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ORIGINAL ARTICLE

C-type natriuretic peptide attenuates LPS-induced endothelial activation: involvement of p38, Akt, and NF-κB pathways

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Abstract Endothelial activation elicited by inflammatory agents is regarded as a key event in the pathogenesis of several vascular inflammatory diseases. In the present study, the inhibitory effects and underlying mechanism of C-type natriuretic peptide (CNP) on LPS-induced endothelial activation were examined in human umbilical vein endothelial cells (HUVECs). The effect of CNP on adhesion molecule expression was assessed using quantitative real-time RT-PCR and western blotting analyses. The nuclear factor-κB (NF-κB), MAPK, and PI3K/Akt signaling pathways in LPS-stimulated HUVECs were investigated using western blotting analyses, and the production of intracellular reactive oxygen species (ROS) was measured using a fluorescence method. Pretreatment with CNP inhibited LPS-induced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, and P-selectin in a concentration-dependent manner. CNP suppressed the phosphorylation of p65 and NF-κB activation in LPS-stimulated cells. Moreover, CNP reduced ERK1/2 and p38 phosphorylation induced by LPS but not JNK. Furthermore, CNP induced Akt phosphorylation and activation of hemeoxygenase-1 (HO-1) expression. CNP significantly inhibited the production of intracellular ROS. These results suggest that CNP effectively attenuated LPS-induced endothelial activation by inhibiting the NF-κB and p38 signaling pathways, eliminating LPS-induced intracellular ROS production, and activating the PI3K/Akt/HO-1 pathway in HUVECs; thereby, demonstrating that CNP may be a potential therapeutic target for the treatment of sepsis and inflammatory vascular diseases.

Keywords C-type natriuretic peptide · Endothelial activation · Lipopolysaccharide · Adhesion molecules · Nuclear factor-κB (NF-κB) · Mitogen-activated protein kinase (MAPK) · Hemeoxygenase-1

Abbreviations

CNP	C-type natriuretic peptide
LPS	Lipopolysaccharide
VCAM-1	Vascular cell adhesion molecule-1
ICAM-1	Intercellular adhesion molecule-1
ROS	Reactive oxygen species
HO-1	Hemeoxygenase-1
HUVECs	Human umbilical vein endothelial cells
NF-κB	Nuclear factor-κB
PBS	Phosphate-buffered saline
AREs	Antioxidant response elements
EGM-2	Endothelial cell growth medium-2
DCFH-DA	2′,7-Dichlorodihydrofluorescein diacetate

Introduction

Endothelial activation elicited by inflammatory agents is regarded as a key event in the pathogenesis of several vascular inflammatory diseases, such as sepsis (Aird 2003) and cardiovascular disorders (Beverley and Karen 1998).

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At early stages of sepsis, lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, changes the function of the endothelium and causes endothelial activation. Enhanced expression of cell-surface adhesion molecules, such as ICAM-1, VCAM-1, E-selectin, and P-selectin, is an early marker of endothelial activation (Liao 2013). Furthermore, increased adhesion molecule expression facilitates the recruitment and adherence of leukocytes (Albelda et al. 1994; Liao 2013; Tedder et al. 1995), consequently contributing substantially to the progression of inflammation.

Previous studies have indicated that the enhanced expression of adhesion molecules on endothelial cells is associated with the activation of pro-inflammatory transcriptional programs, such as the nuclear factor-κB (NF-κB) pathway (Liao 2013) and mitogen-activated protein kinase (MAPK) pathway (Hommes et al. 2003). Inhibition of extracellular signal-regulated (ERK) 1/2 and p38 MAPK activation can reduce the LPS-induced expression of adhesion molecules in endothelial cells (Tamura et al. 1998; Xu et al. 2013). Reactive oxygen species (ROS) induced by inflammatory stimuli also regulate NF-κB activation and the consequential elevation of adhesion molecules (Chiu et al. 1997; Gloire et al. 2006). In addition, the PI3K/Akt signaling pathway plays an important role in negatively regulating the LPS-induced acute inflammation response (Williams et al. 2004; Xu et al. 2010). Inhibition of the PI3K/Akt signaling pathway also enhances the activation of NF-κB by LPS (Zhang et al. 2007). Thus, identification of the molecules that modulate these signaling pathways in the endothelium may provide potential therapeutic targets in the prevention and treatment of inflammation-related disorders.

C-type natriuretic peptide (CNP), one member of the natriuretic peptide family, plays an important role in the regulation of cardiovascular homeostasis (Sandow and Tare 2007). Previous reports have demonstrated that CNP exerts a protective anti-inflammatory effect (Scotland et al. 2005a). Local expression of CNP suppresses inflammation in injured carotid arteries in rabbit by inhibiting the expression of adhesion molecules and the infiltration of macrophages (Qian et al. 2002). CNP also inhibits leukocyte recruitment and platelet-leukocyte interactions (Scotland et al. 2005b). In addition, continuous CNP infusion also attenuates inflammation in an acute myocarditis model (Obata et al. 2007). However, the effect of CNP on LPS-induced inflammation in endothelial cells still requires further verification. In particular, the mechanisms underlying the anti-inflammatory effects of CNP on the human vasculature, specifically on endothelial cells, are still largely unknown. Here, we investigated whether CNP exerts anti-inflammatory effects via the attenuation of LPS-induced endothelial activation. In addition, the underlying mechanisms and intracellular signaling pathways affected by CNP in LPS-stimulated endothelial cells were investigated.

Materials and methods

Reagents and antibodies

CNP was purchased from Calbiochem (La Jolla, CA, USA) and dissolved in sterile 5 % acetic acid. LPS (*Escherichia coli* serotype 055:B5), 2′,7-dichlorodihydrofluorescein diacetate (DCFH-DA), and pyrrolidone dithiocarbamate (PDTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PD98059, SB203580, and rabbit anti-HO-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Wortmannin was purchased from the Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). Antibodies against phospho-p38 MAPK (Thr180/Tyr182), p38, phospho-JNK (Thr183/Tyr185), JNK, ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), Akt, phospho-Akt (Ser473), NF-κB p65, and phospho-NF-κB p65 (Ser536) were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against ICAM-1 and GAPDH were purchased from Proteintech (Chicago, IL, USA). Rabbit anti-VCAM-1 antibody was purchased from Epitomics (Burlingame, CA, USA).

Cell culture

Primary HUVECs were obtained from ScienCell Research Laboratories (ScienCell, Carlsbad, CA, USA) and cultured in endothelial cell growth medium-2 (EGM-2) supplemented with 2 % FCS and growth factors (Lonza, Walkersville, MD, USA) at 37 °C and 5 % CO_2. Primary cells between passages 2 and 6 were used for experiments. All experiments were performed in triplicate.

Treatment conditions

To assess the effect of CNP on gene expression and signaling pathways, the cells were pretreated with the indicated concentrations of CNP for 120 min prior to incubation with LPS (1 μg/ml) for specific time periods. During the treatment, ascorbic acid and heparin were removed from the culture medium.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from HUVECs using an Ultrapure RNA Kit (CoWin Biotech, Beijing, China). Next, first strand

Table 1 Primer information for genes in this study

Gene	Forward primer	Reverse primer	Amplicon size (bp)
ICAM-1	CCGGAAGGTGTATGAACTGA	GGCAGCGTAGGGTAAGGTT	182
VCAM-1	CGAAAGGCCCAGTTGAAGGA	GAGCACGAGAAGCTCAGGAGAAA	141
P-selectin	ACCTTCAGGACAATGGACAGCAG	CCCAGAGGTTGGAGCAGTTCA	104
E-selectin	CACTCAAGGGCAGTGGACACA	CAGCTGGACCCATAACGGAAAC	132
HO-1	ATGACACCAAGGACCAGAGC	GTGTAAGGACCCATCGGAGA	153
GAPDH	GTCAGCCGCATCTTCTTTTG	GCGCCCAATACGACCAAATC	100

cDNA was synthesized using the RevertAid™ first strand cDNA synthesis kit (Fermentas Life Sciences, Vilnius, Lithuania) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed as previously described (Chen et al. 2013). Further details of the primer sequences used in this study are provided in Table 1.

Western blotting analysis

To determine the expression of VCAM-1, ICAM-1, and HO-1, and phosphorylation of the cytosolic signal transducers (ERK1/2, p38 MAPK, JNK, and Akt), HUVECs were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer supplemented with a protease cocktail and phosphatase inhibitors (Roche, Mannheim, Germany). The cell lysates were separated using 8–12 % SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5 % bovine serum albumin in PBS containing 0.5 % Tween 20 (PBST) and then incubated with primary antibodies (1:1000) followed by incubation with an HRP-conjugated secondary antibody (Proteintech, Chicago, IL, USA; 1:5000). The blots were developed using the Immobilon western chemiluminescent HPR substrate (Millipore Corporation, Billerica, MA, USA).

Assay of NF-κB activity

NF-κB activity was measured using an enzyme-linked immunosorbent assay-based colorimetric oligonucleotide-binding assay (TransAM NF-κB p65, Active Mitif, Carlsbad, CA, USA). After the NF-κB-specific oligonucleotide was immobilized, the whole cell extract (20 μg) was added to a 96-well plate in triplicate and incubated for 1 h with mild agitation. The plate was washed three times, and NF-κB antibody (1:1000) was subsequently added. After several washes, the HRP-conjugated antibody (1:1000) was added to the plate. After color development according to the manufacturer's instruction, the absorbance was measured at 450 nm using a SpectraMax® M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Determination of intracellular ROS

Intracellular ROS were detected using the oxidation sensitive fluorescent probe DCFH-DA. HUVECs in black 96-well plates were pretreated with CNP or PDTC for 120 min prior to incubation with LPS (1 μg/ml) for 1 h, and the cells were then incubated with 10 μM DCFH-DA in serum-free medium at 37 °C for 20 min. The fluorescence was measured at wavelengths of 488 nm for excitation and 525 nm for emission on a SpectraMax® M5 plate reader. The results were expressed as the fluorescence intensity.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Multiple comparisons were compared using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

Results

CNP inhibited LPS-induced mRNA levels of adhesion molecules

In this study, nontoxic concentrations of CNP (0.01, 0.1, and 1 μM) were examined for its ability to inhibit the LPS-induced mRNA levels of adhesion molecules (Fig. S1). Unstimulated HUVECs produced low levels of ICAM-1, VCAM-1, E-selectin, and P-selectin, and after a 4-h incubation with LPS, a significant increase in mRNA levels was induced. This increase was markedly inhibited by treatment with CNP (1 μM) (Fig. 1).

CNP inhibited LPS-induced protein expression of VCAM-1 and ICAM-1

Western blotting analysis revealed that the protein expression of VCAM-1 and ICAM-1 was very low in unstimulated HUVECs and was significantly increased by LPS treatment for 6 h. CNP inhibited the protein expression of

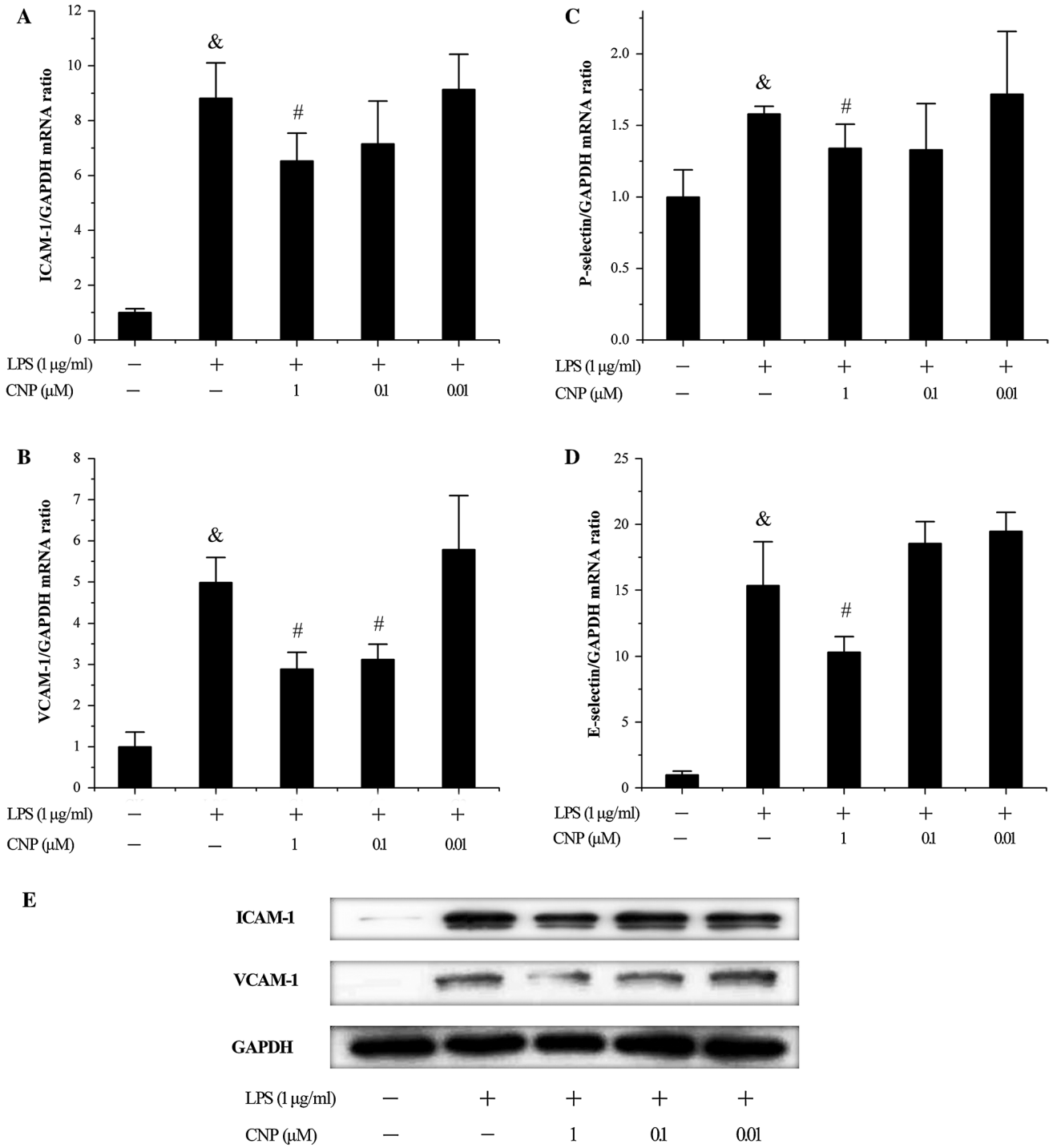

Fig. 1 CNP attenuated LPS-induced adhesion molecule mRNA and protein expression. HUVECs were pretreated with the indicated concentrations of CNP for 120 min and then stimulated with LPS (1 μg/ml) for 4 or 6 h. The mRNA expression levels of adhesion molecules (4 h) were analyzed using qRT-PCR. GAPDH was used as an internal control. (**a–d**) mRNA levels of ICAM-1, VCAM-1, P-selectin, and E-selectin, respectively. Protein expression (6 h) was analyzed using western blotting analyses (**e**). GAPDH was used as a loading control. Representative data are shown from three independent experiments. Data are plotted as the mean ± SD. $^{\&}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group

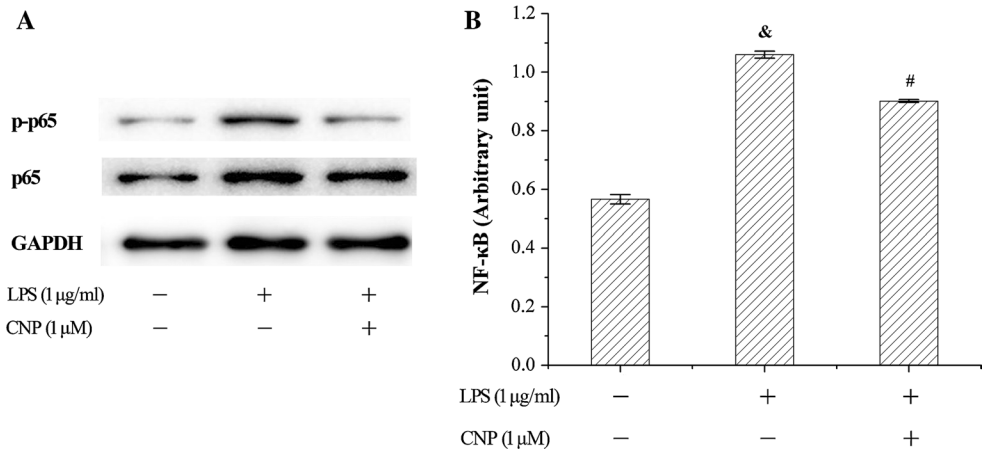

Fig. 2 CNP inhibited LPS-induced phosphorylation of p65 and NF-κB activation. **a** HUVECs were pretreated with CNP (1 μM) for 120 min prior to incubation with LPS (1 μg/ml) for 1 h, and phosphorylation of p65 was determined in whole cell lysates using western blotting analyses. A representative blot of three independent experiments is shown. **b** HUVECs were pretreated with CNP (1 μM) for 120 min prior to incubation with LPS (1 μg/ml) for 1 h. The NF-κB p65 DNA binding activity was measured using ELISA-based DNA binding analysis in whole cell extraction. Representative data are shown from three independent experiments. Data are plotted as the mean ± SD. $^{\&}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group

VCAM-1 and ICAM-1 in a dose-dependent manner at concentrations ranging from 0.01 to 1 μM (Fig. 1e).

CNP suppresses LPS-induced phosphorylation of NF-κB p65 and NF-κB activation

Phosphorylation of the NF-κB p65 subunit, specifically on Ser536, is associated with an increase in NF-κB activity (Hoesel and Schmid 2013) and plays an important role in regulating the transcription of adhesion molecules in response to inflammatory stimuli (Schubert et al. 2002; Viatour et al. 2005). Thus, the phosphorylation levels of the NF-κB p65 subunit were determined. As shown in Fig. 2a, LPS significantly induced phosphorylation of the NF-κB P65 subunit on Ser536 in HUVECs after a 1-h incubation. CNP markedly reduced the phosphorylation of the NF-κB P65 subunit (Fig. 2a).

To investigate whether CNP could affect NF-κB DNA binding activity, an NF-κB activity assay was performed. As shown in Fig. 2b, LPS induced increased DNA binding of NF-κB. CNP attenuated the DNA binding of NF-κB.

Effects of CNP on LPS-induced MAPK phosphorylation

MAPKs play a crucial role in the control of cellular responses to inflammatory stimuli. In addition, MAPKs also play an important role in the activation of NF-κB (Liu et al. 2008). To investigate whether inhibition of endothelial activation by CNP is mediated via the MAPK signaling pathway, we determined the effect of CNP on LPS-induced MAPK phosphorylation in HUVECs at specific time points. LPS stimulation resulted in a rapid activation of ERK1/2, p38, and JNK, with the peak levels of each

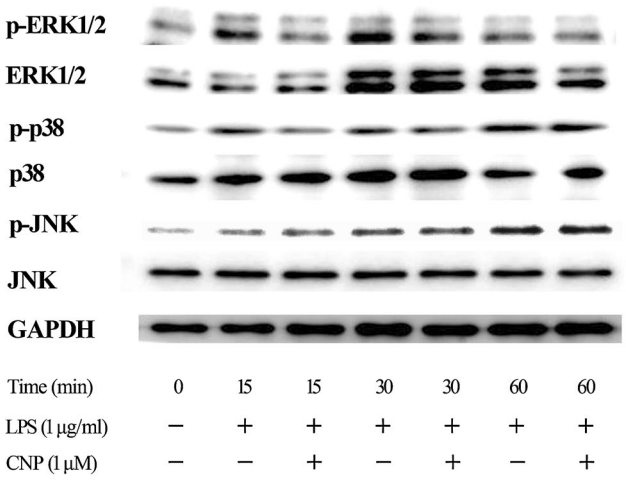

Fig. 3 CNP inhibited LPS-induced phosphorylation of ERK1/2 and p38 MAPK but not JNK. HUVECs were pretreated with CNP (1 μM) for 120 min, followed by incubation with LPS (1 μg/ml) for specific time periods as indicated. Representative immunoblots are shown from three independent experiments

phospho-MAPK occurring at 15–60 min after incubation with LPS. CNP suppressed LPS-induced phosphorylation of ERK1/2 and p38 MAPK at 15 and 30 min after incubation with LPS (Fig. 3). However, CNP did not affect the LPS-induced phosphorylation of JNK.

Effect of inhibition of ERK and p38 signaling pathways on LPS-induced expression of adhesion molecules

To further confirm whether ERK1/2 and p38 MAPK critically contributed to the inhibitory activity of

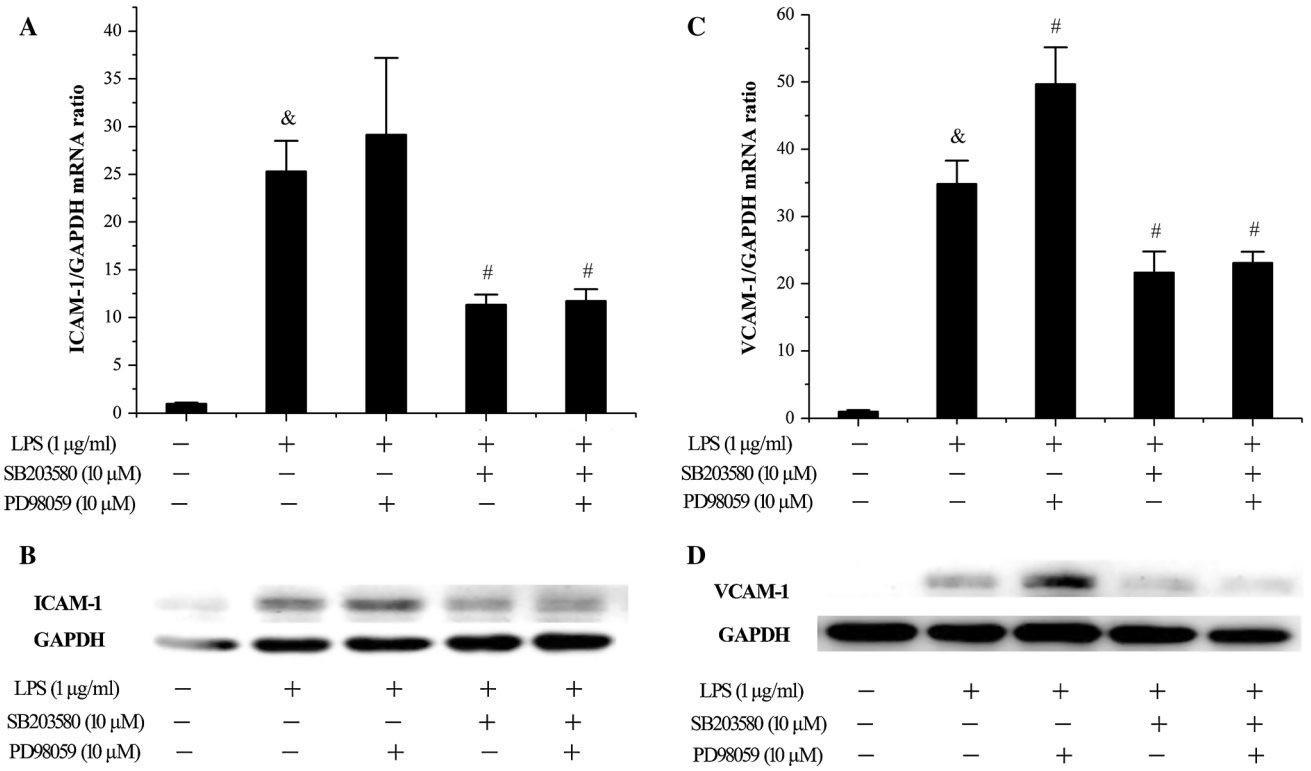

Fig. 4 Effect of p38 and ERK1/2 inhibition on LPS-induced adhesion molecule mRNA and protein expression. HUVECs were pretreated for 40 min with vehicle (DMSO), SB203580 (p38 MAPK inhibitor, 10 μM), PD98059 (ERK1/2 inhibitor, 10 μM), or a combination of the two inhibitors before incubation with LPS (1 μg/ml) for 4 or 6 h. ICAM-1 and VCAM-1 mRNA (4 h) and protein expression (6 h) were analyzed using qRT-PCR (**a**, **c**) and western blotting analyses (**b**, **d**), respectively. Representative data are shown from three independent experiments. Data are plotted as the mean ± SD. $^{\&}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group

CNP, the cells were pretreated with an ERK1/2 inhibitor (PD98059, 10 μM) or p38 MAPK inhibitor (SB203580, 10 μM) for 40 min prior to exposure to LPS (1 μg/ml). The mRNA and protein expression of VCAM-1 and ICAM-1 were significantly inhibited when SB203580 was used alone or in combination with PD98059 (Fig. 4). However, PD98059 did not inhibit the mRNA and protein expression of ICAM-1. Moreover, PD98059 enhanced the mRNA and protein expression of VCAM-1.

CNP enhanced Akt phosphorylation in LPS-stimulated HUVECs

The PI3K/Akt pathway has been shown to negatively regulate the response to inflammatory stimuli. We investigated whether CNP could affect LPS-induced Akt phosphorylation in HUVECs. LPS stimulation resulted in a rapid phosphorylation of Akt, with peak levels occurring 15 min after incubation with LPS (Fig. 5a). Pretreatment with CNP prior to LPS stimulation initially elevated Akt phosphorylation and further increased steadily at 30 min after incubation with LPS (1 μg/ml).

CNP up-regulated HO-1 expression in LPS-stimulated HUVECs

Hemeoxygenase-1 (HO-1) exhibits protective biological activities, including antioxidant, anti-inflammatory, and anti-apoptotic properties (Lee et al. 2009). HO-1 induction is thought to represent an adaptive response that provides cytoprotection to cells and tissues against oxidative stress. The PI3 K/Akt pathway regulates HO-1 expression (Hsu et al. 2009; Kim et al. 2009), and thus, we investigated the effect of CNP on HO-1 expression in LPS-stimulated HUVECs. Incubation with LPS slightly elevated HO-1 expression. CNP increased HO-1 expression in LPS-stimulated cells in a concentration-dependent manner. Thus, the expression of HO-1 induced by CNP may negatively regulate LPS-induced inflammatory responses in HUVECs (Fig. 5b, c).

Inhibition of PI3K/Akt signaling reversed the inhibitory activity of CNP on LPS-induced expression of adhesion molecules

To investigate whether Akt phosphorylation is involved in the inhibitory activity of CNP, the cells were pretreated

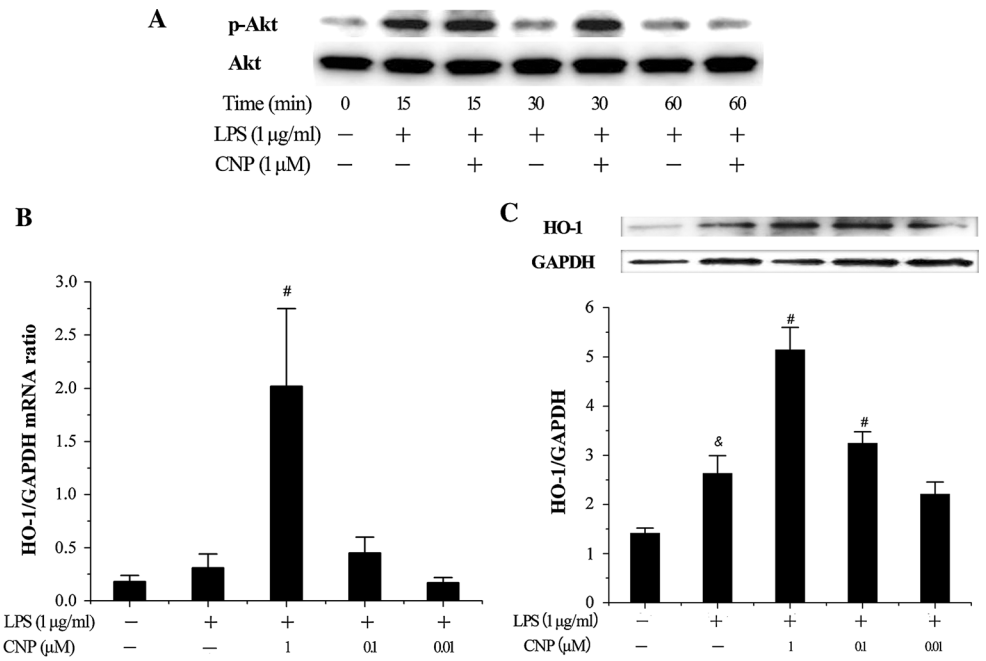

Fig. 5 CNP activated the PI3 K/Akt/HO-1 pathway in LPS-stimulated HUVECs. **a** HUVECs were pretreated with CNP (1 μM) for 120 min, followed by incubation with LPS (1 μg/ml) for specific time periods as indicated. The phosphorylation of Akt was determined in whole cell lysates using western blotting. Representative blots of three independent experiments are shown. **b** HUVECs were pretreated with the indicated concentrations of CNP for 120 min prior to incubation with LPS (1 μg/ml) for 2 h. Total RNA was isolated and subsequently analyzed using qRT-PCR. Data are expressed as the fold change relative to the nonstimulated cells. **c** HUVECs were pretreated with specific concentrations of CNP for 120 min prior to incubation with LPS (1 μg/ml) for 6 h, and HO-1 expression was analyzed using western blotting analyses. GAPDH was used as a loading control. Representative blots and densitometric evaluations are shown from three independent experiments. Data are plotted as the mean ± SD. $^{\&}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group

with 100 nM wortmannin (a PI3K/Akt pathway inhibitor) prior to treatment with 1 μM CNP and LPS (1 μg/ml). The addition of wortmannin reversed the inhibitory activity of CNP on LPS-induced mRNA and protein expression of ICAM-1 and VCAM-1 (Fig. 6). In addition, wortmannin alone also can increase the LPS-induced ICAM and VCAM expression, and the increased ICAM and VCAM expression induced by wortmannin can be inhibited by CNP.

CNP reduced intracellular ROS production in LPS-stimulated HUVECs

ROS are critical for LPS-induced inflammatory responses via activation NF-kB signaling (Haddad and Land 2002), and thus, we investigated the effects of CNP on LPS-induced ROS signaling in HUVECs. Treatment with LPS significantly elevated the production of intracellular ROS after 1 h incubation. Moreover, CNP significantly reduced LPS-induced intracellular ROS production (Fig. 7). In addition, the antioxidant PDTC (10 μM) also significantly reduced LPS-induced upregulation of ROS.

Discussion

Endothelial activation is typically induced by inflammatory agents and facilitates the recruitment and adherence of circulating leukocytes, consequently contributing to the progression of inflammatory diseases (Liao 2013). Thus, inhibition of endothelial activation may be a potential therapeutic approach to regulate inflammatory diseases. In this study, we demonstrated that CNP attenuates LPS-induced endothelial activation and that these effects are mediated by inhibiting NF-κB and p38 signaling pathways, eliminating LPS-induced intracellular ROS production, and activating the PI3K/Akt/HO-1 pathway in HUVECs.

In the present study, we found that CNP (1 μM) attenuates LPS-induced upregulation of adhesion molecules in HUVECs, indicating that CNP exerts its anti-inflammatory effects by directly inhibiting endothelial activation. The concentrations of CNP used in this study were consistent with the local concentration of CNP released from the vascular endothelium (Chauhan et al. 2003; Scotland et al. 2005b). Previous studies have also indicated that CNP modulates the response of leukocytes and platelets in the presence of inflammatory agents such as IL-1β and

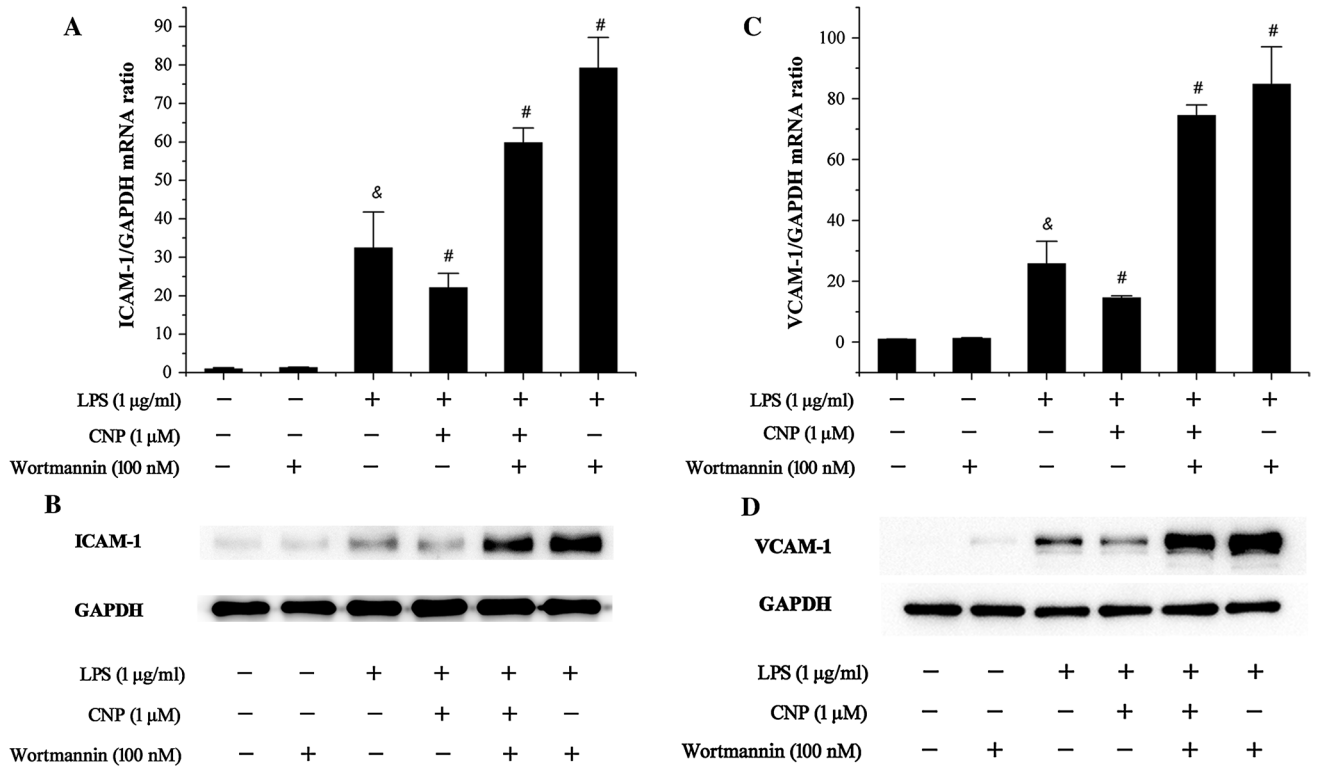

Fig. 6 Inhibition of the PI3K/Akt pathway reversed the inhibitory activity of CNP on LPS-induced expression of adhesion molecules. HUVECs were pretreated with CNP (1 μM) in the presence or absence of wortmannin (PI3K/Akt pathway inhibitor, 100 nM) prior to incubation with LPS (1 μg/ml) for 4 or 6 h. ICAM-1 and VCAM-1 mRNA (4 h) and protein expression (6 h) were analyzed using qRT-PCR (**a**, **c**) and western blotting analyses (**b**, **d**), respectively. Representative data are shown from three independent experiments. Data are plotted as the mean ± SD. $^{\&}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group

histamine (Scotland et al. 2005b). These findings suggest that CNP exerts its anti-inflammatory effects via multiple mechanisms, including inhibiting endothelial activation and modulating the response of leukocytes and platelets.

NF-κB is a transcription factor that exhibits crucial roles in inflammation (Viatour et al. 2005). Activation of NF-κB in response to inflammatory agents induces a large number of genes, including adhesion molecules and inflammatory cytokines (Checker et al. 2012), thereby contributing to endothelial activation (Liao 2013). NF-κB activation requires the phosphorylation of NF-κB proteins, such as p65 (Hu et al. 2004; Viatour et al. 2005). These findings indicate that the inhibitory effect of CNP on endothelial activation is mediated via the suppression of LPS-triggered phosphorylation of NF-κB p65 and subsequent NF-κB activation.

MAPKs are involved in the intracellular signaling pathway in response to inflammatory stimuli and are required for adhesion molecule expression and endothelial activation (Binion et al. 2009; Roussel et al. 2010). As expected, exposure of HUVECs to LPS increased the activation of MAPKs, including ERK1/2, p38 MAPK, and JNK. Interestingly, pretreatment with CNP abolished LPS-induced ERK1/2 and p38 MAPK phosphorylation, but not JNK. Next, we demonstrated that p38 MAPK, but not ERK1/2 inhibition, mediated the downregulation of adhesion molecule expression in LPS-stimulated HUVECs. We concluded that ERK1/2 activation in HUVECs induced by LPS may not be involved in the expression of adhesion molecules, and this result was consistent with previous studies (Cho et al. 2013; Tsoyi et al. 2009). P38 MAPK plays an essential role in LPS-induced endothelial activation. Previous studies have indicated that p38 MAPK positively regulated several transcription factors, including NF-κB, in LPS-induced inflammation (Carter et al. 1999; Liu et al. 2008). Thus, inhibition of p38 MAPK activation by CNP may partially explain the suppression of NF-κB activation.

ROS are involved in LPS-induced inflammation. ROS have been shown to activate the NF-κB and p38 MAPK signaling pathways (Haddad and Land 2002; Kim et al. 2007). Our data demonstrated that CNP significantly suppressed LPS-induced increases in intracellular ROS accumulation. Although the precise mechanism underlying this phenomenon remains unclear, enhanced HO-1 expression induced by CNP may be involved. HO-1 expression is considered to be an adaptive and beneficial response

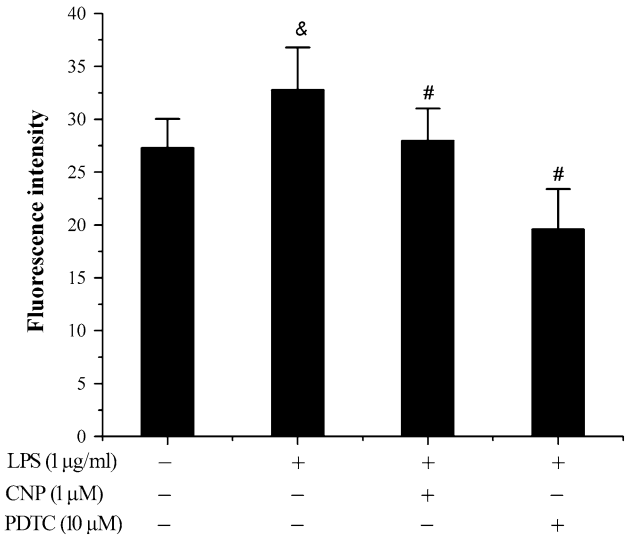

Fig. 7 CNP inhibits the production of intracellular ROS in LPS-stimulated HUVECs. HUVECs were pretreated with CNP (1 μM) or PDTC (10 μM) for 120 min prior to incubation with LPS (1 μg/ml) for 1 h, and the cells were subsequently incubated with 10 μM DCFH-DA in serum-free medium for 20 min. PDTC was used as a positive control. Data are plotted as the mean ± SD of three independent experiments, performed in triplicate. $^{\&}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group

to oxidative stress in specific cells (Seldon et al. 2007; Takahashi et al. 2004). HO-1 also inhibits the expression of adhesion molecules associated with endothelial activation via the inhibition of NF-κB p65 phosphorylation (Seldon et al. 2007; Soares et al. 2004). In addition, CNP may induce Nrf2 activation and subsequently enhance Nrf2-mediated antioxidant response elements (AREs), thereby contributing to chemoprotective gene expression, including HO-1. Previous studies have suggested that the PI3K/Akt signaling pathway can positively regulate Nrf2 activation (Dai et al. 2007; Pugazhenthi et al. 2007; Yang et al. 2013). However, the effect of CNP on the Nrf2/ARE pathway requires further investigation.

The PI3K/Akt pathway plays a crucial role in a wide variety of biological responses, including cellular proliferation and survival. A growing body of evidence suggests that the PI3K/Akt pathway plays a dual role in LPS-induced inflammation because it both positively and negatively regulates pro-inflammatory pathways (Dauphinee and Karsan 2006). We found that CNP activation of the PI3 K/Akt pathway negatively regulates the pro-inflammatory response in endothelial cells. These results are consistent with a previous study (Schabbauer et al. 2004). Akt phosphorylation dampens LPS-induced endothelial activation via inhibition of the p38 MAPK pathway. Previous studies have indicated that inhibition of the PI3K/Akt pathway enhances LPS activation of p38 MAPK (Schabbauer et al. 2004). In addition, the PI3K/Akt pathway might also inhibit NF-κB activation (Guha and Mackman 2002; Hwang et al. 2013). However, other studies have demonstrated that PI3 K is required for LPS-induced NF-κB activation (Dauphinee and Karsan 2006). These results indicate that further investigation is required to elucidate the role of PI3K/Akt signaling in the LPS-induced inflammation response.

Conclusion

In summary, these results imply that CNP effectively attenuates LPS-induced endothelial activation by inhibiting NF-κB and p38 signaling pathways, eliminating LPS-induced intracellular ROS production, and activating the PI3K/Akt/HO-1 pathway in HUVECs, thereby demonstrating that CNP may be a potential therapeutic target for the treatment of sepsis and inflammatory vascular diseases.

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Conflict of interest The authors declare that they have no competing interests.

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