Decrease of Intracellular Chloride Concentration Promotes Endothelial Cell Inflammation by Activating Nuclear Factor-κB Pathway

Hui Yang, Lin-Yan Huang, De-Yi Zeng, Er-Wen Huang, Si-Jia Liang, Yong-Bo Tang, Ying-Xue Su, Jing Tao, Fei Shang, Qian-Qian Wu, Li-Xiong Xiong, Xiao-Fei Lv, Jie Liu, Yong-Yuan Guan, Jia-Guo Zhou

Abstract—Recent evidence suggested that CIC-3 channel/antiporter is involved in regulation of nuclear factor (NF)-κB activation. However, the mechanism explaining how CIC-3 modulates NF-κB signaling is not well understood. We hypothesized that CIC-3-dependent alteration of intracellular chloride concentration ([Cl^-]) underlies the effect of CIC-3 on NF-κB activity in endothelial cells. Here, we found that reduction of [Cl^-], induced tumor necrosis factor-α (TNFα)-induced expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 and adhesion of monocytes to endothelial cells (P<0.05; n=6). In Cl^- reduced solutions, TNFα-evoked IkB kinase complex β and inhibitors of κBα phosphorylation, inhibitors of κBα degradation, and NF-κB nuclear translocation were enhanced. In addition, TNFα and interleukin 1β could activate an outward rectifying Cl^- current in human umbilical vein endothelial cells and mouse aortic endothelial cells. Knockdown or genetic deletion of CIC-3 inhibited or abolished this Cl^- conductance. Moreover, Cl^- channel blockers, CIC-3 knockdown or knockout remarkably reduced TNFα-induced intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 expression, monocytes to endothelial cell adhesion, and NF-κB activation (P<0.01; n=6). Furthermore, TNFα-induced vascular inflammation and neutrophil infiltration into the lung and liver were obviously attenuated in CIC-3 knockout mice (P<0.01; n=7). Our results demonstrated that decrease of [Cl^-], induced by CIC-3-dependent Cl^- efflux promotes NF-κB activation and thus potentiates TNFα-induced vascular inflammation, suggesting that inhibition of CIC-3-dependent Cl^- current or modification of intracellular Cl^- content may be a novel therapeutic approach for inflammatory diseases. (Hypertension. 2012;60:1287-1293.) ● Online Data Supplement

Key Words: basic science • membrane transport/ion channels • cytokines • endothelium • inflammation • ion channels
evidence has demonstrated that intracellular chloride concentration ([Cl−]) was dynamically regulated. Obvious changes in [Cl−], have been observed in hippocampal neurons during development, in T cells undergoing apoptosis, and in macrophages during foam cell formation.12,14,22 Moreover, previous work in Jurkat T cells showed that depletion of intracellular chloride blocked UV-C induced cytochrome c release from mitochondria and thus inhibited cell apoptosis.22 These results suggested that the alterations of [Cl−] play a critical role in a variety of physiological and pathological processes.

CIC-3 has been found to be an essential regulator of [Cl−],15,16; however, whether the alteration of [Cl−] contributes to the functional roles of CIC-3 is not clear. Our present study, therefore, aimed to investigate the effects of [Cl−], as well as CIC-3 genetic deficiency on TNFα-induced inflammatory response and NF-κB activation in endothelial cells. Our results demonstrated that decrease of [Cl−] underlies, at least in part, the proinflammatory effects of CIC-3-dependent Cl− efflux.

Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

All of the experimental procedures were approved by the Sun Yat-Sen University Committee for Animal Research and conformed to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in China. CIC-3 heterozygous mice (CIC-3+/−) were kindly provided by Dr Dean Burkin (University of Nevada School of Medicine). CIC-3 knockout (KO) (CIC-3−/−) mice were prepared as described previously.5,16,27 Chloride-reduced medium was prepared by replacing chloride with gluconate.

Results

Reduction of [Cl−], Potentiated TNFα-Induced Inflammatory Response and NF-κB Activation in HUVECs

We found that TNFα (10 ng/mL) treatment decreased [Cl−] from 33.3±2.6 (n=52) to 23.1±1.9 mmol/L (n=30) in the mice were examined by polymerase chain reaction on tail DNA (Figure S1 in the online-only Data Supplement).

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously.25 The study protocol was approved by the medical research ethics committee of Sun Yat-Sen University. Informed consent was obtained from all subjects, and the experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Perforated whole-cell patch experiments were performed as described previously.14 Adhesion of monocytes to endothelial cells was examined by using Calcein-AM labeled human acute mononcytic leukemia cells or mouse monocytes. Quantitative real-time-polymerase chain reaction was performed using SYBR green fluorescence. Immunohistochemistry was performed by using the streptavidin-biotin-peroxidase complex system, according to the manufacturer’s instructions (SABC peroxidase kit). Myeloperoxidase activity was measured as described previously.26 Small interfering RNA transfection, Western blot, and [Cl−], measurement were performed as described previously.15,16,27 Chloride-reduced medium was prepared by replacing chloride with gluconate.

All data were expressed as mean±SEM. Statistical analysis was determined by an unpaired 2-tailed Student t test or 1-way ANOVA followed by Bonferroni multiple comparison post hoc test with a 95% CI. Values of P<0.05 were considered significant.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Tumor necrosis factor-α (TNFα)–induced inflammatory response and NF-κB activation in human umbilical vein endothelial cells (HUVECs) were enhanced in Cl−-reduced solution. A and B, HUVECs were preincubated in normal Cl−-, medium Cl−-, or low Cl−-solution before treatment with TNFα (10 ng/mL). Normal Cl−-solution without TNFα was used as control. Twenty-four-hours later, the expression of intercellular adhesion molecule 1 (ICAM-1) (A) and vascular cell adhesion molecule 1 (VCAM-1) (B) was determined by Western blot (**P<0.01 vs control, #P<0.05 vs TNFα+normal Cl−; n=6). C, adhesion of calcein-labeled human acute mononcytic leukemia cell monocytes to HUVECs was investigated with confocal microscopy in HUVECs incubated in culture medium with different Cl−-concentration (**P<0.01 vs control, #P<0.05 vs TNFα+normal Cl−; n=5). D and E, HUVECs in normal Cl−-or low Cl−-solution were treated with TNFα (10 ng/mL) for different times as indicated. The nuclear fractions were isolated and the nuclear translocation of p65 was detected by Western blot (**P<0.05, #P<0.01 vs control; n=6).
HUVECs (P<0.05). To examine whether the drop of [Cl−] is involved in TNFα-induced inflammation, we prepared the cell culture medium with reduced chloride concentration to decrease the intracellular chloride content and investigated the inflammatory response in these solutions. In HUVECs, medium Cl− and low Cl− solution decreased the [Cl−] to 26.8±2.1 (n=36) and 16.5±1.6 mmol/L (n=40), respectively (Figure S2). In the medium Cl− or low Cl− solution, TNFα-induced expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) was enhanced obviously compared with that in normal Cl− solution (Figure 1A and 1B and Figure S3; n=4–6). Meanwhile, TNFα-induced adhesion of human acute monocytic leukemia cell monocytes to HUVECs was also increased in Cl− reduced solution (Figure 1C; n=6).

We next investigated whether reduction of [Cl−] affected NF-κB activation. Nuclear translocation of NF-κB subunit p65 was triggered within 30 minutes after TNFα stimulation in normal Cl− solution (Figure 1D; n=5). In low Cl− medium, p65 translocation started from 15 minutes and reached a maximum at 30 minutes after TNFα treatment (Figure 1E; n=6). These findings suggested that reduction of [Cl−] enhanced NF-κB activation and thus increased TNFα-induced inflammation.

Lowering [Cl−], Promoted NF-κB Activation through Modulating IKKβ-IκBa Signaling Pathway

To explore the mechanism how decreases of [Cl−] enhanced NF-κB activation, we investigated the effects of Cl− reduced medium on TNFα-induced degradation of inhibitors of xBα (IxBα) and phosphorylation of IxBα and IkB kinase complex β (IKKβ). Our results showed that TNFα induced IxBα degradation from 15 minutes and reached a maximum at 30 minutes in normal Cl− solution. In low Cl− solution, TNFα induced more significant degradation of IxBα, which started from 5 minutes and reached a maximum at 15 minutes (Figure 2A, n=6). TNFα induced phosphorylation of IxBα in low Cl− solution from 5 minutes after treatment, which preceded the increase of IxBα phosphorylation in normal Cl− solution (Figure 2B, n=6). In addition, TNFα-induced IKKβ phosphorylation in low Cl− solution also preceded the onset of IKKβ phosphorylation in normal Cl− solution. Moreover, IKKβ phosphorylation in low Cl− solution maintained longer than that in normal Cl− solution (Figure 2C, n=5). These results indicated that the increases of IKKβ and IxBα phosphorylation and IxBα degradation contributed to the enhanced inflammatory response in low Cl− solution.
TNFα Activated a CIC-3-Dependent Chloride Current in Endothelial Cells

In HUVECs, perfusion of TNFα (10 ng/mL) induced an outwardly rectifying current under isotonic solutions. This current showed slow inactivation at positive potentials (≥ +60 mV). The reversal potential of this current was −0.4±1.8 mV, which was near to the equilibrium potential for Cl− (0 mV) in our experimental conditions (Figure S4). Reduction of extracellular Cl− concentration from 133 to 44 mmol/L shifted the reversal potential to 25.3±2.1 mV, indicating that this current is mainly carried by Cl− (n=6). TNFα-activated chloride current was remarkably inhibited by the chloride channel blockers 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; 100 μmol/L), 5-Nitro-2-(3-henylpropylamino) benzoic Acid (NPPB; 100 μmol/L), and tamoxifen (10 μmol/L) (Figure S4; n=6).

One recent study in vascular smooth muscle cells suggested that cytokine-activated chloride current is dependent on CIC-3 expression.14 Here, we also found that knockdown of CIC-3 with CIC-3 small interfering RNA transfection remarkably decreased TNFα-activated chloride current in HUVECs (Figure 3A and Figures S5 and S6A). Moreover, our results showed that TNFα can activate an outwardly rectifying chloride current in mouse aortic endothelial cells (MAECs) isolated from CIC-3+/+ mice; however, this chloride current was not observed in CIC-3−/− MAECs (Figure 3B and Figures S5 and S6B). These results demonstrated that CIC-3 is necessary for TNFα-activated chloride currents in endothelial cells. This CIC-3-dependent chloride current was also observed after interleukin-1β (Figure S7) and angiotensin II (data not shown) treatment.

Chloride Channel Blockers Inhibited TNFα-Induced Inflammatory Response in HUVECs

To understand the functions of TNFα-activated chloride current in endothelial cells, we examined the effects of the chloride channel blockers on TNFα-evoked inflammation. In HUVECs, DIDS (100 μmol/L), NPPB (100 μmol/L), or tamoxifen (10 μmol/L) pretreatment significantly inhibited TNFα-induced expression of ICAM-1 and VCAM-1 both at mRNA (Figure S8) and protein levels (Figure S9A and S9B; n=6 in each group). In addition, DIDS, NPPB, or tamoxifen remarkably reduced TNFα-induced adhesion of monocyte to HUVECs (Figure S9C and S9D; n=5). These observations suggested that TNFα-activated chloride current is involved in endothelial cell inflammation.

CIC-3 Deficiency Attenuated TNFα-Induced Inflammatory Response in Endothelial Cells

CIC-3 is required for TNFα-activated chloride current, so we next examined whether TNFα-induced inflammation was altered in CIC-3-deficient endothelial cells. In HUVECs, knockdown of CIC-3 had no significant effects on basal ICAM-1 and VCAM-1 expression; however, CIC-3 knockdown remarkably attenuated TNFα-induced expression of ICAM-1 and VCAM-1 (Figure 4A and 4B, n=6; Figure S10A and S10B; n=4) and adhesion of monocytes to HUVECs (Figure 4C; n=6). To further determine the functional role of CIC-3 in vascular inflammation, we compared TNFα-induced inflammatory response in MAECs isolated from CIC-3+/+ mice with those from CIC-3−/− mice. The expression of ICAM-1 and VCAM-1 and the adhesion of monocyte to MAECs in basal condition were not significantly different between the 2 groups. However, CIC-3 KO dramatically attenuated TNFα-evoked increases of ICAM-1 and VCAM-1 expression and monocyte to MAEC adhesion (Figure 4 and Figure S10C and S10D; n=4–6). Moreover, we found that heterogenous expression of CIC-3 in CIC-3+/− MAECs restored TNFα-induced expression of ICAM-1 and VCAM-1 and adhesion of monocyte to MAECs (Figure S11; n=5).

These findings highlighted that CIC-3 plays an important role in modulating TNFα-induced endothelial cell inflammation.

Figure 3. Tumor necrosis factor-α (TNFα) activated a CIC-3-dependent chloride current in endothelial cells. A, CIC-3 knockdown reduced TNFα-activated chloride current in human umbilical vein endothelial cells (**P<0.01 vs control, #P<0.05, ##P<0.01 vs negative siRNA+TNFα; n=6–10). Neg. RNA means negative siRNA. B, TNFα-activated chloride current in mouse aortic endothelial cells isolated from CIC-3−/− and CIC-3−/− mice (**P<0.01 vs corresponding control, ###P<0.01 vs WT+TNFα; n=10). WT indicates wild type; KO, knockout.
CIC-3 Deficiency Decreased NF-κB Activation in Endothelial Cells

In HUVECs, TNFα treatment for 30 minutes induced remarkable translocation of p65 subunit to the nuclei (Figure 5). Knockdown of CIC-3 decreased p65 in the nuclei after TNFα incubation (Figure 5A and Figure S12A). To further confirm the effects of CIC-3 expression on NF-κB activation, we compared the TNFα-induced p65 nuclear translocation in CIC-3−/− MAECs with that in CIC-3+/+ MAECs. Consistent with the results in CIC-3 knockdown HUVECs, CIC-3 KO drastically reduced TNFα-induced p65 translocation from the cytoplasm to the nuclei in MAECs (Figure 5B and Figure S12B). These data suggested that modulation of the NF-κB activation underlies, at least in part, the proinflammatory effects of CIC-3 in endothelial cells.

CIC-3 Deletion Reduced Vascular Inflammation In Vivo

Similar to the results in MAECs in vitro, the basal expression levels of ICAM-1 and VCAM-1 in the aorta were no differences between CIC-3+/+ mice and their CIC-3−/− littermates. After intraperitoneal injection of TNFα (30 µg/kg) for 72 hours, the expression of ICAM-1 and VCAM-1 in aorta

Figure 4. CIC-3 deletion inhibited tumor necrosis factor α (TNFα)-induced inflammatory response in endothelial cells. A and B, TNFα (10 ng/mL) induced expression of intercellular adhesion molecule 1 (ICAM-1) (A) and vascular cell adhesion molecule 1 (VCAM-1) (B) from human umbilical vein endothelial cells (HUVECs) transfected with negative siRNA (Neg. RNA) or CIC-3 siRNA (**P<0.01 vs control, ##P<0.01 vs Neg. RNA group; n=6). C, Adhesion of calcein-labeled human acute monocytic leukemia cell monocytes to HUVECs transfected with negative siRNA (Neg. RNA) or CIC-3 siRNA (**P<0.01 vs control, ##P<0.01 vs Neg. RNA group; n=6). D and E, TNFα (10 ng/mL) induced expression of ICAM-1 (D) and VCAM-1 (E) in mouse aortic endothelial cells isolated from CIC-3−/− mice wild-type (WT) and CIC-3−/− mice knockout (KO) (*P<0.05, **P<0.01 vs control, ###P<0.01 vs wild-type+TNFα; n=6). F, Adhesion of calcein-labeled mouse monocytes to MAECs from CIC-3−/− (WT) and CIC-3−/− mice (KO) (*P<0.05, **P<0.01 vs control, ###P<0.01 vs WT+TNFα; n=5).

Figure 5. CIC-3 deficiency repressed nuclear factor (NF)-κB activation in endothelial cells. NF-κB activation was determined by the nuclear translocation of p65 subunit of NF-κB. A and B, Nuclear (A) and cytoplasmic (B) fractions were isolated from human umbilical vein endothelial cells transfected with negative siRNA (Neg. siRNA) or CIC-3 siRNA; activation of NF-κB was detected by Western blot using antibody against p65 (**P<0.01 vs control, ##P<0.01 vs Neg. siRNA+TNFα; n=5). C and D, Nuclear and cytoplasmic lysates from CIC-3−/− mouse aortic endothelial cells; wild-type (WT) and CIC-3−/− mouse aortic endothelial cell knockout (KO) was analyzed by Western blot to detect the expression of p65 (**P<0.01 vs control, ##P<0.01 vs Neg. siRNA+TNFα; n=6). TNFα indicates tumor necrosis factor-α.
was remarkably increased in CIC-3−/− mice. However, TNFα-induced increases of these adhesion molecules in CIC-3−/− mice were reduced obviously (Figure 6 and Figures S13 and S14; n=5−6). Moreover, histological examination revealed that the inflammation in lung and liver was dramatically attenuated in CIC-3−/− mice (Figure S15). Myeloperoxidase activity, an indicator of neutrophil infiltration, in the lung tissue was also significantly decreased in CIC-3−/− mice (Figure S15; n=6). The data further supported the critical role of CIC-3 in the regulation of vascular inflammatory response.

Discussion

Chloride was once supposed to be an inert ion; however, recent growing evidence suggested that [Cl−]i is dynamically modulated, and alteration of intracellular Cl− is involved in several physiological processes, such as T-cell apoptosis and neuron excitability.12,22 In HUVECs, we observed that TNFα treatment decreased [Cl−]i. Reduction of [Cl−]i dramatically potentiated TNFα-induced expression of ICAM-1 and VCAM-1 and adhesion of monocytes to HUVECs. The data suggested that reduction of [Cl−]i underlies, at least in part, TNFα-induced inflammatory response.18 Consistent with this study, we found that TNFα, interleukin-1β, and angiotensin II could activate a chloride conductance, and this current is dependent on CIC-3 expression.23 In the vessels, CIC-3-dependent chloride current has been documented to be a critical regulator of cell volume, proliferation, migration, and apoptosis in vascular smooth muscle cells.3,9,13,15 The expression of CIC-3 has been detected in endothelial cells.33 However, its functions remain elusive. CIC-3 is required for cytokines-induced chloride current in endothelial cells, so we examined the effects of this chloride conductance on vascular inflammation. Our results showed that inhibition of the chloride current with chloride channel blockers or knockdown of CIC-3 remarkably reduced TNFα-induced ICAM-1 and VCAM-1 expression and monocytes to HUVECs adhesion. Moreover, we further observed that TNFα-induced endothelial inflammation was attenuated obviously in MAECs isolated from CIC-3−/− mice. Heterogenous expression of CIC-3 in CIC-3−/− MAECs restored TNFα-induced inflammatory responses. Furthermore, TNFα-induced ICAM-1 and VCAM-1 expression in aorta and neutrophil infiltration into the lung and liver were drastically reduced in CIC-3−/− mice compared with their wild-type littermates. Importantly, we found that CIC-3 deficiency profoundly suppressed TNFα-induced nuclear translocation of p65 subunit of NF-κB. Our results were in agreement with a recent study that showed that CIC-3 KO inhibited NF-κB activity in mouse aortic smooth muscle cells.19 All these findings indicated that CIC-3-dependent chloride conductance is involved in regulating NF-κB activation and thus the inflammatory responses.

Perspectives

Our present study provided evidence that CIC-3-dependent Cl− efflux contributes to TNFα-induced endothelial cell inflammation. CIC-3-dependent Cl− efflux evokes decrease of [Cl−]i, which underlies the proinflammatory effects of CIC-3-dependent Cl− conductance by activating the NF-κB pathway. The data suggested that modulation of intracellular Cl− content may be a novel strategy to prevent inflammatory diseases.
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Disclosures

None.

References


Novelty and Significance

What Is New?

- Reduction of [Cl−]i activates IK(Cl)−lKChx-NF-κB signaling pathway and promotes endothelial cell inflammatory response.
- CIC-3 deficiency inhibits NF-κB activation and attenuates vascular inflammation.

What Is Relevant?

- Vascular inflammation contributes to the development of hypertension and hypertension-associated cardiovascular diseases.
- Inhibition of CIC-3-dependent chloride efflux reduces vascular inflammation.

Summary

Our findings demonstrate that CIC-3-dependent Cl− efflux induces decrease of [Cl−], which underlies the proinflammatory effects of CIC-3-dependent Cl− conductance by activation of NF-κB pathway. The data suggested that inhibition of the CIC-3-dependent Cl− current or modulation of intracellular Cl− content may be a novel strategy to prevent inflammatory diseases.
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Decrease of intracellular chloride concentration promotes endothelial cell inflammation by activating NF-κB pathway

Yang et al. Chloride and endothelial cell inflammation

Hui Yang1,3*, Lin-Yan Huang1*, De-Yi Zeng 1*, Er-Wen Huang 1,2,4*, Si-Jia Liang1, Yong-Bo Tang1, Ying-Xue Su1, Jing Tao1, Fei Shang1, Qian-Qian Wu1, Li-Xiong Xiong1, Xiao-Fei Lv1, Jie Liu1, Yong-Yuan Guan1, Jia-Guo Zhou1

1Department of Pharmacology, Cardiac & Cerebral Vascular Research Center and 2Department of Forensic Pathology, Zhongshan School of Medicine, Sun Yat-Sen University. Guangzhou, 510080, China.
3Cardiovascular Institute of Guangdong Academy of Medical Sciences, Medical Research Center of Guangdong General Hospital. Guangzhou, 510080, China.
4Guangzhou Forensic Science Institute, Guangzhou, 510030, China

Correspondence to:

Jia-Guo Zhou, Department of Pharmacology, Cardiac and Cerebral Vascular Research Center, Zhongshan School of Medicine, Sun Yat-Sen University, 74 Zhongshan 2 Rd, Guangzhou, 510080, China. Tel: 86-20-87331857, Fax: 86-20-87331209. E-mail: zhoujg@mail.sysu.edu.cn

* These authors contribute equally to this work
Supplemental Methods

CIC-3 knockout mice

CIC-3 heterozygous mice (CIC-3+/−) were kindly provided by Dr. Dean Burkin (Nevada Transgenic Center, University of Nevada School of Medicine, Nevada, USA) 1. CIC-3 knockout (CIC-3−/−) mice were prepared by mating heterozygous breeders (CIC-3+/−) to their CIC-3+/− siblings as previously described in the Animal Center of Sun Yat-Sen University. Genotypes of the mice were examined by polymerase chain reaction (PCR) on tail DNA using the following primers as previously described 1, 2,

ClC-3 2F 5’- TGTAGCTCTTGTATTCTGTGT -3’
ClC-3 3R neo 5’- TTGGCTACCGATATTGCTGAAG -3’
ClC-3 4R 5’- TTATAGACATGCACACTATGCC -3’

which resulted in a 746 bp band for ClC-3−/−, a 467 bp band for ClC-3+/+, and both bands for ClC-3+/−. All animals were maintained in pathogen-free facilities with a 12-hour light/dark cycle.

All of the experimental procedures were approved by the Sun Yat-Sen University Committee for Animal Research and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China.

Endothelial cell isolation and culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described 3. In brief, HUVECs were harvested by the umbilical vein digested with 0.125% trypsin with 0.01% EDTA, then the cells were cultured in complete M199 medium which supplemented with 20% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 25 U/ml heparin, 2 mM L-glutamine and 5 ng/ml recombinant human endothelial growth factor β (β-ECGF) at 37°C, 5% CO₂ atmosphere. The study protocol was approved by the Medical Research Ethics Committee of Sun Yat-sen University. Informed consent was obtained from all subjects and the experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Mouse aortic endothelial cells (MAECs) were isolated and cultured using the primary explant technique as previously described 4. Briefly, male 6-8 week-old wild type or CIC-3 knockout mice were anaesthetised, the aorta was dissected and immersed immediately in the Kreb’s solution containing (mmol/L): NaCl 137, KCl 5.4, CaCl₂ 2.0, MgCl₂ 1.1, NaH₂PO₄ 0.4, Glucose 5.6, NaHCO₃ 11.9, 10⁵ U/L penicillin and 100 mg/L streptomycin. After fat and connective tissue was carefully cleaned, the vessel was opened longitudinally and cut into small pieces about 1-2mm² and plated with the intima side down in a fibronectin-coated culture dish. A small amount of culture medium (containing: DMEM/F12, 20% FCS, 25 U/ml heparin, 100 U/ml penicillin, 100 U/ml streptomycin and 10 ng/ml ECGs) was added into the dish. The explants were placed in an incubator at 37°C in 5% CO₂ atmosphere. After 24 h, more medium was added. About 5–7 days, the endothelial cells began to migrate from the aortic segments. When reaching confluence, cells were then subcultured.
with 0.25% trypsin with 0.02% EDTA.

Confluent HUVECs and MAECs presented cobblestone appearance and were identified by positive immunocytochemical staining with von Willebrand factor and platelet-endothelial cell molecule 1 (CD31). Passage 4 to 8 of endothelial cells were used in this study.

**Monocyte isolation and culture**

The human monocytotic cell line THP-1 was provided by Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin at 37 °C under 5% CO₂.

To prepare mouse monocytes, peripheral blood was harvested from mouse abdominal aorta and was then separated by using Nycoprep 1.077 A (AX-IS-SHIELD) to obtain peripheral blood mononuclear cells (PBMC). Monocytes were then isolated from the PBMC by CD11b antibody using a magnetic activated cell sorting system (Miltenyi Biotech).

**Small interfering RNA transfection**

The sequences of siRNA against human ClC-3 mRNA (GeneBank Accession No. NM_001829, 5’-CAGAAAGTCCTCGGCCATTGA-3’) was synthesized by Qiagen. A scrambled RNA (Qiagen) was used as negative control. HUVECs were transfected with ClC-3 and control siRNA oligonucleotides by using HiPerfect transfection reagent according to the manufacturer’s instructions (QIAGEN). Briefly, HUVECs were seeded at 2 x 10⁵ cells/ml in 6-well plate in 700 µl normal culture medium. The siRNA was diluted in 100 µl culture medium without serum (the final siRNA concentration was 20 nM), and then 12 µl HiPerFect Transfection Reagent was added to the diluted siRNA. The samples were incubated for 20 min at room temperature to form transfection complexes. The complexes were added to the cells and all were swirled gently to ensure uniform distribution. After 3 h, 1600 µl culture medium was added to each well, and the cells were incubated with transfection complexes under normal culture conditions for 48 h.

**ClC-3 cDNA transfection**

ClC-3/pcDNA3.1 plasmid was transfected as previously described⁵. Briefly, ClC-3⁻/⁻ MAECs were plated on 24-well plate at a density of 1-1.5 x 10⁵/ml. 24 hours later, ClC-3/pcDNA3.1 plasmid were transfected into the cells with LipofectAMINE²⁰₀⁰ reagent (Invitrogen, Life Technologies, Inc.) in OPTI-MEM® reduced serum medium (GIBICO) according to the manufacturer’s instructions.

**Electrophysiological recording**

Perforated whole-cell patch experiments were performed as described previously⁶. Patch pipettes were pulled from borosilicate glass with p-97 puller (Sutter Instrument Co., USA). The resistance of the pipettes used in this study was 3-6 MΩ after filling with pipette solution. The currents were elicited with voltage steps from −100 mV to +120 mV in +20 mV increment for 400 ms at an interval of 5 s from a hold-ing potential of −40 mV. Currents were sampled at 5
kHz using pCLAMP8.0 software (Axon Instruments) and filtered at 2 kHz. To minimize the changes of liquid junction potentials, a 3 mM KCl-agar salt bridge between the bath and the Ag-AgCl reference electrode was used. All experiments were performed at room temperature (25°C).

The extracellular solution contained (mM): 107 N-methyl-d-glucamine chloride (NMDG-Cl), 1.5 MgCl2, 2.5 MnCl2, 0.5 CdCl2, 0.05 GdCl3, 10 glucose, 10 HEPES, and 70 D-mannitol, pH 7.4 with NMDG. The osmolarity was 300 mosmol/kg·H2O. To examine the effects of Cl− concentration on the reversal potential of the current, Cl− in the extracellular medium was replaced by equimolar aspartate− to obtain the extracellular solution containing 39 mM [Cl−]. The internal pipette solution (300 mosmol/kg · H2O) contained (mM): 95 CsCl, 20 TEACl, 5 ATP-Mg, 5 EGTA, 5 HEPES, and 80 D-mannitol, pH 7.2 with CsOH. 60 mg/ml amphotericin stock solution was prepared by dissolving in DMSO. Before experiment, 4 µl stock solution was mixed with 1 ml pipette solution.

**Monocyte adhesion assay**

THP-1 cells or mouse monocytes were labelled with 3 µM Calcein-AM for 30 min at 37°C in 5% CO2. HUVECs pretreated with chloride channel blockers or ClC-3 siRNA transfection and MAECs isolated from ClC-3+/+ or ClC-3−/− mice were plated in 35 mm culture dishes at a density of 2 ×10^5 cells/ml. After incubation with TNF-α (10 ng/ml) for 24 h. The cells were washed twice with 1640, then the Calcein-AM labelled monocytes were added to each culture dish for 1h at 37°C, 5% CO2. To remove non-adherent cells, the dishes were gently washed with prewarmed RPMI-1640. This was repeated twice and then 1ml RPMI-1640 medium was added into each dish and image were captured with Olympus Fluoview 500 laser confocal scanning microscopy with an excitation wavelength of 485 nm and emission at 530 nm. At least 6 fields randomly selected in each dish were observed.

**Nuclear translocation of NF-κB**

Nuclear translocation of NF-κB was determined by western blot with RelA (p65)-specific antibody (Cell Signaling Technology, USA). Nuclear and cytoplasmic proteins were extracted with NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Thermo Scientific, USA). Briefly, HUVECs or MAECs were harvested with trypsin, then centrifuged at 500 g for 5 min. The cell pellet was washed and resuspended with PBS. 10^6 cells were transferred into a 1.5 ml eppendorf tube, centrifuged at 500 g for 2-3 min. Use a pipette to carefully remove the supernatant and make the pellet as dry as possible. Then 100 µl ice-cold CERI containing protease inhibitors was added into the pellet, the tube was vortexed vigorously for 15s to resuspend the cell pellet thoroughly and incubated on ice for 10 min. After that 11 µl ice-cold CERII was added to the tube, vortexed vigorously for 5s and incubated on ice for 1 min. The tube was vortexed for another 5s and centrifuged at 16000g for 5 min. The supernatant was the cytoplasmic protein and would be transferred to a pre-chilled eppendorf tube to store at -80°C. The
pellet which contained nuclei was resuspended with 50 µl ice-cold NER containing protease inhibitors, vortexed vigorously for 15s and incubated on ice, vortexed the tube 15 s for every 10 min, which would last for 40 min. At last the tube was centrifuged at 16000 g for 10 min and the supernatant (nuclear protein) was transferred into a ice-cold tube to store at -80°C.

**Western blot analysis**

Western blot was performed as previously described. Briefly, HUVECs or MAECs were rinsed with ice-cold PBS and lysed with RIPA lysis buffer containing protease inhibitor cocktail (Merk, Germany). The protein content was quantified with BCA kit. Protein was separated with 10% SDS-PAGE and was transformed to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked in 5% non-fat dry milk diluted with TBST (in mM: Tris–HCl 20, NaCl 150, pH 7.5, 0.1% Tween 20) at room temperature for 1h. The membranes were incubated with primary antibody: rabbit anti-ClC-3 (1:400 dilution; Alomone, Jerusalem, Israel), rabbit polyclonal anti VCAM-1 (1:1000 dilution; Santa Cruz Biotechnology Inc., USA), and rabbit polyclonal anti-ICMA-1 (1:800 dilution), anti-IκBα (1:1000 dilution), p65 (1:1000 dilution), pIKKβ (1:500 dilution), IKKβ (1:1000 dilution) (Cell Signaling Technology, USA) at 4 °C overnight, and then were incubated for 1h with anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution; Cell Signaling Technology, USA) at room temperature. Incubation with polyclonal rabbit α-tubulin antibody (1:1000 dilution; Santa Cruz Biotechnology Inc., USA) was performed as the loading sample control. Bands were detected with Pierce ECL western blotting substrate (Thermo Scientific, USA) and quantified with the computer-aided 1-D gel analysis system.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from HUVECs, MAECs or mouse aorta using Trizol reagent according to the manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed in a total volume of 20 µl, and real-time PCR was performed using SYBR green fluorescence. Samples were run in duplicate with RNA preparations from three to five independent experiments. Each real-time PCR reaction consisted of 1 µl RT product, 10 µl SYBR Green PCR Master Mix, and 500 nM forward and reverse primers. Reactions were carried out on a MyiQ Single Color Real-time PCR Detection System (Bio-Rad) for 40 cycles (95°C for 10 seconds, 60°C for 1 min) after an initial 3 min incubation at 95°C. The fold change in expression of each gene was calculated using the 2^-ΔΔCT method with 18S rRNA as an internal control.

**In vivo inflammation assay**

The animal model of acute inflammation was established as previously described. The 6-8 week-old wild type or ClC-3 knockout male mice were intra-peritoneal injected with 30 µg/kg recombinant mouse tumor necrosis factor-α (R&D, USA). After 72h, the thoracic aorta were used for immunohistochemistry and western blot analysis.

**Immunohistochemistry**
Mice were anesthetized with 2% pentobarbital sodium and were perfused intracardiacly with Kreb’s solution containing (mmol/L): NaCl 137, KCl 5.4, CaCl₂ 2.0, MgCl₂ 1.1, NaH₂PO₄ 0.4, Glucose 5.6, NaHCO₃ 11.9, heparin 10 U/ml, followed by 4°C fixative solution containing 4% freshly depolymerized paraformaldehyde in 0.1 mol/L phosphate buffer for 10-15 min. After fat and connective tissue was cleaned, the thoracic aorta was embedded and frozen using optimal cutting temperature compound (OCT, Sakura, Japan). Immunohistochemistry was performed by using the streptavidin-biotin-peroxidase complex system, according to the manufacturer’s instructions (SABC Peroxidase Kit).

Cryostat sections (8 μm) were pretreated with the solution of 3% hydrogen peroxide and methanol at the ratio of 1:50 for 30 min at room temperature, and then blocked with 5% bovine serum albumin in PBS for 30 min. Then the sections were incubated with ICAM-1 (1:200 dilution; Santa Cruz Biotechnology Inc., USA) or VCAM-1 (1:200 dilution; Santa Cruz Biotechnology Inc., USA) polyclonal antibody at 4 °C overnight, and then were treated with a biotinylated secondary anti-rabbit antibody for 30 min at room temperature. After that the sections were incubated with streptavidin biotin-peroxidase complex for 30 min at room temperature. Staining was performed with DAB chromogen. Slides were counterstained with hematoxylin.

**MPO assay**

Neutrophil infiltration in lung was quantified by measuring tissue myeloperoxidase (MPO) activity as previously described 9-11. Tissues were homogenized in 300-500 μl homogenisation buffer containing 0.5% HTAB, 50 mM KH₂PO₄, 5 mM EDTA, PH 6.0, followed by sonicati on for three times. Then the suspension was subjected to three cycles of freezing and thawing and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was then assayed for MPO activity. Briefly, this reaction was carried out in a 96-well plate by adding 290 μl substrate solution containing 0.167mg/ml O-Dianisidine dihydrochloride, 0.0005% H₂O₂, 50mM KH₂PO₄, PH6.0. 10 μl sample was added to each well to start the reaction. Optical density at 460 nm was read immediately, the OD value was called A₁, the second OD value was read 5 min later, called A₂. The changes in absorbance in 5 min represented the change of the MOP activity. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25°C.

**Measurement of [Cl⁻]**

[Cl⁻] was measured with 6-Methoxy-N-ethylquinolinium iodide (MEQ) as we described previously 5, 12. Briefly, MEQ was first reduced to a cell-permeable form, 6-methoxy-N-ethyl-1,2-dihydroquinoline (diH-MEQ). Then cells were incubated with 100-150 μmol/L diH-MEQ in a Ringer’s buffer solution containing (mmol/L): 119 NaCl, 2.5 KCl, 1.0 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃ and 11 Glucose, pH 7.4 at room temperature in the dark for 30 min. [Cl⁻] was monitored by MetaFluor Imaging software (Universal Imaging Systems, Chester, PA) with 350 nm excitation wavelength and 435 nm emission wavelength. Relationship between fluorescence intensity of MEQ and chloride
concentration is given by the Stern–Volmer equation: 
$$\frac{F_0}{F} - 1 = K_{SV} [Q].$$
Where $F_0$ is the fluorescence intensity without halide or other quenching ions; $F$ is the fluorescence intensity in the presence of quencher; $[Q]$ is the concentration of quencher; and $K_{SV}$ is the Stern–Volmer constant.

**Preparation of chloride reduced medium**

M199 cell culture medium lacking KCl and NaCl was initially made from Invitrogen, which account for 99.5% of the total chloride content. The normal chloride medium was prepared by adding 5 mM KCl and 105 mM NaCl. The medium chloride solution was prepared by adding 2.5 mM KCl plus 2.5 mM potassium gluconate and 52.5 mM NaCl plus 52.5 mM sodium gluconate. The chloride free medium was prepared by adding 5 mM potassium gluconate and 105 mM sodium gluconate. The osmolarities of the solutions ranged between 303.4 to 310.2 mosmol/kg-H$_2$O measured by a freezing point depression osmometer (OSMOMAT030, Germany).

**Supplemental References**

8. Dwight D, Henninger JP, Michael Eppihimer, Janice Russell, Mary Ger-


Supplemental Figures

Figure S1

Identification of ClC-3 knockout mice. A, Genotyping of mice by PCR. Heterozygous mice (+/-), homozygous wild type mice (+/+) and homozygous knockout mice (-/-) were identified by PCR amplified from mouse genomic DNA using primers specific for the wild type Clcn3 gene and the neomycin resistance gene, respectively. B, Western blot analysis of ClC-3 protein expression in aortic endothelial cells from wild type and ClC3−/− mouse.

Figure S2

Effects of medium chloride or low chloride solution on intracellular chloride concentration (n=36 or 40).

Figure S2. Effects of medium chloride or low chloride solution on intracellular chloride concentration (n=36 or 40).
Figure S3. TNFα-induced expression of ICAM-1 and VCAM-1 in HUVECs were enhanced in Cl⁻ reduced medium. HUVECs were preincubated in normal Cl⁻, medium Cl⁻ or low Cl⁻ solution before treatment with TNFα (10 ng/ml). 24 hours later, the cell lysates were isolated and the expression of ICAM-1 (A) and VCAM-1 (B) mRNA was determined by qRT-PCR (**p<0.01 vs. normal Cl⁻ solution, n=4).

Figure S4. TNFα activated a chloride current in HUVECs. Chloride channel blockers, DIDS (100 µmol/L), NPPB (100 µmol/L) and tamoxifen (10 µmol/L), remarkably inhibited TNFα-activated current (**p<0.01 vs control, #p<0.05, ##p<0.01 vs TNFα, n=6).
Figure S5

Figure S5. CIC-3 siRNA inhibited CIC-3 expression in HUVECs. A, HUVECs were treated with 10, 20nM CIC-3 siRNA for 48h. CIC-3 mRNA expression was detected by RT-PCR. #p<0.05, ##p<0.01 vs. con (n=6). B, HUVECs were treated with 10, 20, 40nM CIC-3 siRNA for 48h. CIC-3 protein expression was detected with Western blot. *p<0.05, **p<0.01 vs. con (n=6).

Figure S6

Figure S6. Effects of CIC-3 knockdown with siRNA transfection (A) and CIC-3 knockout (B) on TNFα-evoked chloride current in endothelial cells. The current densities were measured at +100 mV and -100 mV (**p<0.01 vs corresponding control, ###p<0.01 vs WT+TNFα, n=10).
Figure S7. IL1β-activated chloride current in mouse aortic endothelial cells (MAECs) isolated from CIC-3+/+ and CIC-3−/− mice. A, representative traces of IL1β-activated current recorded in MAECs isolated from CIC-3+/+ and CIC-3−/− mice, B, I-V curves of the currents from experiments as those shown in A, C, current densities of the currents at +100 mV and -100 mV from experiments as those shown in A (n=8-10, **p<0.01 vs corresponding control, ###p<0.01 vs WT+ IL1β).
Figure S8. Chloride channel blockers reduced TNFα-induced mRNA expression of ICAM-1 and VCAM-1 in HUVECs. HUVECs were preincubated with chloride channel blockers DIDS (100 µmol/L), NPPB (100 µmol/L) or tamoxifen (10 µmol/L) for 5 minutes and TNFα (10 ng/ml) induced expression of ICAM-1 (A) and VCAM-1 (B) was then determined by qRT-PCR (**p<0.01 vs TNFα only group, n=5 in each group).
Figure S9. Chloride channel blockers reduced TNFα-induced expression of ICAM-1 and VCAM-1 and adhesion of monocytes to HUVECs. A and B, HUVECs were preincubated with chloride channel blockers DIDS (100 µmol/L), NPPB (100 µmol/L) or tamoxifen (10 µmol/L) and TNFα (10 ng/ml) induced expression of ICAM-1 (A) and VCAM-1 (B) was determined by western blot (**p<0.01 vs control, ##p<0.01 vs TNFα only group, n=6). C, HUVECs were treated with DIDS, NPPB or tamoxifen and then TNFα was added into the cell culture. 24 hours later, the calcein-labeled THP-1 monocytes was coincubated with the HUVECs for 1 hour at 37 °C and the cell adhesion was analyzed with confocal microscope (**p<0.01 vs control, ##p<0.01 vs TNFα only group, n=5).
Figure S10. ClC-3 knockdown or knockout inhibited TNFα-induced ICAM-1 and VCAM-1 expression in endothelial cells. A and B, TNFα (10 ng/ml) induced expression of ICAM-1 (A) and VCAM-1 (B) from HUVECs transfected with negative siRNA (Neg. RNA) or ClC-3 siRNA was determined by qRT-PCR (*p<0.05, **p<0.01 vs Neg. RNA group, n=4). C and D, TNFα (10 ng/ml) induced expression of ICAM-1 (C) and VCAM-1 (D) in MAECs isolated from ClC-3+/+ mice (WT) and ClC-3−/− mice (KO) was analyzed by qRT-PCR (**p<0.01 vs wild type+TNFα, n=4).
Figure S11

Figure S11. Heterogenous expression of ClC-3 in ClC-3<sup>-/-</sup> MAECs restored TNFα-induced expression of ICAM-1 and VCAM-1 and adhesion of monocytes to MAECs. A and B, TNFα (10 ng/ml) induced expression of ICAM-1 (G) and VCAM-1 (H) in ClC-3<sup>-/-</sup> MAECs transfected with ClC-3 cDNA or pcDNA3.0 vector (*p<0.05 vs control, #p<0.05, ##p<0.01 vs TNFα, n=5). C, the adhesion of calcein-labeled mouse monocytes to ClC-3<sup>-/-</sup> MAECs transfected with ClC-3 cDNA or pcDNA3.0 vector (*p<0.05 vs control, #p<0.05, ##p<0.01 vs TNFα, n=5).

Figure S12

Figure S12. ClC-3 knockdown or knockout repressed p65 nuclear translocation in endothelial cells. A, cytoplasmic fractions were isolated from HUVECs transfected with negative siRNA (Neg. siRNA) or ClC-3 siRNA, activation of NF-κB was detected by western blot using antibody against p65 (**p<0.01 vs control, ###p<0.01 vs Neg. siRNA + TNFα, n=5). B, cytoplasmic lysates from ClC-3<sup>+/+</sup> MAECs (WT) and ClC-3<sup>-/-</sup> MAECs (KO) was analyzed by western blot to detect the expression of p65 (**p<0.01 vs control, ###p<0.01 vs Neg. siRNA + TNFα, n=6).
**Figure S13.** CIC-3−/− mice (KO) and their CIC-3+/+ littermates (WT) were intraperitoneally injected with TNFα (30 µg/kg) for 72 hours, the expression of ICAM-1(A) and VCAM-1(B) in the aorta were analyzed by immunohistochemistry.

**Figure S14.** ICAM-1 and VCAM-1 expression were reduced in CIC-3−/− mice response to TNFα in vivo. CIC-3−/− mice (KO) and their CIC-3+/+ littermates (WT) were intraperitoneally injected with TNFα (30 µg/kg) for 72 hours, the expression of ICAM-1 and VCAM-1 in the aorta were analyzed by qRT-PCR (**p<0.01 vs. WT+TNFα, n=5).
**Figure S15.** Histological analysis of the inflammation in the lungs (A) and livers (B) from CIC-3−/− mice (KO) and their CIC-3+/+ littermates (WT) after intraperitoneal injection of TNFα (30 µg/kg) for 72 hours. Representative images from 5 different experiments were shown. C, the myeloperoxidase (MPO) activity was determined in the lung tissues from CIC-3−/− mice (KO) and CIC-3+/+ mice (WT) after intraperitoneal injection of TNFα (30 µg/kg) for 72 hours (*p<0.05 vs control, #p<0.05 vs WT, n=5).