Original Article

Lowering intracellular chloride concentration promotes endothelial cells apoptosis by increasing ROS generation derived from mitochondria

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Received September 1, 2015; Accepted October 19, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Accumulating evidences suggest that intracellular chloride concentration ([Cl–]) is involved in several pathological process of cardiovascular disease. However, the role of [Cl–] in endothelial cells apoptosis remains unknown. Therefore, this study aims to test the function of [Cl–] in human umbilical vein endothelial cells (HUVECs) apoptosis induced by lipopolysaccharide (LPS). Cell viability and apoptosis were assessed by CCK-8 assay and flow cytometry. Western blot was used to detect the mitochondria-dependent pathway. Mitochondrial membrane potential (MMP) was measured by a membrane-permeant dye JC-1. Moreover, reactive oxygen species (ROS) generation was determined by 2′,7′-dichlorofluorescin diacetate (H2DCF-DA). Our results showed that LPS significantly induced a decrease of cell viability and [Cl–] both dose-dependently and time-dependently in HUVECs. Compared with control, treatment with LPS induced cell injury and apoptosis, concomitantly with declined Bcl-2/Bax ratio, depressed MMP, increased cytochrome c release and cleaved caspase-9 and -3 protein expressions, which were all significantly potentiated in HUVECs cultured in low Cl– medium. However, lowering [Cl–]-induced reactive oxygen species (ROS), cell injury and apoptosis were inhibited by mitochondrial electron transport complex inhibitor rotenone. In conclusion, decrease of [Cl–] promotes LPS-induced HUVECs apoptosis via increasing ROS generation derived from mitochondria, suggesting modulation of [Cl–] is a novel approach to prevent endothelial cells apoptosis.

Keywords: Cl–, apoptosis, endothelial cells, mitochondria, ROS

Introduction

Vascular endothelial cells are the first cells in the body to expose to blood, sensing directly the changes of the components in the blood [1]. Due to its anatomical localization lining the smooth muscle cells, endothelial dysfunction has been implicated in the development of a variety of vascular diseases, such as atherosclerosis, thrombosis and hypertension [2]. Many studies have demonstrated that vascular endothelial cells apoptosis was closely associated with endothelial dysfunction and cardiovascular diseases. For instance, endothelial cells apoptosis alters the structure and function of the vascular, and eventually results in hypertension and vascular remodeling [2, 3]. Moreover, during apoptosis, the gap between the endothelial cells will be widened and macrophage will be aggregated and foamed inside the vascular walls, leading to hypertension and atherosclerosis [4]. Thus, how to protect endothelial cells injury is an effective method to prevent cardiovascular diseases.

Oxidative stress has been demonstrated to be capable of causing a number of disorders through injuring the endothelia [5, 6]. In biological systems, oxidative stress is induced by reactive oxygen species (ROS), which has been implicated as potential modulator of apoptosis [7]. Excessive ROS generation in the vasculature can lead to endothelial apoptosis and dysfunction, reduction of nitric oxide (NO) production and increase of active substances, resulting in the development of several cardiovascular disorders such as hypertension, atherosclerosis, diabetes and heart failure [8-10]. Therefore, protection of the endothelia against the damage caused by the oxidative stress has
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become a general and effective way to fight the vascular diseases.

Chloride (Cl-) movement plays a critical role in the regulation of diverse physiological functions [11]. Previous study has been shown that the intracellular Cl- concentration ([Cl-]) is decreased in macrophages after ox-LDL treatment, indicating [Cl-] is involved in the foam cell formation [12]. Additionally, lowering [Cl-] results in NF-κB pathway activation and promotes inflammation in endothelial cells [13]. Some physiological and genetic studies have suggested that Cl- movement is strictly regulated by several Cl- channel genes, including CFTR, ClC-2, ClC-3, CLCA, Bestrophin and Ano1 [14]. Recent study demonstrated that Bestrophin-3 attenuated TNF-α-induced endothelial inflammation through inhibition of NF-κB activation [15]. Importantly, blockade of ClC-3 protects endothelial progenitor cells against oxidative damage and thus prevents apoptosis induced by angiotensin II, suggesting that there may be a close relationship between [Cl-] and apoptosis [16]. However, little is known whether [Cl-] is directly involved in the process of endothelial apoptosis.

Therefore, in this study, we prepared the low Cl- culture medium to decrease intracellular chloride content and determined whether Cl- plays a functional role in apoptosis in human umbilical vein endothelial cells (HUVECs), and to further explore the precise mechanisms.

Materials and methods

Cells and reagents

M199 cell culture medium, fetal bovine serum (FBS), endothelial growth factors (EGF), penicillin and streptomycin were purchased from Invitrogen (CA, USA). Rotenone and protease and phosphatase inhibitor cocktail were from Calbiochem (Darmstadt, Germany). RIPA lysis buffer, secondary HRP-conjugated antibodies, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-benzamidazolcarbocyanin iodide (JC-1) dye, 2',7'-dichlorofluorescin diacetate (H2DCF-DA), FITC-Annexin V Apoptosis Detection Kit and ECL Kit were from Beyotime (Jiangsu, China). Antibodies against Bcl-2, Bax, cytochrome c, caspase-3, caspase-9 and PARP were purchased from Cell Signaling Technology (MA, USA). Antibodies against COX IV and β-actin were obtained from Santa Cruz Biotechnology (CA, USA). All other reagents utilized were obtained from Sigma Chemical Co. (MO, USA) unless otherwise specified.

Human umbilical vein endothelial cells (HUVECs) culture

Primary HUVECs (Allcells, Shanghai, China) were cultured in M199 supplemented with 20% FBS, 100 μg/mL EGF, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37°C with 5% CO2.

Cell viability assay

Cell viability was determined by cell counting kit-8 (CCK-8; Dojindo Laboratories, MD, Japan) according to the manufacturer's protocols. HUVECs were seeded in 96-well plates at a density of 5×10^3 cells/well for 24 h. After treatment with LPS in the presence or absence of low chloride medium for another 24 h, 10 μl of CCK8 was added to each well for additional 3 h at 37°C. The absorbance of each well was measured with a SPECTRA MAX190 spectrophotometry (Sunnyvale, CA, USA) at 450 nm wavelength.

Measurement of intracellular chloride concentration ([Cl-])

[Cl-] was measured as previously described [13]. 6-methoxy-N-ethyl-1,2-dihydroquinoline (dihydro-MEQ) is the precursors of MEQ. HUVECs were incubated with dihydro-MEQ (100 μmol/L) in a Ringer’s buffer solution containing (in mmol/L: 119 NaCl, 2.5 KCl, 1.0 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 26 NaHCO3, 11 glucose, pH 7.4) at room temperature in the dark for 30 min. Then dihydro-MEQ is oxidized to MEQ, and the fluorescence of MEQ is quenched by Cl-, which was monitored by MetaFluor Imaging software (Universal Imaging Systems, Chester, PA) with 350-nm excitation and 435-nm emission wavelength. Chloride concentration is calculated by the Stern-Volmer equation: (F_o/F)=1=Ksv [Q]. F_o is the fluorescence intensity without quencher; F is the fluorescence intensity in the presence of quencher; [Q] is the concentration of quencher; and Ksv is the Stern-Volmer constant.

Low chloride medium preparation

Low chloride medium was prepared as previously described [13]. Briefly, M199 medium
lacking NaCl and KCl was initially purchased from Invitrogen. Actually, the normal chloride medium was prepared by adding 5 mmol/L KCl and 105 mmol/L KCl, while the low chloride medium was prepared by adding 5 mmol/L potassium gluconate and 105 mmol/L sodium gluconate (pH=7.2). The osmolarities of the solutions measured by a freezing point depression osmometer (OSMOMAT030, Germany) were ranged from 303.4 to 310.2 mosmol/kgH2O.

**Apoptosis quantification by flow cytometry**

The HUVECs apoptosis detection was measured with the FITC-Annexin V Apoptosis Detection Kit using a flow cytometry. In brief, cell at a density of 1×10^6 cells/mL were trypsinized, harvested by centrifugation, washed with PBS twice, and then the cell pallets were re-suspended and incubated with Annexin V and propidium iodide (PI) at room temperature in dark for 20 min. The stained cells were analyzed by a flow cytometry (BD Bioscience).

**Preparation of mitochondrial fractions**

Mitochondrial and cytoplasmic proteins were isolated using the Mitochondria Isolation Kit (Thermo Fisher Scientific Inc., IL, USA) according to the manufacturer’s protocol. These fractions were analyzed by western blot analysis. COX IV was used as a loading control for the mitochondria fraction.

**Western blot analysis**

After treatment, HUVECs were lysed in RIPA lysis buffer containing protease and phosphatase inhibitor cocktail. Protein concentrations were quantified with the Bio-Rad Protein Assay (Bio-Rad, MA, USA), separated by 8%-12% SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA), blocked in 5% non-fat milk in TBST for 1 h at room temperature, and then incubated with appropriate primary antibodies in this buffer overnight. After incubation with the appropriate secondary HRP-conjugated antibodies for 1 hour, the signals were detected by an ECL Kit and the intensity of the protein bands was analyzed by ImageJ software (ImageJ, Version 1.41, NIH, Maryland, USA).

**Mitochondrial membrane potential (MMP) detection**

JC-1 is a sensitive dye to the loss of MMP. The JC-1 exhibits potential-dependent accumulation in mitochondria emitting red fluorescence (excitation, 550 nm; emission, 600 nm) in viable cells, JC-1 appears in cytoplasm as a monomer form emitting green fluorescence (excitation, 485 nm; emission, 535 nm) in apoptotic cells. Consequently, the green/red fluorescence intensity ratio will increase in apoptotic cells and can be used to indicate the change of MMP. In this study, HUVECs were incubated with JC-1 at 37°C for 10 min in dark. After washing with phosphate buffer saline (PBS) twice, the images were captured by a fluorescence microscope (Axiovert 200, Zeiss, Oberkochen, Germany) and the ratio of green/red fluorescence intensity was analyzed by ImageJ software.

**Reactive oxygen species (ROS) detection**

ROS in HUVECs was visualized by 2',7'-dichlorofluorescin diacetate (H2DCF-DA) as previously described [16]. HUVECs were seeded in 6-well plates, and then applied to different treatments. After treatment, cells were incubated with H2DCF-DA (10 μmol/L) in serum-free medium for 30 min at 37°C. Then cells were washed twice with PBS and images were captured with a fluorescence microscope (Axiovert 200, Zeiss) at 488-nm excitation and 525-nm emission wavelengths. To quantify the fluorescence intensity of H2DCF-DA, stained cells was quantified by ImageJ software.

**Statistical analysis**

Data were presented as mean ± SEM. Comparisons were analyzed by an unpaired two-tailed Student’s test or one-way ANOVA followed by the Bonferroni multiple comparison post hoc test using SPSS 17.0 statistical software (SPSS Inc., IL, USA). Values of P<0.05 was considered statistically significant.

**Results**

**Changes of [Cl-], parallels with the rate of cell viability in the presence of LPS**

We firstly investigated whether [Cl-], correlates with the rate of cell viability after LPS treat-
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Following treatment with different concentrations of LPS (0.01, 0.1, 0.5, 1 and 5 μg/mL) for 24 h, cell viability and [Cl⁻] were measured, respectively. As shown in Figure 1A, cell viability was gradually decreased by an increase in LPS concentration from 0.01 to 5 μg/mL. The degree of cell injury reached the maximum at 1 μg/mL. Interestingly, we found that [Cl⁻] was also decreased by LPS treatment dose-dependently, which was paralleled to LPS-decreased cell viability (Figure 1B). Compared to control group, 1 μg/mL LPS decreased [Cl⁻] from 41.03±3.66 mmol/L to 25.33±2.15 mmol/L. On the other hand, we measured the time-dependent losses in cell viability. CCK-8 assay showed that cell viability was reduced to approximately 40% after LPS (1 μg/mL) treatment for 24 h (Figure 1C). Similarly, [Cl⁻] was also decreased by LPS in a time-dependent manner (Figure 1D). Based on these results, we used a 24 h exposure of 1 μg/mL LPS in the following experiments.

Lowering [Cl⁻] enhances LPS-induced apoptosis in HUVECs

To investigate whether the reduced [Cl⁻] participates endothelial cells apoptosis, we prepared the low Cl⁻ medium to decrease [Cl⁻] and observed its effects on cell viability and apoptosis in the presence or absence of LPS. As expected, [Cl⁻] was dramatically decreased when the normal culture medium was replaced by low Cl⁻ solution (Figure 2A). Moreover, lowering [Cl⁻] decreased cell viability at basal level and further enhanced LPS-induced the decrease in cell viability in HUVECs (Figure 2B). To test whether the decreased cell viability in low Cl⁻ solution results from HUVECs apoptosis, cell apoptosis was analyzed by Annexin V-FITC/PI flow cytometry. Cell apoptotic rate was signifi-
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Significantly increased in low Cl⁻ solution. Incubation with 1 μg/mL LPS for 24 h induced an apoptotic rate of 29.8±2.6%, which was further enhanced in low Cl⁻ solution (Figure 2C and 2D). These data suggest the increased apoptosis may underlie the cell injury in low Cl⁻ solution.

Low Cl⁻ solution potentiates apoptosis through activation of intrinsic pathway

To elucidate the mechanism of low Cl⁻ solution-induced apoptosis, we first examined the protein expression of Bcl-2 and Bax. LPS treatment remarkably decreased Bcl-2 expression and increased Bax expression, resulting in a decrease in Bcl-2/Bax ratio (Figure 3A). Moreover, the Bcl-2/Bax ratio in HUVECs treated with or without LPS were both significantly decreased in low Cl⁻ solution, resulting from a decrease in Bcl-2 expression but an increase in Bax expression (Figure 3B). To investigate whether reducing [Cl⁻]i promotes HUVECs apoptosis through the intrinsic (mitochondria) pathway, we examined the effects of low Cl⁻ solution on mitochondrial membrane potential (MMP) and cytochrome c release. Fluorescence microscope revealed that LPS treatment dramatically decreased red fluorescence and increased green fluorescence of JC-1 dye, which resulted in a decline in red/green fluorescence intensity ratio. Lowering [Cl⁻]i not only induced loss of MMP

Figure 2. Lowering [Cl⁻]i exacerbated LPS-induced apoptosis in HUVECs. (A) Cells were incubated in normal medium or low Cl⁻ solution for 24 h. [Cl⁻]i was examined using MEQ fluorescence probe. (B) HUVECs were cultured in low Cl⁻ solution with or without LPS (1 μg/mL) treatment for 24 h. Cell viability was evaluated by CCK-8 kit. (C) After treatment mentioned in (B), Cell apoptosis was determined by Annexin V/PI staining followed by flow cytometry. (D) Quantitative analysis of the percentage of apoptotic cells. **P<0.01 vs. control, ##P<0.01 vs. LPS alone, n=6.
Figure 3. Lowering [Cl–] induced mitochondrial dysfunction. HUVECs were treated with low Cl– solution in the presence or absence of LPS (1 μg/mL) treatment for 24 h. A. Bcl-2 and Bax expression were detected by western blot. Representative western blot images are shown. B. Densitometric analysis of Bcl-2, Bax and Bcl-2/Bax ratio. C. Mitochondrial membrane potential (MMP) was measured using JC-1 staining. Representative merged images of JC-1 derived fluorescence in HUVECs. D. Quantitative analysis of the ratio of red/green fluorescence. E. Cytochrome c protein expression in the mitochondrial and cytosol was examined by western blot. Cox IV was used as a loading control of mitochondrial protein. F. Densitometric analysis of the release of cytochrome c. **P<0.01 vs. control, ##P<0.01 vs. LPS alone, n=6.
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but also enhanced the effect of LPS on MMP, as evidenced by an augmented ratio of green/red fluorescence intensity (Figure 3C and 3D). The increase of mitochondria membrane permeability can induce cytochrome c release from mitochondria to cytoplasm. As shown in Figure 3E and 3F, cytoplasmic cytochrome c level was increased basally in low Cl⁻ solution. LPS treatment caused cytochrome c to release into the cytoplasm, which was further enhanced by reducing [Cl⁻]. These data indicate that the mitochondrial dysfunction contributes the pro-apoptotic effects in low Cl⁻ solution.

Effect of lowering [Cl⁻] on LPS-induced caspase activation

Cytochrome c release sequentially activates the downstream caspase cascades, which are the key signaling steps during apoptosis. Western blot results showed that procaspase-9 and procaspase-3 cleaved into the characteristic active fragments in low Cl⁻ solution. Additionally, LPS treatment significantly increased caspase-9 and caspase-3 cleavage, and lowering [Cl⁻] further enhanced LPS-induced activation of caspases (Figure 4A and 4B). Since cleavage of PARP is a hallmark of cells during apoptosis, we next examined the PARP in low Cl⁻ solution with or without LPS treatment. Similarly, PARP cleavage with accumulation of the characteristic 89-kDa fragments in HUVECs treated with or without LPS were both significantly increased in low Cl⁻ solution (Figure 4C).

Inhibition of mitochondria-derived ROS generation attenuates the increased apoptosis in low Cl⁻ solution

Since excessive reactive oxygen species (ROS) has been show to play an essential role in endothelial cells dysfunction and apoptosis [17], we then examined whether reducing [Cl⁻] induces apoptosis by influencing ROS generation. Compared with the control group, lowering [Cl⁻] resulted in an obvious increase in ROS generation. This induction of ROS generation was remarkably attenuated by pretreatment with N-acetyl-L-cysteine (NAC, 10 μmol/L) in low Cl⁻ solution. It has been document that mitochondrial electron transport chain is one of the main sources for generation of intracellular ROS [18]. Pretreatment with 10 μmol/L rotenone, a specific inhibitor of mitochondrial electron transport chain, for 30 min significantly prevented low Cl⁻ solution-induced ROS generation (Figure 5A and 5B), suggesting the elevation of ROS generation induced by low Cl⁻ solution may be ascribed to a mitochondria origin.

To explore whether the low Cl⁻ solution-induced ROS generation contributes to the increased apoptosis of HUVECs, cells were pretreated with different antioxidants before culturing in low Cl⁻ medium and cell viability was measured by CCK-8 assay. As shown in Figure 5C, low Cl⁻ solution-induced the decrease in cell viability was significantly inhibited by NAC and rotenone. In line with its restoration on cell viability, the specific inhibitor of mitochondria-derived ROS,
rotenone also remarkably attenuated low Cl⁻ solution-induced apoptosis in HUVECs (Figure 5D and 5E), indicating that the inhibition in mitochondria-derived ROS generation results in an attenuation of cell apoptosis in low Cl⁻ solution.

Discussion

Endothelial dysfunction, which is mainly caused by cell injury and apoptosis, would trigger numbers of pathophysiologic processes, such as inflammatory cell infiltration, vascular smooth muscle cell hyperplasia and ultimate atheromatous plaque formation [19, 20]. Because of high morbidity and mortality of the cardiovascular diseases, the understanding of vascular endothelial apoptosis has been a long-term focus of research. Here, we reported for the first time that LPS concentration-dependently and time-dependently decreased [Cl⁻] in HUVECs. Given that changes of [Cl⁻] in HUVECs paralleled with cell viability after LPS treatment, we hypothesized that [Cl⁻] may affect HUVECs viability and apoptosis. In the present study, we found that lowering [Cl⁻] induced cell injury and apoptosis at basal level and exacerbated LPS-induced cell apoptosis in HUVECs, concomitantly with mitochondrial dysfunction and ROS generation. These findings indicate a critical role of [Cl⁻] in endothelial cells apoptosis. Our results are, at least partially, consistent with a previous clinical study, which reports that reduced chloride in serum is an independent predictor of mortality in hypertensive patients [21].

Apoptosis, a programmed cell death resulting from acute cellular injuries, is a crucial process under physiological conditions, whereas dysregulation occurs in pathological state [22, 23]. Apoptosis can be modulated by various pathways, including ROS generation [17], mitochondria [5] and endoplasmic reticulum (ER) stress [24]. Several studies have shown that endothelial cells undergo apoptosis in response to sepsis-related factors such as LPS [25]. Along with apoptosis in sepsis, mitochondrial dysfunction
is observed and seems to have a great influence on sepsis patients, because it has been closely linked to programmed cell death [26]. Bcl-2 and Bax are the two important proteins in the apoptotic machinery, determining the fate of the cells [17]. In the apoptotic process, the balance of Bcl-2 and Bax is broken and mitochondrial dysfunction consequently occurs following MMP depolarization, which results in a release of cytochrome c from mitochondrial to cytosol [27]. Consistent with previous studies, LPS indeed caused mitochondria dysfunction, as evidenced by an increase of Bax/Bcl-1 ratio and a loss of MMP. These effects were further enhanced in low Cl- medium. Notably, lowering [Cl-] without LPS stimulation also induced mitochondrial dysfunction, suggesting that decrease of [Cl-] promotes endothelial cells through mitochondria-dependent pathway.

The release of cytochrome c can cleave and activate caspase-9, then in turn activate caspase cascade characteristic of the apoptotic pathway, such as caspase-3 and PARP, leading to initiate the mitochondria-dependent apoptosis [28, 29]. Here, we found that lowering [Cl-] increased the cleavages of caspase-9, caspase-3 and PARP, resulting in cellular disassembly. Caspase-mediated mitochondrial pathway dysfunction has been notably involved in the pathogenesis of most ROS-induced endothelial cells apoptosis [30]. Based on our results, we may hypothesize that treatment of HUVECs in low Cl- medium will lead to mitochondrial dysfunction and subsequent mitochondrial-derived ROS generation, which itself might be a trigger for apoptosis. In the present study, we provided an interesting finding that pretreatment with rotenone, a specific inhibitor of mitochondrial electron transport chain, inhibited cell injury and apoptosis induced by low Cl- solution, accompanied by reduced ROS generation in HUVECs. Our results demonstrate a novel correlation between [Cl-], and oxidative stress. Excessive mitochondria-derived ROS generation underlies, at least partially, the enhanced effect of lowering [Cl-] on apoptosis in HUVECs.

In conclusion, our present study demonstrate that reduction of [Cl-] causes mitochondrial dysfunction, increases ROS generation derived from mitochondrial, and thus contributes to endothelial cells apoptosis. These data indicate the modulation of [Cl-] may be a rational therapeutic approach to prevent oxidative stress and endothelial dysfunction in cardiovascular diseases.

Disclosure of conflict of interest

None.

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