

CFTR ameliorates high glucose-induced oxidative stress and inflammation by mediating the NF- κ B and MAPK signaling pathways in endothelial cells

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Abstract. Diabetic cardiovascular diseases are characterized by progressive hyperglycemia, which results in excessive production of oxidative stress and pro-inflammatory cytokines. Cystic fibrosis (CF) is characterized by chronic inflammation due to mutations in CF transmembrane conductance regulator (CFTR). However, little information is available about the role of CFTR in hyperglycemia-induced endothelial cell oxidative stress and inflammation. In the present study, a high glucose-treatment was applied in human umbilical vein endothelial cells with CFTR overexpression or inhibition, and the oxidative and inflammatory characteristics were measured. It was shown that CFTR protein and mRNA expression were reduced by glucose in a concentration-dependent manner. Overexpression of CFTR via adenoviral infection significantly inhibited the production of reactive oxygen species and inflammatory biomarkers induced by high glucose. Conversely, pharmacological inhibition of CFTR led to the opposite effects. Mechanistically, nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling were activated following high glucose treatment, which were inhibited by CFTR overexpression and enhanced by CFTR inhibition. The pro-inflammatory effect of CFTR inhibition was abolished by pharmacological inhibition of the NF- κ B or MAPK pathways. Moreover, inhibition of MAPK abrogated CFTR inhibition-induced NF- κ B nuclear translocation, whereas NF- κ B inhibitor produced no effects on MAPK activation. Additionally, antioxidant treatment inhibited the high glucose-induced decrease in CFTR expression and the increase in inflammatory responses. Collectively, these findings revealed that CFTR attenuates high glucose-induced endothelial cell oxidative stress and inflammation through inactivation of NF- κ B and MAPK signaling, indicating that elevation of CFTR expression may be a novel strategy in preventing endothelial dysfunction in diabetes.

Introduction

Diabetes is a chronic macrovascular and microvascular disease induced by hyperglycemia, affecting millions of individuals globally (1). Approximately 80% of diabetes-related mortality is caused by cardiovascular diseases, including atherosclerosis, hypertension and cardiomyopathy (2). Moreover, endothelial dysfunction, characterized by inflammation and oxidative stress, is one of the most common causes of morbidity and mortality in diabetes-related cardiovascular diseases (1,3).

It is known that inflammation serves a critical role in the development and progression of diabetes (3,4). An excessive or inappropriate production of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β), could induce the expression of inflammatory mediators [e.g., intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)] as a positive feedback mechanism and subsequently result in endothelial cell activation, which eventually leads to endothelial dysfunction (1,5). In addition, high glucose-induced oxidative stress, primarily associated with the production of reactive oxygen species (ROS), also serves a pivotal role in endothelial dysfunction during the development of diabetes (6). ROS function as signaling molecules to activate a variety of stress pathways and inflammatory pathways, which in turn further enhance inflammatory responses (7,8). Furthermore, inhibition of ROS with antioxidant agents can attenuate inflammation and rescue endothelial cells (9). However, the mechanisms by which high glucose induces inflammation and oxidative stress are not fully understood.

Mounting evidence has suggested that nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling are the classical pathways to regulate ROS production and inflammation (10-12). Activation of these pathways can increase ROS production and accelerate the inflammation progress, and thus cause endothelial dysfunction (13). It is worthy to note that high glucose can induce NF- κ B nuclear translocation, MAPK activation and inflammatory biomarker production in the endothelial cells of diabetic patients (14). Therefore, these signaling pathways and inflammation may be pivotal pathogenic mechanisms underlying endothelial dysfunction caused by hyperglycemia.

Cystic fibrosis (CF) is a common autosomal recessive disorder due to mutations of the CF transmembrane conductance regulator (CFTR) gene, which is located on the long

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arm of chromosome 7 (15). Previous studies have shown massive infiltration of neutrophils and excessive production of pro-inflammatory cytokines in the airways of CF patients (16,17), suggesting that CF may be a hallmark of pulmonary inflammation. CFTR loss or aberration in the lungs leads to bacterial infection in association with inflammation as a consequence of abnormal reabsorption of sodium and water, and impairment of mucociliary clearance (18). Even if multiple studies have suggested that defective or dysfunctional CFTR can also result in bacterial colonization, inflammation usually occurs in the earliest stage of lung damage prior to bacterial infection in CF patients (19,20), indicating the direct role of CFTR in the inflammatory process. Moreover, NF- κ B and MAPK signaling pathways have been suggested to be implicated in the regulation of the inflammatory response of CF airway epithelia (16,19,21). Although these findings have provided evidence that CFTR defects are likely to contribute to inflammation, little attention has been devoted to the investigation of the role of CFTR in hyperglycemia-induced vascular endothelial cell inflammation. The present study aims to establish whether high glucose regulates CFTR expression in endothelial cells and whether such changes are causally associated with high glucose-induced endothelial cell oxidative stress and inflammation.

Materials and methods

Materials and reagents. Endothelial basal medium (EBM), fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, heparin and vascular endothelial growth factor were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mannitol, glucose, PD98059, SP600125, BAY11, apocynin and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). CFTR_{inh}-172 was purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in EBM supplemented with heat-inactivated 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 25 U/ml heparin and 5 ng/ml vascular endothelial growth factor, in a humidified atmosphere of 5% CO₂ at 37°C.

Western blot analysis. Western blot analysis was performed as previously described (19). HUVECs were washed with phosphate-buffered saline (PBS) and harvested in mammalian protein extraction reagent (Thermo Fisher Scientific, Inc.) containing 1 mM protease inhibitor (Roche Diagnostics, Laval, QC, Canada). To detect the expression of the NF- κ B p65 subunit in the nuclei and cytoplasm, nuclear and cytosolic proteins were isolated with a Nuclear/Cytosol Fractionation kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions. The protein content was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 50 μ g protein was separated on 8 or 10% sodium dodecyl sulfate-polyacrylamide gels according to protein molecular weights and was then electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk at room temperature for 1 h and incubated with the following primary antibodies overnight at 4°C: CFTR

(#78335), p65 (#8242) and lamin B (#12586) (diluted 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated Janus kinase (p-JNK; sc-293136), JNK (sc-572), p-extracellular signal-regulated kinase (ERK), ERK (sc-514302) and β -actin (sc-130300) (diluted 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following incubation with horseradish peroxidase-conjugated anti-rabbit (ab6721) or anti-goat (ab6885) secondary antibodies (diluted 1:5,000; Abcam, Cambridge, MA, USA) for 1 h at room temperature, bands were detected with ECLTM western blot detection reagents (GE Healthcare, Chicago, IL, USA) and quantified by Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc.).

Immunofluorescence staining. To detect NF- κ B nuclear translocation in endothelial cells, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and labeled with rabbit-anti-p65 antibodies (diluted 1:100; #8242; Cell Signaling Technology, Inc.) overnight at 4°C. Subsequent to incubation with the primary antibody overnight at 4°C, the cells were then washed three times with PBS for 3 min and incubated with anti-goat FITC antibody for labeling CFTR or with anti-rabbit Cy3 antibody for labeling p65 (diluted 1:200; Beyotime, Jiangsu, China) for 1 h at room temperature. Fluorescence images were acquired using the Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from HUVECs using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. cDNA was synthesized using the ReverTra ACE qPCR RT kit (Toyobo Life Science, Osaka, Japan) in a 20- μ l reaction mixture. qPCR was performed with a CFX 96 Connect real-time PCR detection system (Bio-Rad Laboratories, Inc.) using SYBR-Green PCR master mix reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.). Samples were denatured at 94°C for 4 min, followed by 32 cycles of 95°C for 10 sec and 60°C for 30 sec. The specific primers (Invitrogen; Thermo Fisher Scientific, Inc.) used were as follows: CFTR forward, 5'-AGAAGGCTGGGGCTCATT-3' and reverse, 5'-GGGCCATCCACAGTCTTCT-3'; ICAM-1 forward, 5'-GCA GACAGTGACCATCTACAGCTT-3' and reverse, 5'-CTTCTG AGACCTCTGGCTTCGT-3'; VCAM-1 forward, 5'-GGCAGG CTGTAAGAAGATTGCA-3' and reverse, 5'-GTCATGGTC ACAGAGCCACCTT-3'; E-selectin forward, 5'-AAGCCACAT GTGAAGCTGT-3' and reverse, 5'-CTCCAATAGGGGAAT GAGCA-3'; IL-1 β forward, 5'-CTGAGCTCGCCAGTGAAA TG-3' and reverse, 5'-TGTCATGGCCACAACAAC-3'; and 18S rRNA (reference gene) forward, 5'-CGGCTACCACAT CCAAGGAA-3' and reverse, 5'-CTGGAATTACCGCGGCT-3'. The data were calculated using the 2^{- $\Delta\Delta$ CT} method (20).

ROS measurement. ROS level in the HUVECs was measured using 2',7'-dichlorofluorescein diacetate (H₂DCF-DA; Invitrogen; Thermo Fisher Scientific, Inc.), as previously described (7). Briefly, HUVECs (2x10⁴ cells/well) were seeded in 96-well clear-bottom black plates and then incubated with different concentrations (10, 15, 20 and 25 mM) of glucose for 24 h at 37°C. Following treatment, the cells were stained with H₂DCF-DA (10 μ M) in serum-free EBM for 30 min at 37°C and washed with PBS three times. ROS production was determined by a fluorescence

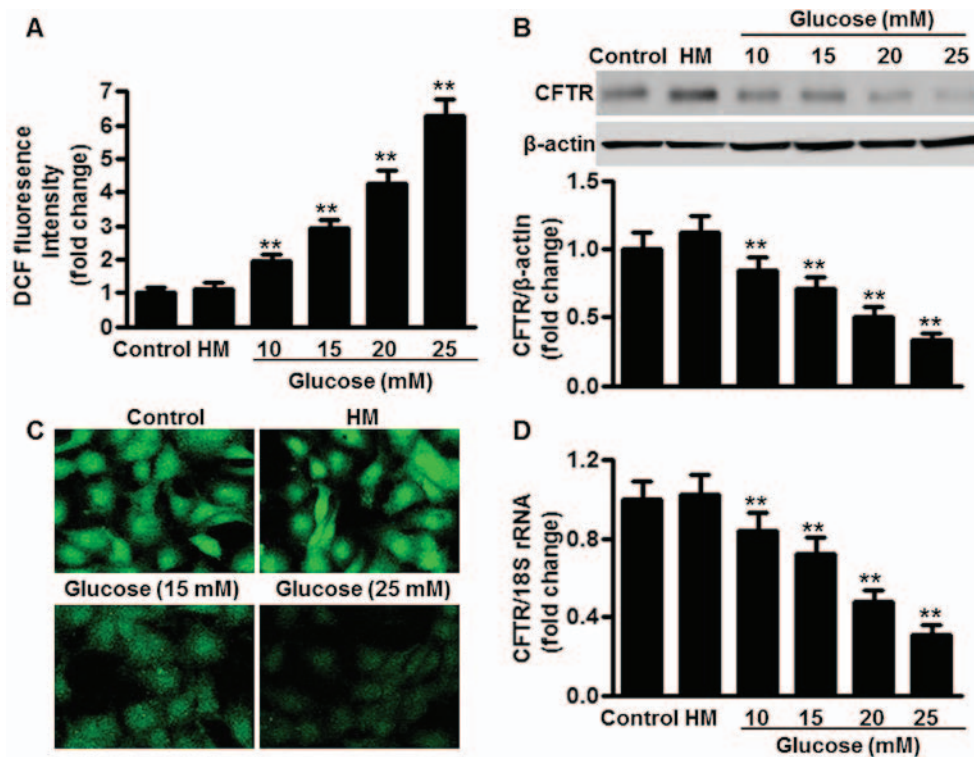


Figure 1. High glucose-induced ROS production is associated with decreased CFTR expression in HUVECs. (A) Cells were incubated with 10, 15, 20 or 25 mM glucose for 24 h. High mannitol (25 mM) served as an osmotic control. ROS production was measured by H₂DCF-DA staining and fluorescence intensity was quantified by a fluorescence microplate reader. (B) Western blot analysis of CFTR protein expression. (C) The expression of CFTR in HUVECs was analyzed by immunofluorescence staining. (D) CFTR mRNA expression was determined by reverse transcription-quantitative polymerase chain reaction analysis. All data are expressed as the mean \pm standard error of the mean. ** $P < 0.01$ vs. control ($n = 6$). ROS, reactive oxygen species; CFTR, cystic fibrosis transmembrane conductance regulator; HUVECs, human umbilical vein endothelial cells; HM, high mannitol; H₂DCF-DA/DCF, 2',7'-dichlorofluorescein diacetate.

microplate reader (Tecan Group, Ltd., Männedorf, Switzerland) at 488 nm excitation and 525 nm emission. The fluorescence intensities were normalized to protein concentrations.

Adenovirus infection. The recombinant adenoviral vector (Ad) encoding human CFTR was constructed by SunBio Biotechnology (Shanghai, China). The recombinant Ad expressing LacZ (Clontech Laboratories, Inc., Mountainview, CA, USA) was used as a control. When cells reached 60-70% confluence, the medium was removed and replaced with serum-free medium containing 50 multiplicity of infection LacZ or Ad-CFTR. HUVECs were infected with LacZ or Ad-CFTR for 24 h prior to high-glucose treatment.

Pharmacological treatment. HUVECs were pretreated with Ad-CFTR or CFTR_{inh}-172 (10 μ M) for 24 h, followed by BAY11 (20 μ M), PD98059 (10 μ M), SP600125 (10 μ M), apocynin (1 μ M) or NAC (5 μ M) treatment for another 24 h in the presence of high glucose (25 mM).

Enzyme-linked immunosorbent assay (ELISA). The concentration of ICAM-1 (EK0370), VCAM-1 (EK0537), E-selectin (EK0501) and IL-1 β (EK0392) was measured in the supernatants of HUVECs using an ELISA kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China). All measurements were performed as recommended by the manufacturer's protocols.

Statistical analysis. All data are presented as the mean \pm standard error of the mean. N represents the number of independent

experiments on different batches of cells. The statistical significance between samples was evaluated by the unpaired two-tailed Student's t-test or by one-way analysis of variance using SPSS 16.0 system (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

High glucose-induced endothelial cell ROS production is associated with decreased CFTR expression. High glucose has been documented to increase endothelial cell oxidative stress (22,23). To confirm these results of previous studies, in the present study, HUVECs were incubated with different concentrations of glucose (10, 15, 20 and 25 mM) for 24 h. As depicted in Fig. 1A, increasing concentrations of glucose enhanced ROS production in the HUVECs. Glucose concentrations of 15 and 20 mM induced ROS production by 3.1- and 4.4-fold, respectively, compared with the control group (5.5 mM glucose). A glucose concentration of 25 mM further stimulated ROS production by 6.3-fold. High mannitol (25 mM), which served as an osmotic control, produced no significant effects on ROS production. Notably, it was found that the glucose-induced ROS production was associated with the inhibition of the expression of CFTR. Glucose at 11 mM decreased CFTR protein expression in the HUVECs to $70.2 \pm 8.3\%$ relative to the control, while at 20 mM glucose, CFTR protein expression was $51.6 \pm 6.8\%$ of the control, and at 25 mM, glucose decreased the CFTR protein expression to $34.0 \pm 4.3\%$ of the control (Fig. 1B). Similar results were obtained in CFTR immunofluorescence staining of HUVECs treated with or without

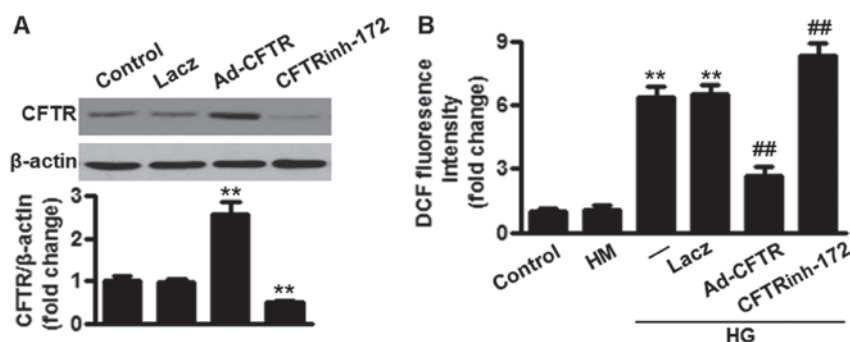


Figure 2. Overexpression of CFTR inhibits high glucose-induced ROS production in HUVECs. (A) Cells were treated with LacZ (50 MOI), Ad-CFTR (50 MOI) or CFTR_{inh}-172 (10 μ M) for 24 h. The infection efficiency and inhibition efficiency were assessed by western blotting and data are shown as the ratio of CFTR/ β -actin. ** P <0.01 vs. control (n =4). (B) Following treatment, HUVECs were incubated with high glucose (HG, 25 mM) for another 24 h and then ROS production was measured. ** P <0.01 vs. control; ## P <0.01 vs. high glucose alone (n =6). ROS, reactive oxygen species; CFTR, cystic fibrosis transmembrane conductance regulator; HUVECs, human umbilical vein endothelial cells; HM, high mannitol; H₂DCF-DA/DCF, 2',7'-dichlorofluorescein diacetate; MOI, multiplicity of infection; Ad, adenoviral vector; HG, high glucose.

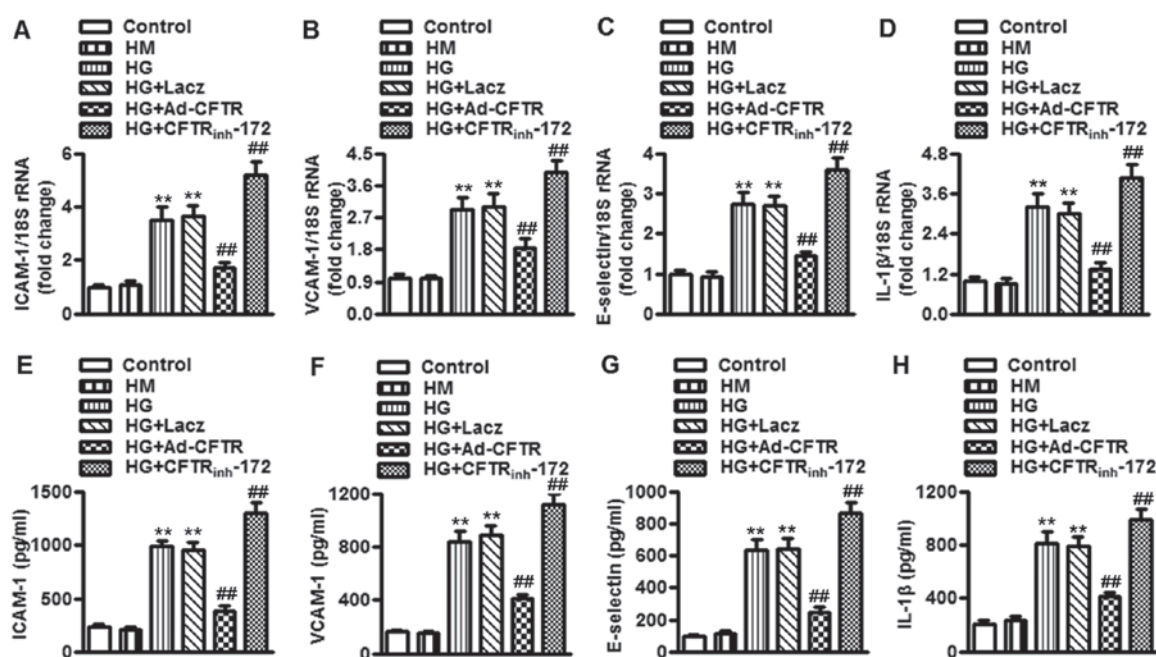


Figure 3. CFTR inhibition enhances high glucose-induced secretion of inflammatory biomediators in endothelial cells. (A-D) HUVECs were pretreated with LacZ (50 MOI), Ad-CFTR (50 MOI) or CFTR_{inh}-172 (10 μ M) for 24 h in prior to incubation of high glucose (25 mM) for another 24 h. The mRNA expression of (A) ICAM-1, (B) VCAM-1, (C) E-selectin and (D) IL-1 β was determined by reverse transcription-quantitative polymerase chain reaction analysis. (E) The concentration of (E) ICAM-1, (F) VCAM-1, (G) E-selectin and (H) IL-1 β in HUVECs were measured by enzyme-linked immunosorbent assay. All data are expressed as the mean \pm standard error of the mean. ** P <0.01 vs. control; ## P <0.01 vs. high glucose alone (n =6). CFTR, cystic fibrosis transmembrane conductance regulator; HUVECs, human umbilical vein endothelial cells; HM, high mannitol; H₂DCF-DA/DCF, 2',7'-dichlorofluorescein diacetate; MOI, multiplicity of infection; Ad, adenoviral vector; HG, high glucose; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; IL, interleukin.

high glucose (Fig. 1C). Furthermore, RT-qPCR results showed that CFTR mRNA expression was also decreased following glucose challenge, in a similar concentration-dependent manner (Fig. 1D).

CFTR attenuates ROS production in endothelial cells under high glucose conditions. To determine the role of CFTR in ROS production in endothelial cells, HUVECs were infected with adenoviral vector encoding human CFTR (Ad-CFTR) for 24 h. Western blotting showed that CFTR protein expression was significantly increased following overexpression of CFTR compared with that in the control group. LacZ exhibited no effect on CFTR expression. By contrast, CFTR protein expression was markedly inhibited following treatment with CFTR inhibitor,

CFTR_{inh}-172 (Fig. 2A). As shown in Fig. 2B, a 25 mM glucose concentration (high glucose) was used to induce endothelial cell ROS production. The H₂DCF-DA fluorescence intensity of LacZ-infected cells was ~6.5-fold higher than that of the control group. When cells were infected with Ad-CFTR, the ability of high glucose to induce ROS production was markedly inhibited. However, CFTR inhibition markedly enhanced high glucose-induced ROS production in the HUVECs. In addition, neither overexpression nor inhibition of CFTR affected ROS production under the basal level (data not shown).

Inhibition of CFTR promotes a high glucose-induced inflammatory response in endothelial cells. In addition to oxidative stress,

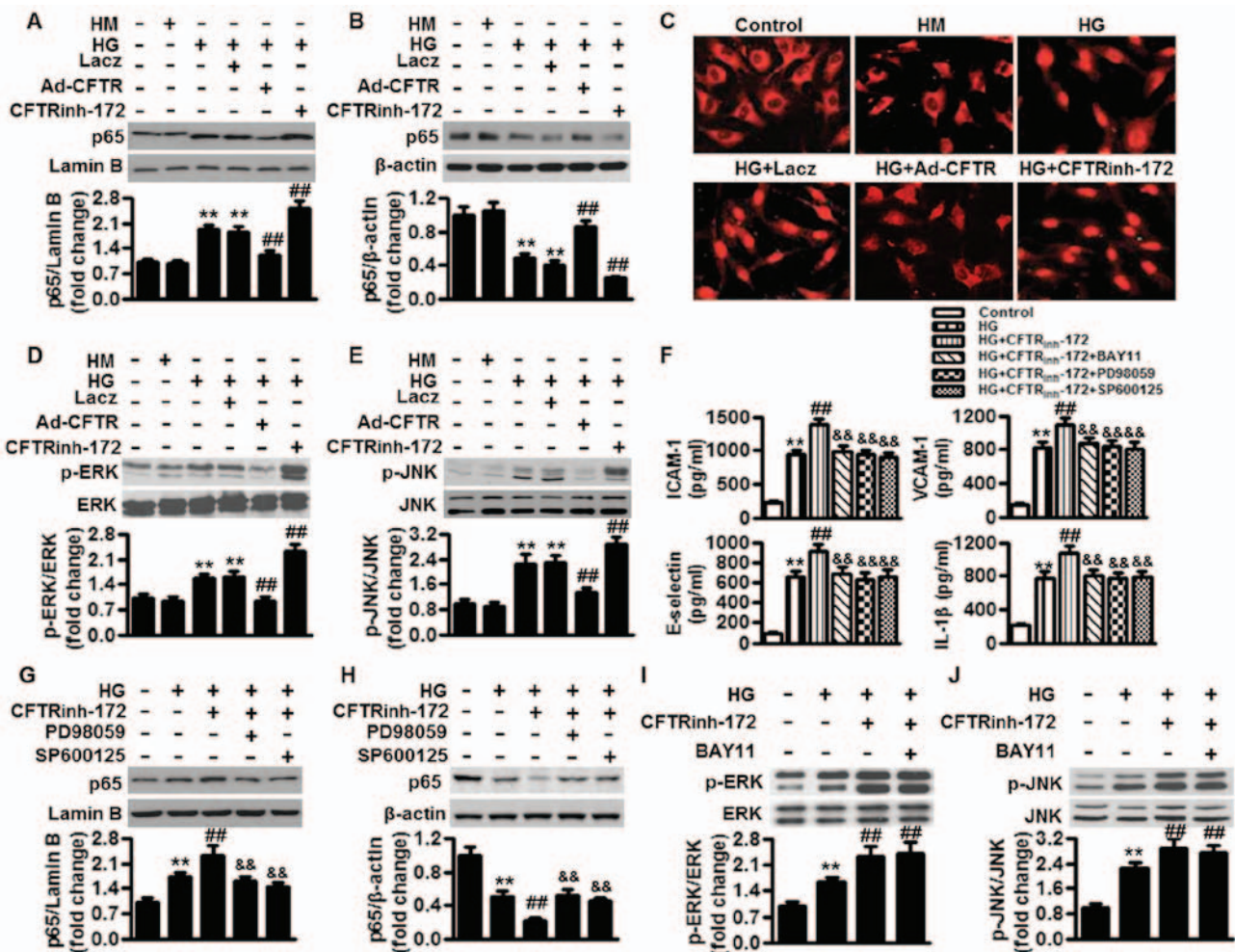


Figure 4. CFTR attenuates inflammatory response under high glucose conditions through the inhibition of NF-κB and MAPK signaling. (A and B) HUVECs were pretreated with LacZ (50 MOI), Ad-CFTR (50 MOI) or CFTR_{inh}-172 (10 μM) for 24 h, followed by high glucose (25 mM) treatment for another 24 h. (A) Nuclear and (B) cytosolic proteins were isolated and detected by western blotting using p65 antibody. **P<0.01 vs. control; ##P<0.01 vs. high glucose alone (n=6). (C) Representative images of p65 distribution in HUVECs. (D) ERK and (E) JNK phosphorylation was determined by western blotting. **P<0.01 vs. control; ##P<0.01 vs. high glucose alone (n=6). (F) Cells were pretreated with CFTR_{inh}-172 (10 μM) for 24 h, followed by incubation with BAY11 (20 μM), PD98059 (10 μM) or SP600125 (10 μM) for another 24 h under high glucose conditions. The concentrations of ICAM-1, VCAM-1, E-selectin and IL-1β were measured by enzyme-linked immunosorbent assay. (G-J) p65 expression in (G) nuclear and (H) cytosolic fractions, and (I) ERK and (J) JNK phosphorylation were assessed by western blotting. **P<0.01 vs. control; ##P<0.01 vs. high glucose alone; &&P<0.01 vs. high glucose + CFTR_{inh}-172 (n=6). CFTR, cystic fibrosis transmembrane conductance regulator; HUVECs, human umbilical vein endothelial cells; HM, high mannitol; MOI, multiplicity of infection; Ad, adenoviral vector; HG, high glucose; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Janus kinase; p-, phosphorylated.

diabetes is also associated with vascular inflammation (22,23). Therefore, the present study next determined the role of CFTR in modulating endothelial cell inflammation. The mRNA expression of the inflammatory mediators ICAM-1 and VCAM-1, endothelial cell activation marker E-selectin, and pro-inflammatory cytokine IL-1β were examined by RT-qPCR. In HUVECs, the mRNA expression of ICAM-1, VCAM-1, E-selectin and IL-1β was significantly increased upon high glucose treatment, and was markedly attenuated following overexpression of CFTR. However, CFTR-specific inhibitor further increased the mRNA expression of ICAM-1, VCAM-1, E-selectin and IL-1β under high glucose condition (Fig. 3A-D). Consistent with these results, overexpression of CFTR inhibited, whereas inhibition of CFTR enhanced, the concentration of these inflammatory biomarkers in HUVECs, as determined by ELISA assay (Fig. 3E-H). Similar to the effect of CFTR on ROS production, overexpression or inhibition of CFTR also did not alter the expression and concentration of ICAM-1, VCAM-1, E-selectin and IL-1β

mRNA under the basal level (data not shown). These data indicate that overexpression of CFTR will be effective in reducing high glucose-induced inflammation in endothelial cells.

Role of NF-κB and MAPK signaling in CFTR-attenuated inflammation induced by high glucose in endothelial cells. Since NF-κB and MAPK signaling pathways serve important roles in the regulation of inflammation (11,12), the present study proceeded to investigate whether CFTR regulates high glucose-induced inflammation via NF-κB and/or MAPK signaling. Western blotting results showed that the translocation of p65 from the cytoplasm to nucleus was increased following high glucose treatment for 24 h, indicating the activation of NF-κB. Following CFTR overexpression, the ability of high glucose to induce p65 nuclear translocation was almost abolished. However, inhibition of CFTR further enhanced NF-κB nuclear accumulation in the presence of high glucose (Fig. 4A and B). These results were further confirmed

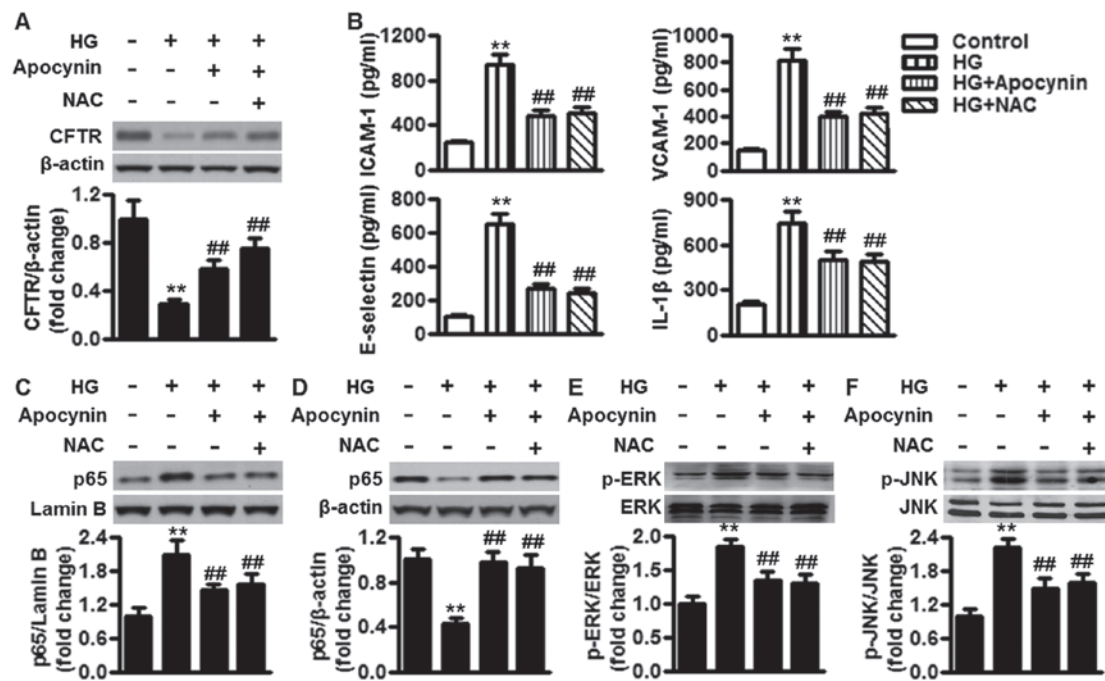


Figure 5. Inhibition of oxidative stress ameliorates high glucose-induced inflammation by reducing CFTR expression. (A) HUVECs were treated with apocynin (1 μ M) or NAC (5 μ M) in the presence of high glucose (25 mM) for 24 h. CFTR protein expression was examined by western blotting. (B) The concentrations of ICAM-1, VCAM-1, E-selectin and IL-1 β were measured by enzyme-linked immunosorbent assay. (C and D) p65 nuclear translocation and (E) ERK and (F) JNK phosphorylation were determined by western blotting. All data are expressed as the mean \pm standard error of the mean. * P <0.01 vs. control; ** P <0.01 vs. high glucose alone (n=6). CFTR, cystic fibrosis transmembrane conductance regulator; HUVECs, human umbilical vein endothelial cells; HG, high glucose; NAC, N-acetyl-L-cysteine; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; IL, interleukin; ERK, extracellular signal-regulated kinase; JNK, Janus kinase; p-, phosphorylated.

by immunofluorescence staining using anti-p65 antibody (Fig. 4C). In addition, high glucose also activated MAPK signaling, as evidenced by significant phosphorylation of ERK and JNK. In Ad-CFTR-infected cells, ERK and JNK signaling in HUVECs was no longer activated by high glucose, whereas the phosphorylation of ERK and JNK was further enhanced by the addition of CFTR_{inh}-172 (Fig. 4D and E). To further confirm whether NF- κ B and/or MAPK serve a key role in CFTR-mediated inflammation under high glucose conditions, pharmacological inhibitors of NF- κ B, ERK and JNK were employed, and then their effects on the secretion of inflammatory biomediators were measured. The enhanced effects of CFTR inhibition on inflammatory biomediator secretion were completely inhibited by NF- κ B inhibitor (BAY11), ERK inhibitor (PD98059) or JNK inhibitor (SP600125) alone (Fig. 4F), suggesting that CFTR ameliorates the high glucose-induced inflammatory response through inhibition of NF- κ B and MAPK signaling. Moreover, western blotting results revealed that PD98059 and SP600125 blocked CFTR inhibition-induced NF- κ B activation under high glucose conditions, as shown by significantly inhibiting p65 nuclear translocation (Fig. 4G and H). Nevertheless, pharmacological inhibition of NF- κ B with its selective inhibitor, BAY11, did not alter the phosphorylation of ERK and JNK (Fig. 4I and J). These data indicate that MAPK may be upstream of NF- κ B in CFTR-mediated inflammation under high glucose conditions.

Inhibition of oxidative stress mitigates high glucose-induced inflammation by reducing CFTR expression. Previous studies have demonstrated the role of high glucose-induced oxidative

stress in inflammation (3,6). To investigate the role of oxidative stress on high glucose-mediated CFTR expression, in the present study, HUVECs were treated with the antioxidants apocynin (1 μ M) and NAC (5 μ M). Addition of the each antioxidant significantly inhibited high glucose-induced downregulation of CFTR expression, respectively (Fig. 5A). Next, the effects of ROS inhibition on the secretion of inflammatory biomediators were observed. HUVECs treated with apocynin or NAC following high glucose treatment showed a marked reduction in the concentration of inflammatory biomediators, including ICAM-1, VCAM-1, E-selectin and IL-1 β , in comparison to cells treated with high glucose (Fig. 5B). Concomitantly, western blotting results showed that following treatment of high glucose-challenged HUVECs with apocynin or NAC, p65 translocation and ERK and JNK phosphorylation were significantly inhibited as compared with that of the high glucose-treated group without antioxidant treatment (Fig. 5C-F). These results suggested that the anti-inflammatory effect of oxidative stress inhibition was mediated, at least partially, by restoration of CFTR expression.

Discussion

To the best of our knowledge, the current study is the first to present the following novel findings: i) CFTR expression is inhibited by glucose in a concentration-dependent manner and is negatively associated with ROS production in endothelial cells; ii) the downregulation of CFTR induced by high glucose leads to activation of NF- κ B and MAPK signaling pathways, and excessive production of inflammatory biomediators, leading

to endothelial cell inflammation; and iii) inhibition of oxidative stress ameliorates high glucose-induced endothelial cell inflammation, at least partially by elevating CFTR expression.

Endothelial dysfunction in diabetes is characterized by complex changes in the biochemical, mechanical and structural properties of the endothelium, which may be responsible for the development of various cardiovascular diseases (24,25). However, the precise mechanism of endothelial dysfunction induced by hyperglycemia has not been fully investigated. In the present experiment, *in vitro*, it was found that CFTR expression in endothelial cells was inhibited following high glucose challenge, indicating that the changes in CFTR expression may serve as an important factor or mediator of endothelial dysfunction in diabetes. The mechanisms by which high glucose regulates CFTR expression in endothelial cells requires further study.

Multiple hypotheses have been proposed to explain the mechanisms of CF, among which oxidative stress and inflammation are considered to be closely associated with CFTR mutations, as chronic inflammation with excessive production of inflammatory biomediators can be observed in the airways of CF patients (16,17). Moreover, mutation of CFTR or loss of function of CFTR has also been shown to directly affect the intracellular redox status in CF lungs (26,27). Additionally, in CF mouse intestines or CFTR-knockdown intestinal epithelial cells, there is an upregulation of genes involved in oxidative stress and inflammation (19,28). These findings collectively suggest that oxidative stress and inflammation are implicated in the pathophysiology of several disorders in CF subjects. Notably, oxidative stress and inflammation are central in diabetes complications, and vascular endothelial cells are critical in orchestrating these effects (22,23). However, the contributions of CFTR have been poorly investigated in endothelial cells under high glucose conditions. The present study found that high glucose could increase ROS production and induce the secretion of inflammatory biomediators in endothelial cells. Pharmacological inhibition of CFTR markedly augmented the effects of high glucose treatment, while overexpression of CFTR markedly attenuated the high glucose-induced responses. These results clearly suggest that high glucose-induced CFTR downregulation is responsible for high glucose-induced oxidative stress and inflammation.

To further investigate the mechanism of CFTR with regard to protecting endothelial function, the involvement of NF- κ B signaling in damage responses triggered by high glucose in endothelial cells was first investigated, as various studies have indicated the intrinsic activation of this signaling in CF (19,29). Moreover, NF- κ B has been found to be an essential regulator of various genes, including inflammatory biomediators ICAM-1, VCAM-1, E-selectin and IL-1 β (19,30). Once activated, the p65 subunit of NF- κ B is released and translocates into the nucleus to regulate the target genes (31). In the present study, it was found that forced CFTR expression blocked high glucose-induced p65 nuclear translocation in endothelial cells, whereas CFTR inhibition further enhanced the translocation. In addition, the increase in pro-inflammatory cytokines and oxidative stress under diabetic conditions can also activate MAPK signaling (32). Importantly, mutations of CFTR have been suggested to abnormally activate MAPK (16,33). The present data showed that overexpression of CFTR was decreased, whereas inhibition of CFTR augmented the phosphorylation of ERK and JNK under

high glucose conditions. It is noteworthy that the pro-inflammatory effect of CFTR inhibition was almost abolished by NF- κ B, ERK and JNK inhibitors, further supporting the fact that CFTR alleviates high glucose-induced inflammation via inhibition of the NF- κ B and MAPK signaling pathways. It has also been reported that inhibition of the MAPK signaling pathway can block NF- κ B nuclear translocation, and thus attenuate inflammatory response (29). In line with this, pharmacological inhibition of ERK or JNK in the present study markedly blocked CFTR inhibition-induced NF- κ B nuclear translocation under high glucose conditions. However, NF- κ B inhibition failed to inhibit MAPK activation, suggesting NF- κ B is downstream of MAPK in CFTR-mediated inflammatory responses.

Furthermore, to delineate the mechanism behind high glucose-induced inflammation, given the reported involvement of oxidative stress in regulating inflammation (7,8), the role of ROS was examined. The present study showed that high glucose decreased CFTR expression, which exhibited a reciprocal pattern of concentration-dependent ROS production. More importantly, apocynin and NAC, known antioxidants, not only restored the high glucose-induced decrease in CFTR expression, but also attenuated the pro-inflammatory effect of high glucose via inactivation of the NF- κ B and MAPK signaling pathways. These data indicate that the anti-inflammatory effect of antioxidants may be due to their effect on the upregulation of CFTR expression.

In conclusion, the present findings revealed a novel function of CFTR as a regulator of endothelial cell oxidative stress and inflammation, suggesting that CFTR overexpression may be a feasible strategy for alleviating vascular endothelial inflammation and diabetic cardiovascular diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YF supervised the study. LS and CY participated in study design and scientific discussion of the data. YF, MJ and QL contributed to the scientific discussion of the data. YF and YX contributed to the biochemical analysis of the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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