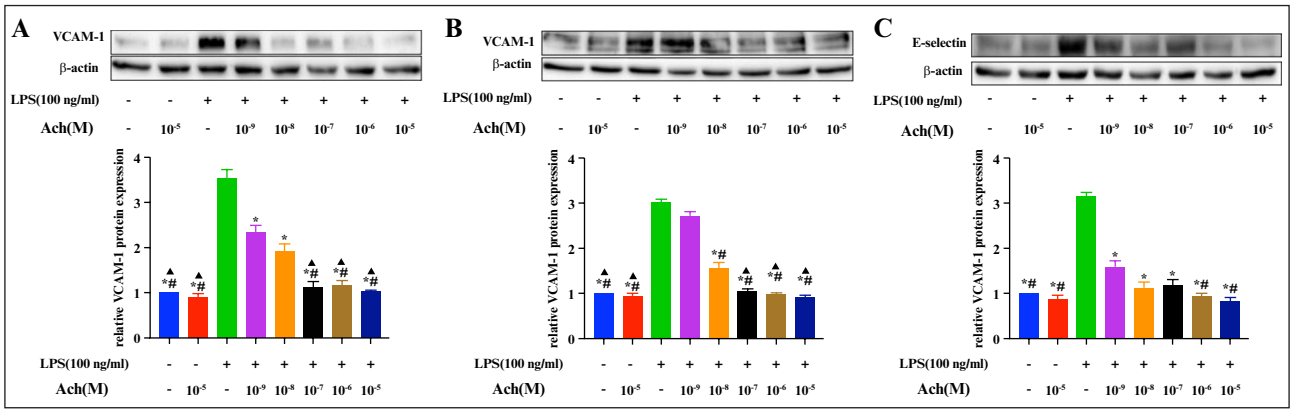

Figure 1
Effect of ACh (10^{-9} – 10^{-5} M) on adhesion molecule expression in HUVECs treated with LPS. Confluent monolayers of HUVECs were untreated, treated with ACh (10^{-5} M) alone for 24 hours, treated with 100 ng/mL LPS alone for 8 hours, or treated with different concentrations of ACh (10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M) for 24 hours before LPS (100 ng/mL, 8 hours) stimulation. Proteins from whole-cell extracts were analysed by immunoblotting with anti-VCAM-1 antibody (**A**), anti-ICAM-1 antibody (**B**), and anti-E-selectin antibody. **C**) Densitometric analysis was performed with normalization to control (β-actin), and the results are expressed as mean ± SEM ($n = 3$); *$p < 0.05$ versus the LPS group, $^{\#}p < 0.05$ versus the LPS + 10^{-9} M ACh group, $^{\blacktriangle}p < 0.05$ versus the LPS+10^{-8} M ACh group.

ACh suppresses inflammatory cytokine production in LPS-treated HUVECs

The endothelium is not only a target of inflammatory mediators but also a source of proinflammatory molecules such as chemokines (MCP-1, CCL5 and CXCL14) and cytokines (IL-6, INFγ and TNF-α). To evaluate the impact of ACh on inflammatory cytokine release, the cells were treated with ACh alone or combined with LPS, and the production of TNF-α, IL-6, IL-8 and MCP-1 was subsequently measured by ELISA. We found that the production of TNF-α, IL-6, IL-8 and MCP-1 (*figure 2A-D*) in HUVECs was significantly augmented after exposure to LPS (100 ng/mL) for 8 hours. HUVECs treated with different doses of ACh (10^{-9}-10^{-5}M) showed a decrease in LPS-induced (100 ng/mL) production of these cytokines in a dose-dependent manner.

ACh suppresses LPS-activated monocyte-endothelial cell adhesion

Adhesion molecules mediate monocyte attachment and rolling on endothelial cells, and are required for recruitment of monocyte to sites of inflammation. To examine the influence of ACh on this process, HUVECs were untreated, treated with LPS alone, treated with ACh+LPS, or treated with ACh alone, and subsequently examined regarding monocyte adhesion (*figure 3A-D*). The results revealed that the number of adhered cells increased significantly after treatment with LPS (100 ng/mL), which was partially abolished by pre-incubation with ACh (10^{-6} M). However, ACh (10^{-6} M) alone had no obvious effect on monocyte-endothelial cell adhesion (*figure 3B*). These results suggested that the effect of ACh on adhesion molecule expression can functionally prevent monocyte-endothelial cell adhesion.

ACh suppresses adhesion molecule expression via nAChR

It has been reported that nAChR, particularly α7 nAChR, mediates the anti-inflammatory action of ACh. To determine whether α7 nAChR is involved in ACh-suppressed endothelial cell activation, experiments were performed with mecamylamine (an nAChR blocker) and methyllycaconitine (a specific α7 nAChR blocker). We found that mecamylamine inhibited ACh-induced suppression of VCAM-1 expression which is increased due to LPS, whereas LPS-induced VCAM-1 expression was not reduced in the presence of methyllycaconitine (*figure 4A-B*). These results suggest that the effect of ACh (10^{-6} M) on LPS-induced (100 ng/mL) expression of adhesion molecules in vascular endothelial cells may be mediated via nAChR rather than α7 nAChR.

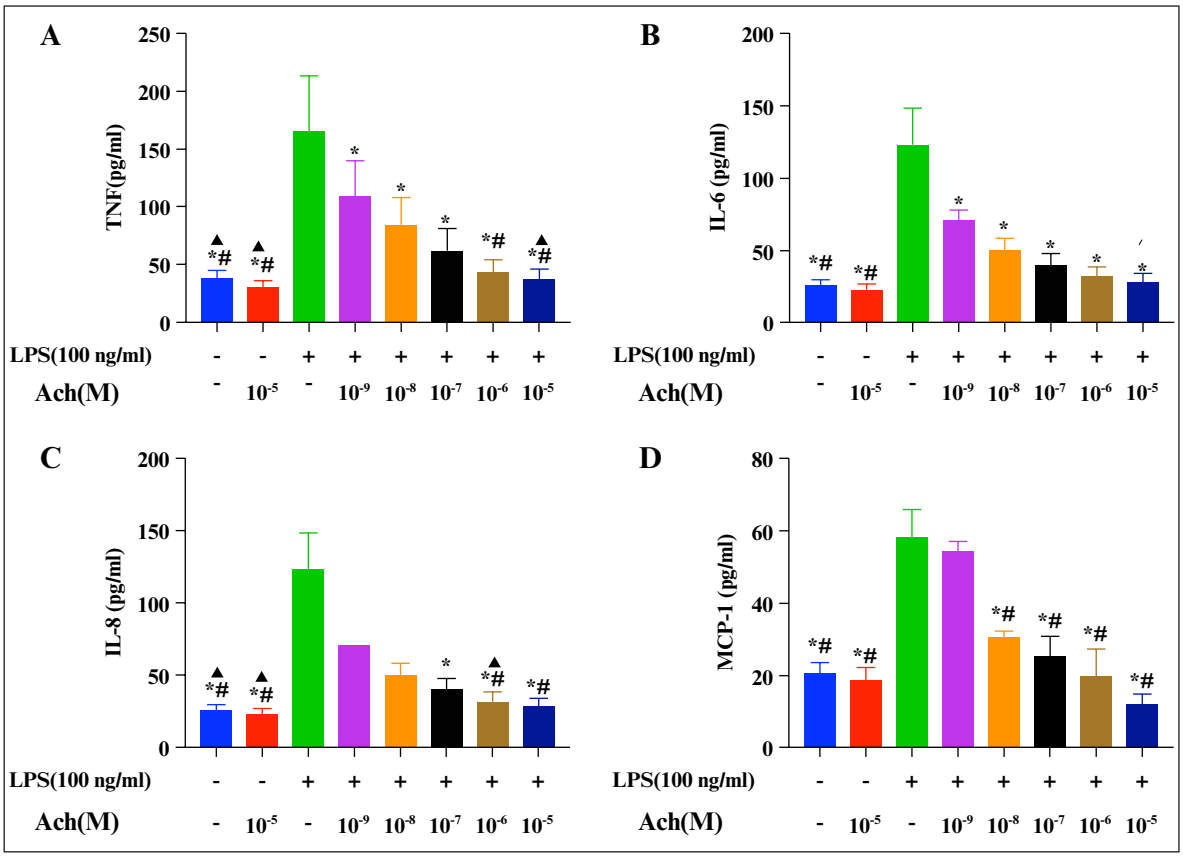

Figure 2
Effect of ACh (10^{-9} –10^{-5} M) on inflammatory cytokine production in HUVECs treated with LPS. Confluent monolayers of HUVECs were untreated, treated with ACh (10^{-5} M) alone for 24 hours, treated with 100 ng/mL LPS alone for 8 hours, or treated with different concentrations of ACh (10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M) for 24 hours before LPS (100 ng/mL, 8 hours) stimulation. Production of TNF-α (**A**), IL-6 (**B**), IL-8 (**C**) and MCP-1 (**D**) was determined by ELISA. The results are expressed as the mean ± SEM (n = 4 –6); *p < 0.05 versus the LPS group, $^{\#}p$ < 0.05 versus the LPS+10^{-9} M ACh group, $^{\triangle}p$ < 0.05 versus the LPS+10^{-8} M ACh group.

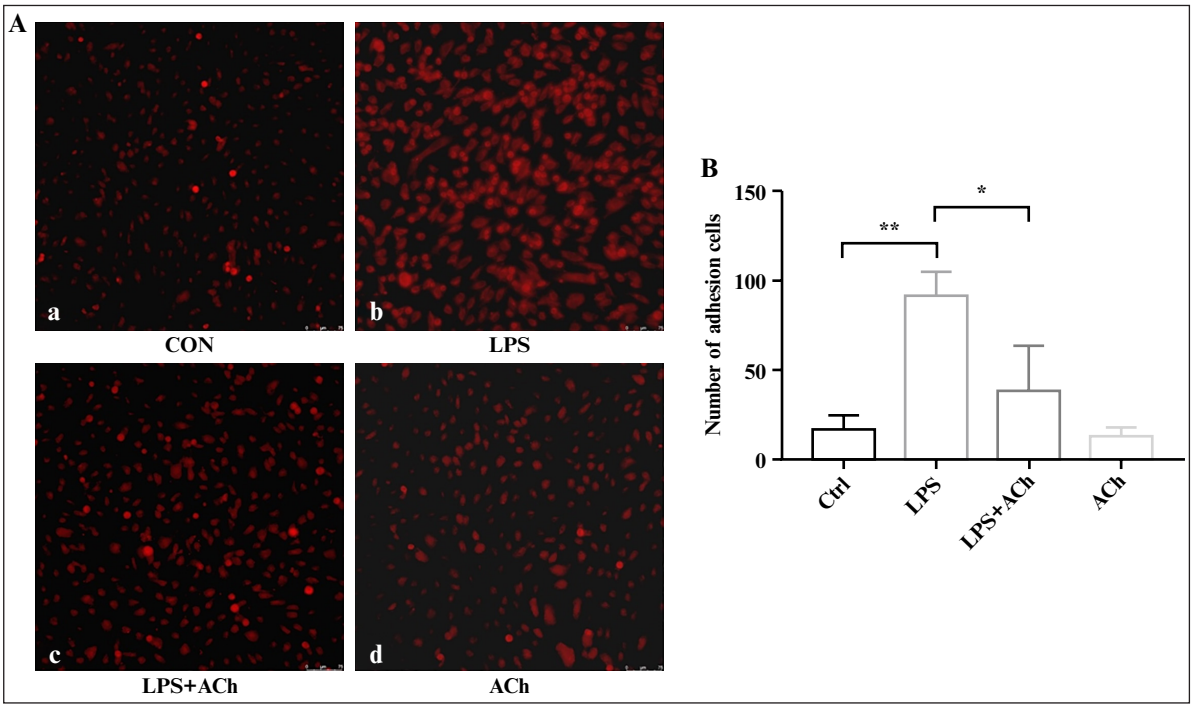

Figure 3
Effect of ACh on the number of adhered cells induced by LPS in TPH-1 and HUVEC co-culture. **A**) fluorescence microscopy; **B**) quantification of fluorescent cells. The co-cultured cells were divided into the following four groups: CON, LPS (100 ng/mL), LPS (100 ng/mL) + ACh (10^{-6} M), and ACh (10^{-6} M). LPS (100 ng/mL, 8 hours) was shown to increase cell adhesion of co-cultured cells, however, the number of adherent cells did not increase significantly after pre-incubation with ACh for 24 hours. The number of adhered cells was calculated based on five fields of view. Results are expressed as mean ± SEM, and experiments were performed three times; $^{**}p < 0.01$, $^{*}p < 0.01$.

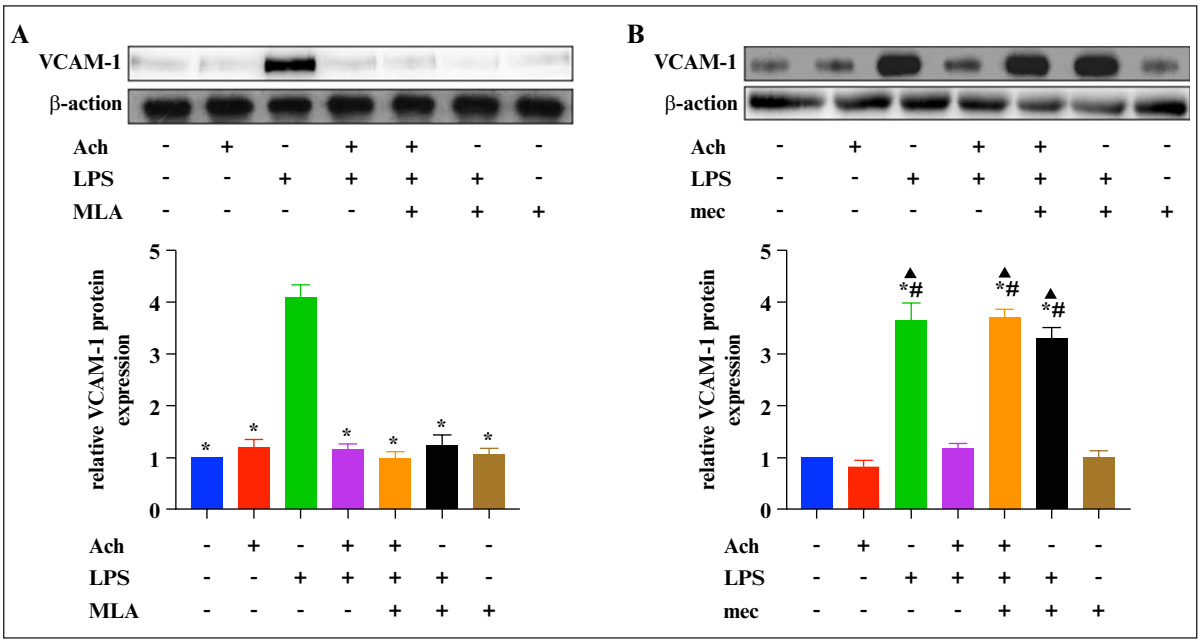

Figure 4
Effect of mecamylamine (mec) and methyllycaconitine (MLA) on ACh-induced suppression of LPS-activated cell adhesion molecule expression. Confluent monolayers of HUVECs were pre-treated with 10^{-6} M ACh (24 hours) with or without mec (10 μM, 60 minutes) (nAChR blocker) and MLA (10 μM, 60 minutes) (specific α7 nAChR blocker) and then incubated with or without LPS (100 ng/mL, 8 hours). Proteins from whole-cell extracts were analysed by immunoblotting with an anti-VCAM-1 antibody. Densitometric analysis was performed with normalization to control (β-actin), and the results are expressed as mean ± SEM ($n = 3$). The data shown are representative of three independent experiments: (**A**) $^{*}p < 0.05$ versus the LPS group; (**B**) $^{*}p < 0.05$ versus the control group, $^{\#}p < 0.05$ versus the ACh + LPS group, $^{\triangle}p < 0.05$ versus the mec group.

ACh suppresses NF-κB/IκBα pathway activation in HUVECs

NF-κB is a central transcriptional factor mediating adhesion molecule expression under inflammatory conditions. To reveal the underlying mechanism of ACh action, we assessed the activation status of the NF-κB signalling pathway upon treatment with LPS alone or combined with ACh. LPS significantly increased the expression of phospho-NF-κB/p65 in cultured HUVECs. ACh reduced the phosphorylation of NF-κB/p65 induced by LPS, resulting in almost normal levels of phospho-NF-κB/p65 (*figure 5A*). Treatment with mecamylamine significantly blocked the effect of ACh. To further clarify the mechanism of ACh blocking the phosphorylation of NF-κB/p65, we examined IκBα and its phosphorylated form, which inhibits the NF-κB signalling pathway (*figure 5B*). The results showed that LPS increased the phosphorylation of IκBα in cultured HUVECs. Preincubation with 10^{-6} M ACh for 24 hours significantly reduced the phosphorylation of IκBα induced by LPS, resulting in almost normal levels of phospho-IκBα. However, Mecamylamine significantly blocked the effect of ACh. These results suggested that ACh (10^{-6} M) significantly reduced activation of the NF-κB/p65 pathway induced by LPS (100 ng/mL), and its effect may be partially mediated by the nAChR receptor.

ACh suppresses LPS-activated nuclear translocation of NF-κB/p65 in HUVECs

To further determine the effect of ACh on NF-κB signalling pathway activation, we examined the nuclear translocation of NF-κB/p65 upon treatment with LPS alone or combined with ACh. The results revealed that NF-κB/p65 was mainly localized to the cytoplasm in the control group (*figure 6A-C*) and ACh group (*figure 6D-F*). After incubation with LPS alone for 8 hours, we found that this treatment significantly increased NF-κB/p65 nuclear translocation (*figure 6G-I*). The effect was eliminated when the endothelial cells were treated with ACh (10^{-6} M) for 24 hours before incubation with LPS (100 ng/mL, 8 hours) (*figure 6J-L*). Moreover, mecamylamine partially reversed the inhibitory effect of ACh on NF-κB/p65 nuclear translocation induced by LPS (*figure 6M-O*).

ACh suppresses MAPK pathway activation in HUVECs

It has been reported that MAPK signalling pathways, including JNK, ERK1/2, and p38 MAPK, are also involved in endothelial cell activation. Thus, the activation status of these signalling pathways was examined in the study. As shown in *figure 7*, LPS increased the phosphorylation of JNK (*figure 7A*), ERK1/2

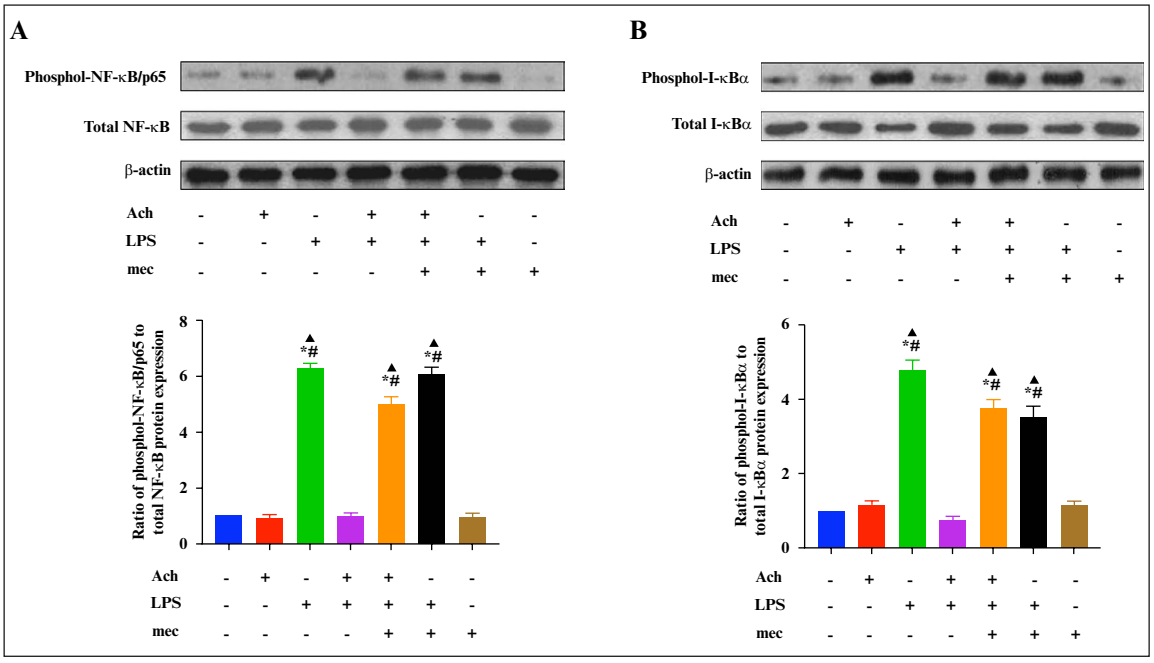

Figure 5

Effect of ACh on the phosphorylation of NF-κB/p65 and IκBα induced by LPS in HUVECs. Confluent monolayers of HUVECs were divided into groups of cells: untreated, treated with ACh (10^{-6} M, 24 hours) alone, treated with LPS (100 ng/mL, 8 hours) alone, treated with ACh (10^{-6} M, 24 hours) before incubation with LPS (100 ng/mL, 8 hours), treated with mecamylamine (mec) (10 μM, 60 minutes) before incubation with ACh (10^{-6} M, 24 hours) and then incubated with LPS (100 ng/mL, 8 hours), treated with mec (10 μM, 60 minutes) before incubation with LPS (100 ng/mL, 8 hours), and treated with mec (10 μM, 60 minutes) alone. The cells were then lysed, and the proteins were analysed by western blotting. **A**) Representative western blotting image and quantification of NF-κB/p65 and phospho-NF-κB/p65. **B**) Representative western blotting image and quantification of IκBα and phospho-IκBα. Results are expressed as mean ± SEM ($n = 3$); $^{*}p < 0.05$ versus the control group, $^{\#}p < 0.05$ versus the ACh+LPS group, $^{\triangle}p < 0.05$ versus the mec group.

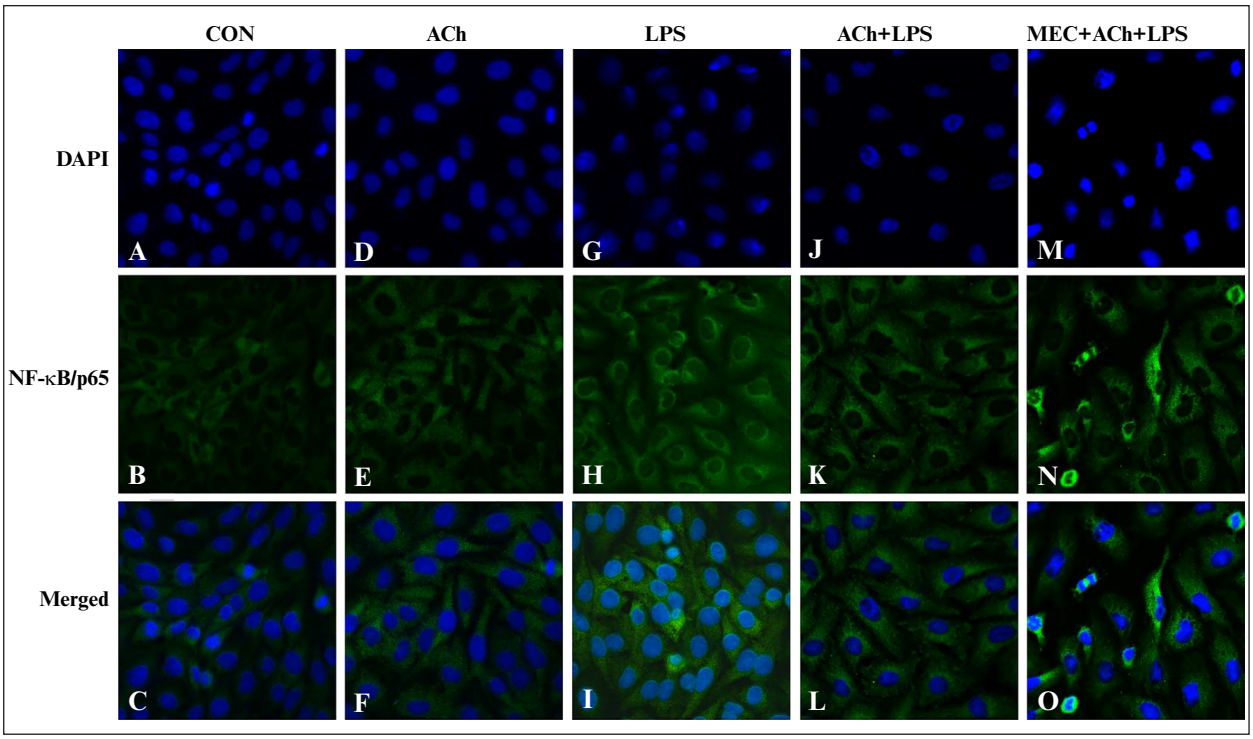

Figure 6
The effect of nAChR blocker, mecamylamine (mec), on nuclear translocation of NF-κB/p65 induced by LPS in HUVECs. Confluent monolayers of HUVECs were divided into groups of cells: untreated (**A**, **B**, **C**), treated with ACh (10^{-6} M, 24 hours) alone (**D, E**, **F**), treated with LPS (100 ng/ml, 8 hours) alone (**G**, **H**, **I**), treated with ACh (10-6 M, 24 hours) before incubation with LPS (100 ng/ml, 8 hours) (**J**, **K**, **L**), or treated with mec (10 μM, 60 min) before incubation with ACh (10^{-6} M, 24 hours) and then incubated with LPS (100 ng/mL, 8 hours) (**M**, **N**, **O**). Nuclear translocation of NF-κB/p65 was assessed by confocal fluorescence microscopy. The cells were immunostained with anti-NF-κB/p65 antibody labelled with TRITC (green), and the nuclei were stained with DAPI (blue). Immunofluorescence was detected by confocal microscopy (20×), and the representative images are shown. NF-κB/p65 activation was evaluated according to green fluorescence intensity. All experiments were repeated three times.

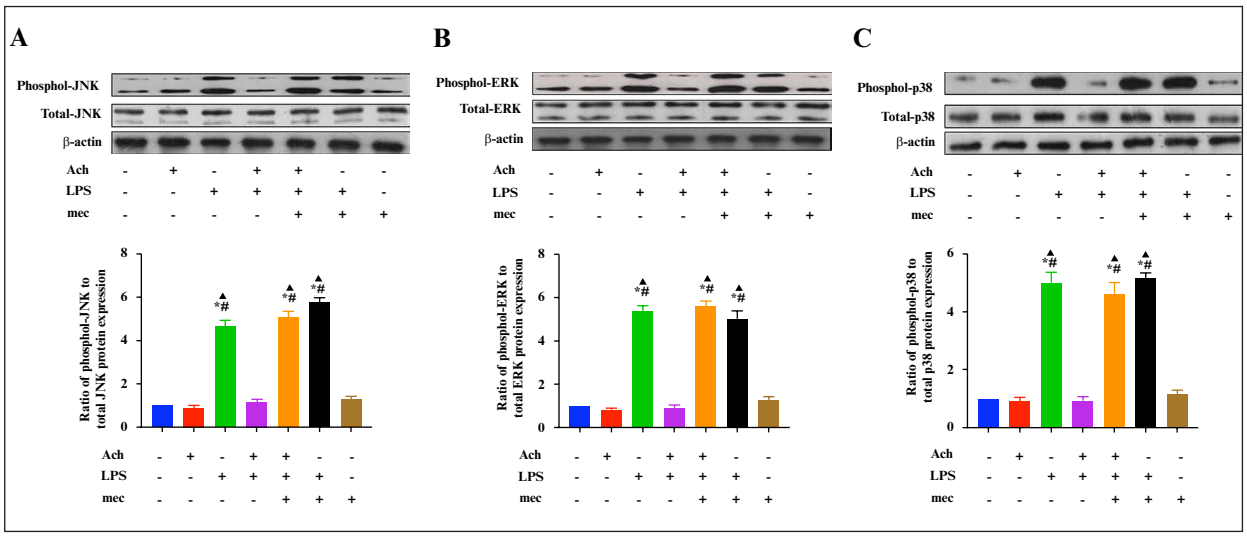

Figure 7
Effect of ACh on phosphorylation of JNK, ERK1/2 and p38 MAPK induced by LPS in HUVECs. Confluent monolayers of HUVECs were divided into groups: untreated, treated with ACh (10^{-6} M, 24 hours) alone, treated with LPS (100 ng/mL, 8 hours) alone, treated with ACh (10^{-6} M, 24 hours) before incubation with LPS (100 ng/mL, 8 hours), treated with mecamylamine (mec) (10 μM, 60 minutes) before incubation with ACh (10^{-6} M, 24 hours) and then incubated with LPS (100 ng/mL, 8 hours), treated with mec (10 μM, 60 minutes) before incubation with LPS (100 ng/mL, 8 hours), and treated with mec (10 μM, 60 minutes) alone. The cells were then lysed, and the proteins were analysed by western blotting. The results for JNK (**A**), ERK1/2 (**B**) and p38 MAPK (**C**) are presented as a representative western blotting image (upper panels) and quantification (lower panels). Results are expressed as mean ± SEM (n = 3); $^{*}p < 0.05$ versus the control group, $^{\#}p < 0.05$ versus the ACh + LPS group, $^{\triangle}p < 0.05$ versus the mec group.

(*figure 7B*), and p38 MAPK (*figure 7C*) in cultured HUVECs. Treatment with 10^{-6} M ACh effectively inhibited the phosphorylation of JNK, ERK1/2, and p38 MAPK induced by LPS and significantly reduced the expression of phospho-JNK (*figure 7A*), phospho-ERK1/2 (*figure 7B*), and phospho-p38 MAPK (*figure 7C*). These effects on JNK, ERK1/2, and p38 MAPK phosphorylation were blocked by mecamylamine. These results suggest that nAChR may be involved in the inhibition of LPS-induced JNK, ERK1/2, and p38 MAPK phosphorylation by ACh.

Discussion

In previous studies [24], we explored the protective role of chronic VNS on the cardiovascular system under conditions of oestrogen deficiency. To mimic postmenopausal conditions, we adopted an ovariectomized (OVX) rat animal model that has been widely used to study many diseases related to the menopause, such as cardiovascular disease and osteoporosis [25, 26]. We found that the administration of chronic VNS during the early stage of oestrogen deficiency protected OVX rats from endothelial impairment and an inflammatory profile. We also observed that the translocation of NF-κB/p65 to the nucleus was significantly reduced. Thus, we deduced that the administration of chronic VNS during the early stage of oestrogen deficiency might decrease the risk of developing cardiovascular disease in postmenopausal women. The intrinsic mechanism may be related to blocking the NF-κB signalling pathway. In this study, we explored the potential role of nAChR, NF-κB/IκBα, and MAPK pathways in endothelial cell activation induced by LPS *in vitro*.

Vascular endothelial cells are not only a target for inflammatory cytokines but also represent an important part of the endocrine system involved in the occurrence and development of inflammatory reactions. When activated, these cells can produce a large number of inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, and IL-12. Studies have shown that human high mobility group protein-1 (HMGB1) and LPS can significantly stimulate the production of the chemokines, IL-8 and MCP-1, in vascular endothelial cells. When various pathway inhibitors of MAPK were added, especially p38 MAPK inhibitors, the expression of IL-8 and MCP-1 was partially inhibited [5, 27-29]. Studies have demonstrated that LPS can induce activation of vascular endothelial cells in a concentration-time dependent manner and upregulate the expression of cell surface adhesion molecules, while inhibitors of the NF-κB pathway can significantly inhibit the expression of LPS-induced adhesion molecules [30]. These results suggest that NF-κB is the main signalling pathway involved in LPS-induced endothelial cell activation and upregulation of adhesion molecule expression. When the cell is inactive, NF-κB usually binds to the inhibitory proteins, I-κBs (I-κBα, I-κBβ, I-κBε), and exists in the cytoplasm in an inactive form. The interaction between the two proteins masks the nuclear localization signal of NF-κB [31]. When cells are stimulated, serine at positions 32 and 36 of IκBα are phosphorylated, leading to dissociation from NF-κB. The free NF-κB in the cytoplasm can then be transported to the nucleus and binds to binding sites in the promoter region of target genes, thus initiating the transcription and translation of these genes [32]. In animal experiments, we found that the nuclear translocation of NF-κB/p65 increased in OVX rats, while chronic VNS significantly inhibited this process. Experiments with cultured HUVECs further confirmed this speculation. We also found that ACh, a neurotransmitter released from the vagus nerve with high activity, significantly inhibited the phosphorylation of NF-κB/p65 and IκBα in vascular endothelial cells, and this effect may be mediated by nAChR. Previous studies in our laboratory also found that ACh significantly inhibited the LPS-induced nuclear translocation of NF-κB/p65 and phosphorylation of IκBα in intestinal epithelial cells [8]. These results suggest that the effect of chronic VNS on alleviating inflammation in OVX rats may be mediated by the NF-κB signalling pathway, which has been further confirmed in cell experiments *in vitro*.

The MAPK signalling pathway is an important signal transduction system in organisms. This pathway can transmit extracellular signals to cells or even nuclei, and participates in many physiological and pathophysiological processes, such as cell proliferation, growth, differentiation, and inflammation. The MAPK pathway has diverse functions and is highly sensitive [33]. At present, there are three subgroups of MAPK identified in mammals: ERK, JNK and p38 MAPK. ERK plays an important role in cell proliferation, transformation and differentiation [34]. JNK and p38 MAPK can be activated by various stress stimuli, which mainly induce the synthesis of inflammatory cytokines and various types of cellular stress signals [35, 36].

Among them, the p38 MAPK signalling pathway is most closely related to vascular endothelial cell injury. This pathway can be activated by various factors, such as hyperlipidaemia, hyperglycaemia, oxidative stress, reactive oxygen species, and proinflammatory cytokines (TNF-α, LPS, IL-1, FGF) [37]. After activation, p-p38 MAPK migrates from the cytoplasm to the nucleus to regulate gene expression. It regulates the production of inflammatory mediators and various cell functions (such as cell proliferation, growth, apoptosis, differentiation, migration, tissue-specific responses, immune responses, *etc*.). The transcriptional and post-transcriptional mechanisms may involve transcription factors, such as NF-κB/p65 or p53 [38]. Other studies have shown that some substances secreted by endothelial cells after activation can further activate p38 MAPK [39]. Li *et al*. [40] showed that the phosphorylation of p38 MAPK increased after endothelial cell activation in human pulmonary microvascular endothelial cells (HPMECs). In contrast, MKK6 (Glu), which constitutively activates p38 MAPK, resulted in endothelial barrier dysfunction.

Our previous results [8] showed that ACh inhibited the phosphorylation of JNK and p38 MAPK in intestinal epithelial cells, which further confirms the present

results. p38 MAPK is the upstream signalling molecule that is most closely related to the nuclear translocation of NF-κB/p65 in the MAPK signalling pathways. However, in multicellular organisms, especially mammalian cells, the body often requires the synergy of multiple signalling pathways for certain cellular and physiological functions. The synergism of the MAPK signalling pathway can occur at all levels of the signal transduction cascade, and different synergistic modes and/or mechanisms can be used [38, 41, 42]. In addition, activation of any one of the MAPK pathways can increase the expression of certain transcription factors, thus providing a substrate for the phosphorylation of a further MAPK pathway to promote the expression of inflammatory response genes. This phenomenon is another important form of synergy between MAPK pathways [33, 43].

Therefore, we speculate that these protein molecules of the MAPK signalling pathway may be the main upstream molecules that activate the NF-κB pathway. Interestingly, we found that LPS stimulation increased the phosphorylation of ERK, JNK and p38 MAPK *in vitro*, and preincubation with ACh eliminated this effect. However, when nAChR was blocked, we found that ACh no longer had this effect.

Some studies have shown that α7 nAChR is an effector of the inflammatory response and plays a key role in regulating the production of inflammatory cytokines [44]. Blocking α7 nAChR could significantly reduce the production and release of inflammatory cytokines, such as TNF-α, IL-6, and IL-8, by macrophages and block the phosphorylation and nuclear translocation of NF-κB/p65 in the inflammatory response. However, these studies generally focused on macrophages. There are few studies on the cholinergic anti-inflammatory pathway in vascular endothelial cells. Numerous reports have shown that endothelial cells can express various subtypes of nAChR [19, 21]. Cortés and other researchers have found that human microvascular endothelial cells can also express α7 nAChR [17, 45]. To further clarify whether α7 nAChR plays a key role in the alleviation of local vascular inflammation by chronic vagal stimulation, the effect of α7 nAChR-specific receptor blocker, methyllycaconitine, and the nAChR non-specific blocker, mecamylamine, was tested in cultured HUVECs. The results showed that blocking nAChR inhibited the expression of adhesion molecules induced by LPS. The result was not achieved by blocking only α7 nAChR. Saeed *et al.* [22] also found that the nAChR blocker, mecamylamine, inhibited the effect of cholinergic receptor agonists (nicotine or CAP55) in reducing TNF-induced expression of ICAM-1. These results were similar to our findings. Therefore, we conclude that cholinergic agonists (ACh) inhibit endothelial cell activation by activating nAChR rather than α7 nAChR.

In conclusion, these findings indicate that cholinergic agonists (ACh) protect against LPS-induced endothelial cell activation by inhibiting the MAPK and NF-κB/IκBα signalling pathways, and this process may be mediated by nAChR rather than α7 nAChR. Our results may provide novel insight into the anti-inflammatory effects and mechanisms of ACh.

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Conflict of interest: *none.*

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