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Propofol attenuates monocyte-endothelial adhesion via modulating connexin43 expression in monocytes

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ABSTRACT

Aims: Monocyte-endothelial adhesion is considered to be the primary initiator of inflammatory vascular diseases, such as atherosclerosis. Connexin 43 (Cx43) has been reported to play an important part in this process, however, the underlying mechanisms are not fully understood. Intravenous anesthetics, propofol is commonly used in the perioperative period and in the intensive care unit, and considered to have good anti-inflammatory and antioxidant effects. Thus, we speculate that propofol could influence monocyte-endothelial adhesion, and explore whether its possible mechanism is relative with Cx43 expression in U937 monocytes influencing cell adhesion of U937 monocytes to human umbilical vein endothelial cells (HUVEC).

Main methods: Cx43-siRNAs or pc-DNA-Cx43 were used to alter Cx43 expression in U937 monocytes. Propofol was given as pretreatments to U937 monocytes. Then, cell adhesion, ZO-1, LFA-1, VLA-4, COX and MCP-1 were determined. PI3K/AKT/NF-κB signaling pathway was explored to clarify the possible mechanism.

Key findings: Alternation of Cx43 expression affects cell adhesion and adhesion molecules significantly, such as ZO-1, LFA-1, VLA-4, COX-2 and MCP-1, the mechanism of which is relative with Cx43 influencing the activation of PI3K/AKT/NF-κB signaling pathway. Preconditioning with propofol at its clinically relevant anesthesia concentration attenuates cell adhesion. Propofol not only decreases Cx43 expression in U937 monocytes, but also depresses the activation of PI3K/AKT/NF-κB signaling pathway.

Significance: Modulation Cx43 expression in U937 monocytes could affect cell adhesion via regulating the activation of PI3K/AKT/NF-κB signaling pathway. Propofol attenuates cell adhesion via inhibiting Cx43 and its downstream signaling pathway of PI3K/AKT/NF-κB.

1. Introduction

Under normal conditions, circulating monocytes interact minimally with vascular endothelial cells, however, monocytes flowing in blood vessels will adhere to the inflamed or damaged vascular endothelial cells, which might initiate inflammatory vascular diseases, even atherosclerosis [1]. Monocyte-endothelial adhesion occurs at the initial stage of inflammatory vascular processes. Adherent monocytes transmigrate into the arterial intima, then propagate, mature and accumulate lipids, and ultimately transform into macrophage foam cells, which is the hallmark of atherosclerotic pathology. Meanwhile, adherent monocytes release a large number of chemoattractants and inflammatory factors, which not only self-reinforce cell adhesion, but also furtherly damage the vascular endothelium [2,3].

Besides atherosclerosis, monocyte-endothelial adhesion is closely related to the occurrence and development of many vascular pathologies, such as acute coronary syndromes, diabetic nephropathy and bacterial endocarditis [4–6]. Thus, it is important to understand the underlying mechanisms of monocyte-endothelial adhesion. This is beneficial to employ reasonable and effective strategies for the prevention and treatment of atherosclerosis, as well as for other inflammatory vascular diseases.

Connexins express in almost all human organs and tissues. Six connexins compose a hemi-channel, two of which in the neighboring cells dock together, thereby creating an extracellular gap, called Gap junction (GJ). Connexin proteins could play roles in the formation of

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intercellular channels, allowing small molecules transfer between neighboring cells and regulating the direct cell-to-cell signaling transfer. From another aspect, connexins themselves could interact with other proteins, exerting totally different biological functions [7,8]). As reported, carboxyl-terminal domain of Cx43 could interact with various phosphorylases [9,10]. Therefore, we explored effects of Cx43 on the classical phosphorylation signal pathway of phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) [11]. As reported, nuclear transcription factor that nuclear factor-kappaB (NF-κB) plays an important role in the regulation of multiple adhesion molecules [12]. The change of phosphorylation status is crucial to the activity of NF-κB [13]. Thus, in the present study, we explored whether alternation of Cx43 expression in monocytes could regulate cell adhesion via mediating PI3K/AKT/NF-κB signaling pathway.

Propofol commonly used in clinical anesthesia and intensive care unit sedation, not only modulates the balance between oxidants and antioxidants, but also provides protective effects on different organs [14]. Our previous study demonstrated that propofol attenuated liver transplantation-induced acute kidney injury and others indicated that propofol reduced X-ray induced cellular toxicity, both of which were relative with the inhibition of propofol on connexin protein [15,16]. Cx43 is expressed widely in different organs and functions in different ways, which had led to its consideration as a potential target to protect against organ damage. Various studies have shown that propofol could protect organs by inhibiting Cx43 protein. Our previous study showed that propofol could attenuate liver transplantation-induced acute lung injury via Cx43 inhibition [14] ; Chang et al. demonstrated that propofol reduced abnormal Cx43 expression in the interlobar arteries to protect against renal ischemia-reperfusion injury [17]; Jovic et al. showed that propofol exerts cardiac protection through regulating Cx43 expression [18]. Based on the abovementioned findings, we explored whether propofol could influence monocyte-endothelial adhesion through mediating Cx43 expression. This viewpoint has never been reported before. The new mechanistic insight of cell adhesion obtained from the present study would provide a new basis for developing valid therapies to combat a series of inflammatory vascular diseases.

2. Material and methods

2.1. Cell culture

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki with the approval of the Institutional Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

Both HUVECs and U937 monocytes were obtained from American Type Culture Collection (Manassas, VA, USA). HUVECs were cultured with human endothelial SFM (Invitrogen, Carlsbad, CA, USA), which contained 20% fetal bovine serum (Invitrogen), 100 μg/ml heparin (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin-streptomycin (Invitrogen), and 150 μg/ml endothelial cell growth supplement (Becton, Dickinson and Company, Frankin Lakes, NJ, USA). U937 monocytes were cultured in RPMI1640 medium (Invitrogen), supplemented with 20% fetal bovine serum (Invitrogen) and 100 U/ml penicillin-streptomycin (Invitrogen). These two cell lines were both cultured at 37 °C in a 5% CO_2 incubator at 90% humidity (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Cells treatments

U937 monocytes were pretreated with Cx43 expression enhancer, retinoic acid (RA) 10 μM, for 24 h (Sigma-Aldrich) before other assays. Corresponding solvent of RA was DMSO. LY294002 (50 μM, for 24 h, Sigma-Aldrich) was used to inhibit PI3K/AKT signaling pathway. Bay 11-7028 (10 μM, for 24 h, Sigma-Aldrich) was used to inhibit NF-κB. The highly selective COX-2 inhibitor, Celecoxib (20 μM, for 24 h, Sigma-Aldrich) and NS-398 (20 μM, for 24 h, Sigma-Aldrich), were used to inhibit COX-2. Propofol was used at the concentration of 15 μM for 24 h, 48 h and 72 h. This concentration of 15 μM was based on our previous study [15], and more importantly, this concentration (15 μM) was in the range of target plasma concentration of propofol 2 to 4 μg/ml (i.e., 11 to 22 μM) as used clinically during major surgeries.

2.3. Cell survival assay

Cell survival was determined with cell counting kit-8 (CCK8) in 96-well plates, according to the introduction (Dojindo, Tokyo, Japan).

2.4. Adhesion assay

U937 monocytes were labeled with 5 μmol/l calcein-acetoxymethyl ester (Invitrogen) and cultured in the incubator for 30 min. After that, the labeled cells were washed twice with PBS (Invitrogen) and re-suspended in the medium without serum. Then, they were added onto confluent monolayers of HUVECs, which had been pretreated with recombinant mouse tumor necrosis factor (TNF-α, 20 ng/ml, Peprotech, Rocky Hill, NJ, USA) overnight. The plates were put back into the incubator. One hour later, the plates were rinsed twice with medium without serum. The adherent U937 monocytes were left and remained on the confluent monolayers of HUVECs. The adherent U937 monocytes, labeled with calcein-acetoxymethyl ester were counted with a fluorescence microscope (Olympus IX71, Tokyo, Japan). For each condition, 8 different 200× visual fields in the middle of the dish were chosen for analysis. Supplemental Fig. 1 shows that U937 monocytes adhere to the HUVEC cell monolayers.

2.5. Cx43 overexpression and knock-down

Cx43 was over-expressed in U937 monocytes with a pcDNA3.1-Cx43 vector (gift of Ryan Jensen and Peter M. Glazer). Two different siRNAs were used to reduce Cx43 expression specifically. SiRNAs targeting to the human Cx43 gene (CAGUCUGCCUUUCGUUGUA) and a nonspecific, control siRNA was used (as NC in the figures) [8]. Transfection into U937 monocytes was carried out using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 72 h, western blotting was used to assess Cx43 expression.

2.6. Protein detection and co-immunoprecipitation (CoIP)

MCP-1 was detected with ELISA kit (Sigma-Aldrich). Other proteins were detected by their own corresponding antibodies with Western blotting described in previous study [19]. The dilution of antibodies as following: anti-Cx43 (1:3000, Sigma-Aldrich), anti-LFA-1 and VLA-4 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-COX-1 and COX-2 (1:2000, Sigma-Aldrich), anti-ZO-1 (1:500; Sigma-Aldrich), anti-p-AKT and p-p65 (1:1000, Sigma-Aldrich), p-IKBα (1:200, Santa Cruz), anti-β-actin (1:10,000, Sigma-Aldrich), anti-AKT, p65 and IKBα (1:1000, Sigma-Aldrich). Each signaling molecule expression was standardized with its corresponding β-actin. Then, the standardized protein was counted according to the formula: phosphorylated/total protein.

To detect protein-protein interaction, soluble proteins were extracted using the Pierce IP Lysis Buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktails and CoIP was performed using the CoIP Kit (Thermo Fisher Scientific). The CoIP result was showed in Supplemental Fig. 3.

Protein band sizes were estimated using Alpha View software (version number: 2.2.14407, Protein Simple, Santa Clara, CA, USA).

2.7. Statistical analysis

Statistical analysis was performed by using SPSS 15.0 software

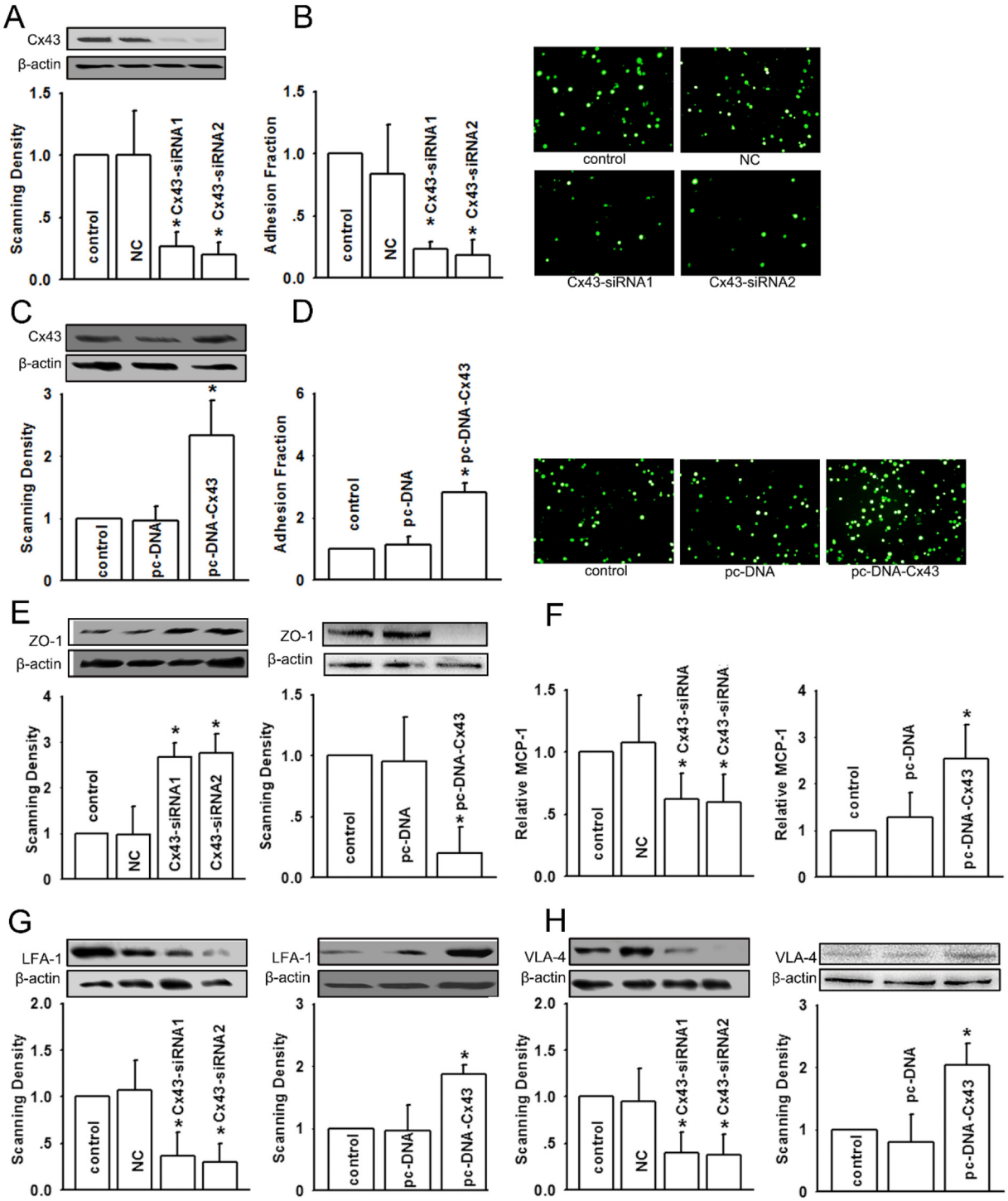

Fig. 1. Specific modulation of Cx43 expression in U937 monocytes modulates U937-HUVEC adhesion, as well as levels of ZO-1, MCP-1, LFA-1 and VLA-4.
(A) Expression of Cx43 in U937 monocytes following treatment with two kinds of Cx43-siRNAs (n = 5, *P < 0.05 vs control). NC: negative control. (B) The change of U937-HUVEC adhesion when Cx43 expression was knocked-down by Cx43-siRNAs in U937 monocytes (n = 4, *P < 0.05 vs control). NC: negative control; U937 monocytes were labeled with calcein-acetoxymethyl ester. (C) Expression of Cx43 in U937 monocytes following treatment with pc-DNA or pc-DNA-Cx43 (n = 5, *P < 0.05 vs control). (D) The change of U937-HUVEC adhesion when Cx43 expression was over-expressed by pc-DNA or pc-DNA-Cx43 in U937 monocytes (n = 4, *P < 0.05 vs control). U937 monocytes were labeled with calcein-acetoxymethyl ester. (E) The change of ZO-1 expression when Cx43 was knocked-down by Cx43-siRNAs or over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 4, *P < 0.05 vs control). NC: negative control. (F) The change of MCP-1 when Cx43 was knocked-down by Cx43-siRNAs or over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 5, *P < 0.05 vs control). NC: negative control. (G) The change of LFA-1 expression when Cx43 was knocked-down by Cx43-siRNAs or over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 4, *P < 0.05 vs control). NC: negative control. (H) The change of VLA-4 expression when Cx43 was knocked-down by Cx43-siRNAs or over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 4, *P < 0.05 vs control). NC: negative control.

(SPSS, Inc., Chicago, IL, USA). Multiple comparisons among groups were analyzed using repeated measures one-way ANOVA, followed by Tukey post hoc comparisons.

3. Results

3.1. Specific modulation of Cx43 expression in U937 monocytes modulates U937-HUVEC adhesion

To investigate the role of Cx43 expression in U937 monocytes on monocyte-endothelial cell adhesion, two different methods: Cx43 knock-down with two kinds of Cx43-siRNAs (Cx43-siRNA1 and Cx43-siRNA2) and Cx43 overexpression with pc-DNA-Cx43, were used to alter Cx43 expression. Fig. 1A showed that Cx43 expression in U937 monocytes was attenuated by Cx43-siRNAs significantly, which was coincident with U937-HUVEC adhesion reduction (Fig. 1B). In contrast, when Cx43 expression was up-regulated with pc-DNA-Cx43 in U937 monocytes, U937-HUVEC adhesion was increased as Cx43 expression was elevated (Fig. 1C and D). These results in Fig. 1 demonstrated that Cx43 expression in U937 monocytes could modulate U937-HUVEC adhesion.

3.2. Cx43 expression alternation in U937 monocytes modulates the levels of ZO-1, MCP-1, LFA-1 and VLA-4

As far as we know, cell adhesion is a very complex process, which contains a series of pathophysiological changes as following in short: destruction of cytoskeletal proteins leads to the changes of cell morphology and polarity, making monocytes more easily attached to endothelial cells [20]; chemokines release results in monocytes aggregating [21]; the increased expression of adhesion proteins greatly enhanced the adhesion characteristics of monocytes [22]. Therefore, we explored changes of ZO-1, anchoring the junctional complex to the cytoskeletal elements of the adjacent cells [23]; MCP-1, an important monocyte chemoattractant protein [24]; cell adhesion molecules: LFA-1 and VLA-4, receptors of ICAM-1 and VCAM-1 [25]. Fig. 1E to H illustrated that as Cx43 expression in U937 monocytes was knocked down with siRNAs, the expression of ZO-1 was increased, while the levels of MCP-1, LFA-1 and VLA-4 were all attenuated at different degrees. In contrast, when Cx43 was over-expressed by pc-DNA-Cx43, the expression of ZO-1 was decreased, but the levels of MCP-1, LFA-1 and VLA-4 were all increased significantly (Fig. 1E to H).

3.3. Cx43 down-regulation attenuates MCP-1 release via inhibiting COX-2 in U937 monocytes

In both in vivo and in vitro experiments, it has been proved that MCP-1 has significant chemotaxis activity to monocytes, which not only activates monocytes, but also leads to monocytes aggregation. MCP-1 is considered to be one of the most important chemokine for promoting adhesion between monocytes and endothelial cells [24]. Therefore, in this part, we have studied the change of cyclooxygenase (COX), which is directly related to MCP-1 secretion [26]. Fig. 2A and B showed that Cx43 alternation in U937 monocytes with Cx43-siRNAs or pc-DNA-Cx43 had no effects on the content of COX-1, but in contrast, the content of COX-2 changed with the alternation of Cx43 expression. Thus, we used highly selective COX-2 inhibitors, Celecoxib and NS-398 to inhibit COX-2 and observed effects on MCP-1 secretion and U937-HUVEC adhesion [27]. The results showed that the secretion of MCP-1 was significantly inhibited and cell adhesion was also attenuated (Fig. 2C and D). Fig. 2E and F showed that NS-398 and Celecoxib could diminish the increase in MCP-1 level and adhesion fraction generated by the overexpression of Cx43. Based on the results above, we concluded that the decrease of Cx43 expression in U937 monocytes attenuated MCP-1 secretion and cell adhesion via inhibiting COX-2, but not COX-1.

3.4. Cx43 down-regulation inhibits PI3K/AKT/NF-κB signaling pathway in U937 monocytes

In order to explore the possible mechanism, we mainly test the change of PI3K/AKT/NF-κB signaling pathway, which is always considered to play an important part in cell adhesion [11,13]. Fig. 3A and B showed that the key molecules of PI3K/AKT/NF-κB signaling pathway, such as p-AKT, p-p65 and p-IKBα were all reduced as Cx43 expression was down-regulated with Cx43-siRNAs, but all of them were increased as Cx43 expression was up-regulated with pc-DNA-Cx43. In Fig. 3C to F, U937 monocytes were pretreated with PI3K inhibitor, LY294002 and NF-κB inhibitor, Bay 11-7082 respectively [28]. These two chemicals inhibited the PI3K/AKT/NF-κB signaling pathway and simultaneously attenuated the expression of adhesion molecules and cell adhesion. Meanwhile, we noticed that NF-κB inhibitor, Bay 11-7082 had no effects on the expression of p-AKT, which demonstrated that NF-κB was located at the lower reaches of the PI3K/AKT signaling pathway (Fig. 3C). Fig. 3D demonstrated that these two inhibitors did not affect the expression of Cx43 and ZO-1. This indicated that the expression of Cx43 and ZO-1 was not affected by PI3K/AKT/NF-κB signaling pathway. Fig. 3G and H showed that both LY294002 and Bay 11-7082 could diminish the increase in MCP-1 level and adhesion fraction generated by the overexpression of Cx43. Through results above (Figs. 1 to 3), we found that Cx43 down-regulation in monocytes could modulated a series of associated adhesion molecules by regulating PI3K/AKT/NF-κB signaling pathways and ultimately affected cell adhesion, such as ZO-1, COX-2/MCP-1, LFA-1 and VLA-4.

3.5. Propofol attenuates U937-HUVEC adhesion via inhibiting Cx43 expression in U937 monocytes

The conclusion we obtained from Figs. 1 to 3 prompt us Cx43 alternation in monocytes might be an effective strategy to improve cell adhesion. Propofol is a commonly used sedative anesthetic. It is widely used in operation room and intensive care unit [15]. Thus, we assess effects of propofol on cell adhesion. The clinically relevant anesthesia concentration was 15 μM (measured as total concentration: protein bound and free) [15]. At this concentration, propofol had no effects on U937 monocytes survival from 0 to 72 h exposure (Fig. 4A), but down-regulated Cx43 expression at the timepoints of 48 h and 72 h, the reductions of which were about 50% and 70%, respectively (Fig. 4B). With the decline of Cx43 expression in U937 monocytes, U937-HUVEC adhesion was also showed a significant decrease (Fig. 4C). Then, we used RA (the enhancer of Cx43) and pc-DNA-Cx43 to restore the expression of Cx43 in U937 monocytes. Fig. 5A and B showed that both RA and pc-DNA-Cx43 (without cytotoxicity) could increase Cx43 expression in U937 monocytes. More importantly, U937-HUVEC adhesion decline caused by propofol was reversed as Cx43 expression increased (Fig. 5C). These results showed that propofol could attenuate cell adhesion via inhibiting Cx43 expression in U937 monocytes, but after the expression of Cx43 was restored, the protective effect of propofol on cell adhesion was weakened.

After pretreatment of propofol, associated adhesion molecules expressed in U937 monocytes, such as ZO-1, COX-2/MCP-1, LFA-1 and VLA-4, were all changed as the influence of Cx43 expression alternation: ZO-1 was increased; COX-2/MCP-1, LFA-1 and VLA-4 were decreased (Fig. 5D to F).Propofol pretreatment also had no effects on COX-1 (Fig. 5F). In order to further prove the effects of propofol on ZO-1, LFA-1, VLA-4 and COX-2 via inhibiting Cx43, U937 cells were pretreated with propofol together with pc-DNA-Cx43 (Supplemental Fig. 2A). The results showed that propofol could prevent the reduction in ZO-1 expression generated by the overexpression of Cx43, but diminish the increase in the levels of MCP-1, LFA-1, VLA-4 and COX-2 provoked by Cx43 overexpression (Fig. 1E to H and Fig. 5D to F showed that pc-DNA or lipid had no effects on the levels of MCP-1, LFA-1, VLA-4 and COX-2 in U937 monocytes).

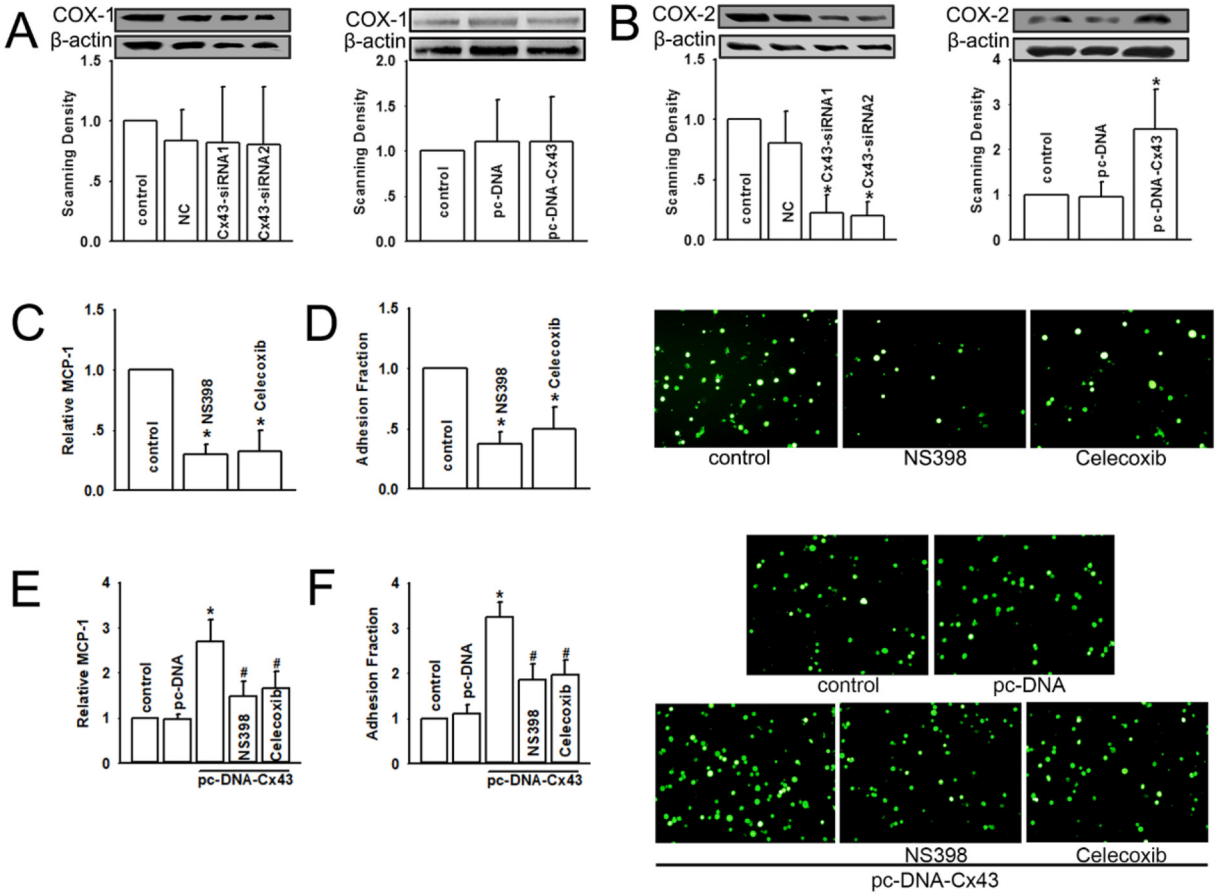

Fig. 2. Cx43 expression alternation in U937 monocytes regulated COX-2, MCP-1 and cell adhesion.
(A) The change of COX-1 expression when Cx43 was knocked-down by Cx43-siRNAs or over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 5, *P < 0.05 vs control). NC: negative control. (B) The change of COX-2 when Cx43 was knocked-down by Cx43-siRNAs or over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 5, *P < 0.05 vs control). NC: negative control. (C) The change of MCP-1 when U937 monocytes were pretreated with two kinds of COX-2 inhibitors: Celecoxib (20 μM, for 24 h) and NS-398 (20 μM, for 24 h) (n = 6, *P < 0.05 vs control). (D) The change of U937-HUVEC adhesion when U937 monocytes were pretreated with two kinds of COX-2 inhibitors: Celecoxib (20 μM, for 24 h) and NS-398 (20 μM, for 24 h) (n = 4, *P < 0.05 vs control; # P < 0.05 vs pc-DNA-Cx43). (E) The change of MCP-1 when U937 monocytes were pretreated with Celecoxib (20 μM, for 24 h), NS-398 (20 μM, for 24 h) and pc-DNA-Cx43 (n = 3, *P < 0.05 vs control; # P < 0.05 vs pc-DNA-Cx43). (F) The change of cell adhesion when U937 monocytes were pretreated with Celecoxib (20 μM, for 24 h), NS-398 (20 μM, for 24 h) and pc-DNA-Cx43 (n = 3, *P < 0.05 vs control; # P < 0.05 vs pc-DNA-Cx43).

3.6. Propofol attenuates PI3K/AKT/NF-κB signaling pathway in U937 monocytes

Figs. 4 and 5 demonstrated that propofol pretreatment in U937 monocytes could attenuate Cx43 expression and cell adhesion. Down-regulation of Cx43 expression lead to the inactivation of PI3K/AKT/NF-κB signaling pathway (Figs. 1 to 3). Thus, we speculated that inhibition of propofol on PI3K/AKT/NF-κB signaling pathways mediated by Cx43 in U937 monocytes might be the underlying mechanism that propofol protecting against cell adhesion. Fig. 6 just showed that propofol pretreatment reduced the expression of p-AKT, p-p65, p-IKBα and COX-2. In supplemental Fig. 2B, U937 cells were pretreated with propofol together with pc-DNA-Cx43. The results showed that propofol could diminish the increase in the levels of p-AKT, p-p65 and p-IKBα provoked by Cx43 overexpression, but had no effects on AKT, p65 and IKBα, which indicated that propofol could affect the activity of PI3K/AKT/NF-κB signaling pathway through regulating Cx43 (Fig. 3B and Fig. 6 showed that pc-DNA or lipid had no effects on the levels of p-AKT, AKT, p-p65, p65, p-IKBα and IKBα in U937 monocytes).

4. Discussion

As far as we know, atherosclerosis is a chronic inflammatory disease with complicated pathogenesis. Monocyte-endothelial adherence is always considered to be one of the most important primary initiator of atherosclerosis, involving in lots of different risk factors, such as inflammation, damage, or exposing to turbulent shear-stress [29]. Therefore, the study of monocyte-endothelial adhesion will contribute to the prevention and treatment of atherosclerosis or other inflammatory vascular diseases, especially in the early stage. In our previous studies, we have demonstrated that changes of Cx43 expressed in endothelial cells could modulate monocyte-endothelial adhesion through regulating cell adhesion proteins: VCAM-1 and ICAM-1 [7]; Cx43 channel function enhancement in monocytes rapidly reduced monocyte-endothelial adhesion via ATP release and conversion to ADO, but in contrast Cx43 expression itself increased monocyte-endothelial adhesion [8]. Pity that mechanisms about increase of Cx43 expression in monocytes resulting in cell adhesion had not been reported, which was the key problem to be solved in this study.

In present investigation, we confirmed that Cx43 expression alternation could regulate cell adhesion. A series of related adhesion molecules and signaling pathways also altered along with the change of Cx43 expression in U937 monocytes. ZO-1 anchors the junctional complex to the cytoskeletal elements of the adjacent cells, which is essential for maintaining cell morphology and polarity [30]. We found that the change of Cx43 expression in U937 monocytes directly affected

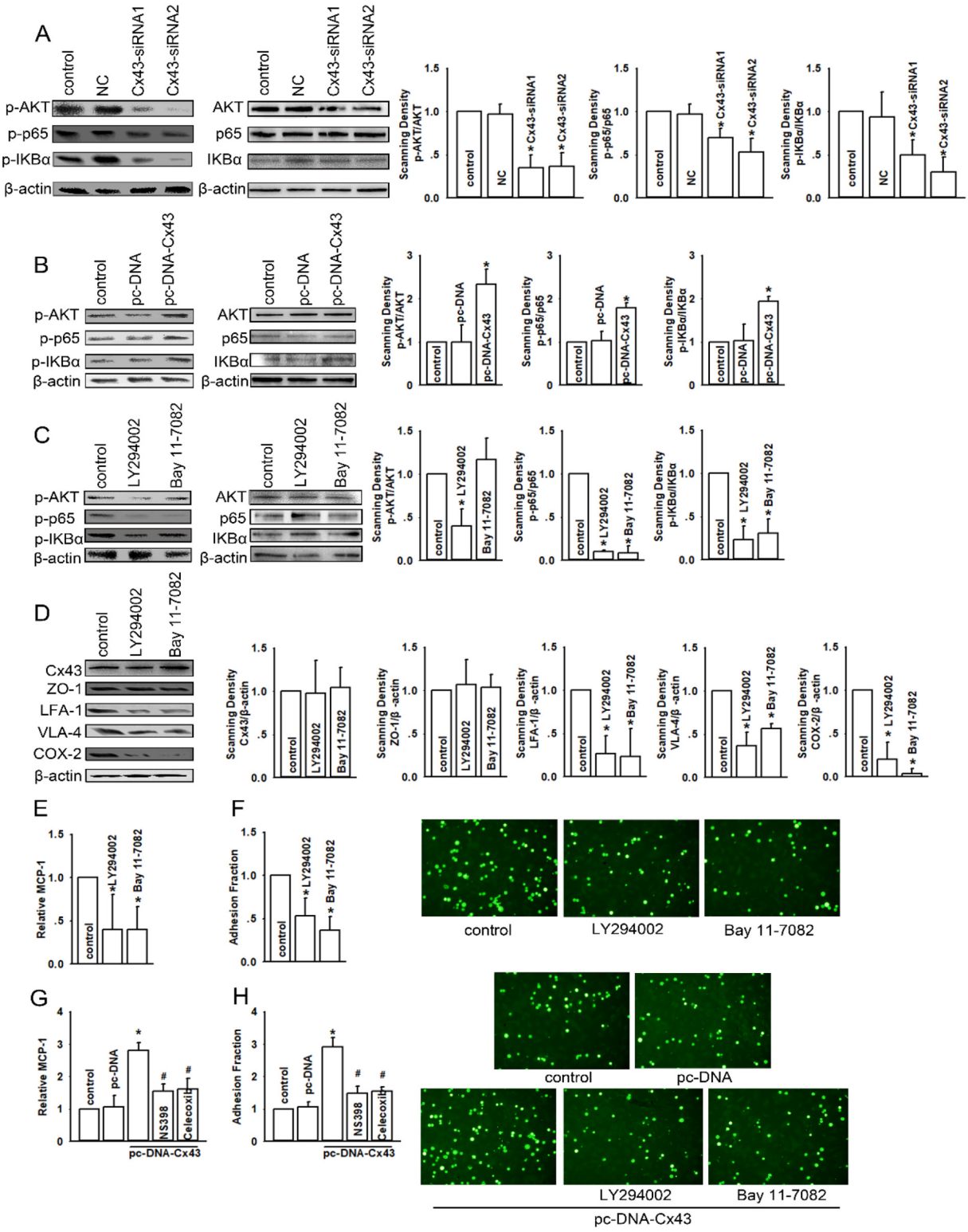

Fig. 3. Cx43 alternation in U937 monocytes modulated cell adhesion via PI3K/AKT/NF-κB signaling pathway.
(A) The change of p-AKT/AKT, p-p65/p65 and p-IKBα/ IKBα expression in U937 monocytes when Cx43 was knocked-down by Cx43-siRNAs (n = 4, *P < 0.05 vs control). NC: negative control. (B) The change of p-AKT, p-p65 and p-IKBα expression in U937 monocytes when Cx43 was over-expressed by pc-DNA-Cx43 in U937 monocytes (n = 4, *P < 0.05 vs control). NC: negative control. (C) The change of p-AKT, p-p65 and p-IKBα expression in U937 monocytes when U937 monocytes were pretreated with LY294002 and Bay 11–7082 (n = 5, *P < 0.05 vs control). (D) The change of Cx43, ZO-1, LFA-1, VLA-4 and COX-2 expression in U937 monocytes when U937 monocytes were pretreated with LY294002 and Bay 11–7082 (n = 3–5, *P < 0.05 vs control). (E) The change of MCP-1 release when U937 monocytes were pretreated with LY294002 and Bay 11–7082 (n = 3, *P < 0.05 vs control). (F) The change of cell adhesion when U937 monocytes were pretreated with LY294002 and Bay 11–7082 (n = 5, *P < 0.05 vs control). LY294002 (PI3K inhibitor, 50 μM, for 24 h), Bay 11–7028 (NF-κB inhibitor, 10 μM, for 24 h). (G) The change of MCP-1 release when U937 monocytes were pretreated with LY294002, Bay 11–7082 and pc-DNA-Cx43 (n = 3, *P < 0.05 vs control; # P < 0.05 vs pc-DNA-Cx43). (H) The change of cell adhesion when U937 monocytes were pretreated with LY294002, Bay 11-7082 and pc-DNA-Cx43 (n = 3, *P < 0.05 vs control; # P < 0.05 vs pc-DNA-Cx43).

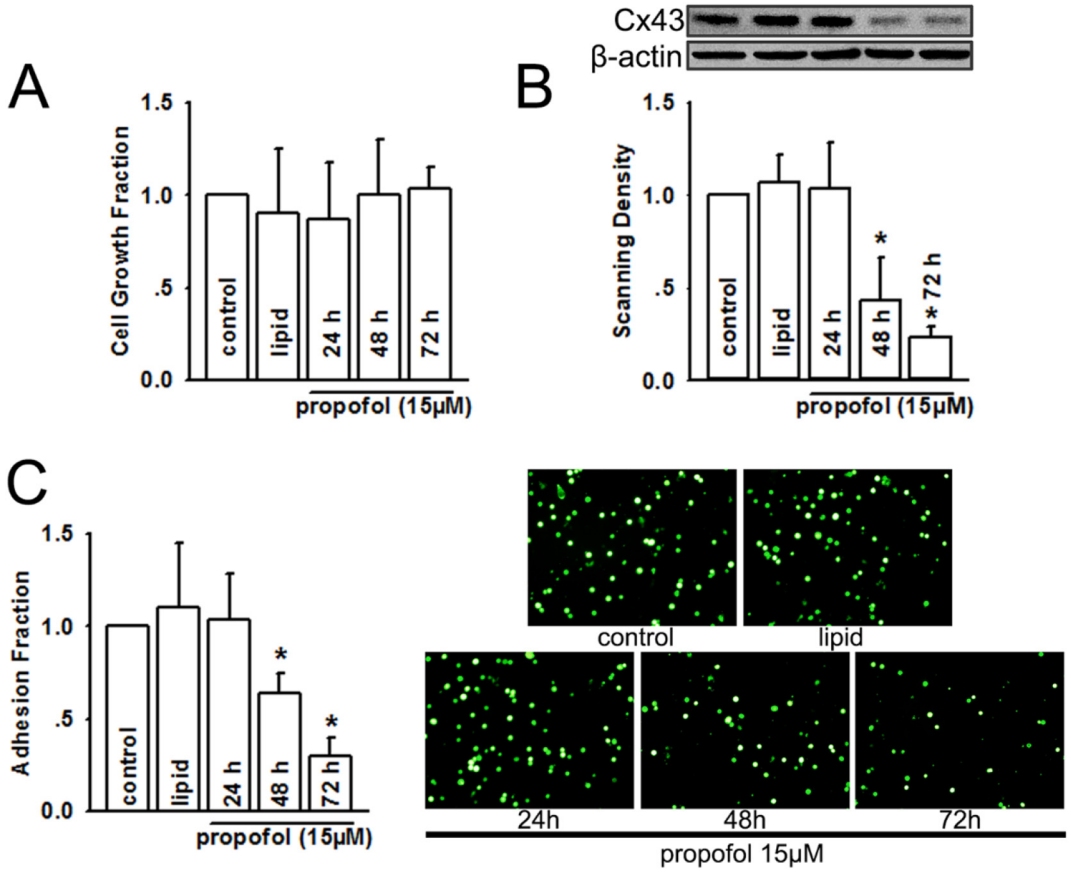

Fig. 4. Propofol attenuated Cx43 expression in U937 monocytes, as well as cell adhesion.
(A) Propofol (15 μM) had no effects in U937 monocytes survival. Corresponding solvent of propofol was lipid. Cell survival was determined with CCK8. (B) Effects of propofol on Cx43 expression in U937 monocytes (n = 4, *P < 0.05 vs control). Corresponding solvent of propofol was lipid. (C) Effects of propofol on U937-HUVEC adhesion when U937 monocytes were pretreated with propofol (n = 5, *P < 0.05 vs control). Corresponding solvent of propofol was lipid. U937 monocytes were labeled with calcein-acetoxymethyl ester.

ZO-1 expression, not through PI3K/AKT/NF-κB signaling pathway, which was different from other associated adhesion molecules, such as MCP-1, LFA-1 and VLA-4. As reported, ZO-1 is directly connected to the carboxyl-terminal domain of Cx43 ([31]. Thus, we speculated that alternation of ZO-1 expression mediated by Cx43 resulted in the destruction of cytoskeletal proteins and the changes of cell morphology and polarity, making monocytes more easily attached to endothelial cells.

Besides ZO-1, the carboxyl-terminal domain of Cx43 also could interact with some special elements of cellular signaling pathways, such as Src, PKC and PKA, which provides the possibility that changes of Cx43 expression affecting other signaling pathways [9]. Our results demonstrated that both PI3K/AKT and NF-κB signaling pathways participated in this process of Cx43 modulating cell adhesion, manifested as (1) p-AKT, p-p65 and p-IKBα could be regulated by Cx43 expression in U937 monocytes (Fig. 3A and B); (2) PI3K inhibitor, LY294002 or NF-κB inhibitor, Bay 11-7082 decreased cell adhesion effectively (Fig. 3E). We noticed that LY294002 could attenuate expression of p-p65 and p-IKBα, but Bay 11-7082 had no effects on the expression of p-AKT, which suggested that NF-κB was located at the lower reaches of the PI3K/AKT signaling pathway. Thus, we concluded that the regulation of Cx43 on PI3K/AKT/NF-κB signaling pathways was crucial for cell adhesion.

This signaling pathway of PI3K/AKT/NF-κB could regulate the expression of many related adhesion molecules, the most important one of which is MCP-1. Our results indicated that Cx43 expression could regulate COX-2, but not COX-1, via PI3K/AKT/NF-κB signaling pathways; highly selective COX-2 inhibitors, Celecoxib and NS-398 [32,33], attenuated MCP-1 release and U937-HUVEC adhesion effectively. Thus, we believed that the effect of Cx43 in U937 monocytes on COX-2/MCP-1 through modulating PI3K/AKT/NF-κB signaling pathways, was also one of the important factors to regulate cell adhesion. MCP-1 is the first discovered and most extensively studied CC chemokine, mainly produced in monocytes, and has a strong chemotactic effect on monocytes, which could induce the migration, aggregation, adhesion and activation of monocytes to the injury site of arterial wall, and ultimately initiated atherosclerosis [34,35]. COX is an important enzyme in prostaglandin synthesis. Its activity could be inhibited by most known NSAIDs. There are two mainly related isoforms of COX, including COX-1 and COX-2. COX-1 is always considered to be a “constitutive isoform” and has mainly cytoprotective effects, such as in the maintenance of renal blood flow and the production of gastric mucus, but in contrast, COX-2 is always considered to be an “inducible isoform” and plays an important part in inflammatory response [36,37]. Our results suggest that inhibition of Cx43 mediated COX-2/MCP-1 via PI3K/AKT/NF-κB signaling pathways in U937 monocytes might be another important way to reduce cell adhesion.

We also found that cell adhesion molecules in U937 monocytes: LFA-1 and VLA-4, could be regulated by Cx43 via PI3K/AKT/NF-κB signaling pathways. LFA-1 and VLA-4 were just the receptors of ICAM-1 and VCAM-1 respectively. Their interaction mediated the recruitment of circulating monocytes to endothelial cells directly [25]. Combining our previous findings that changes of Cx43 expression in endothelial cells could modulate monocyte-endothelial adhesion through regulating cell adhesion proteins: VCAM-1 and ICAM-1 [7], we believed that Cx43 might be a key factor regulating the expression of adhesion

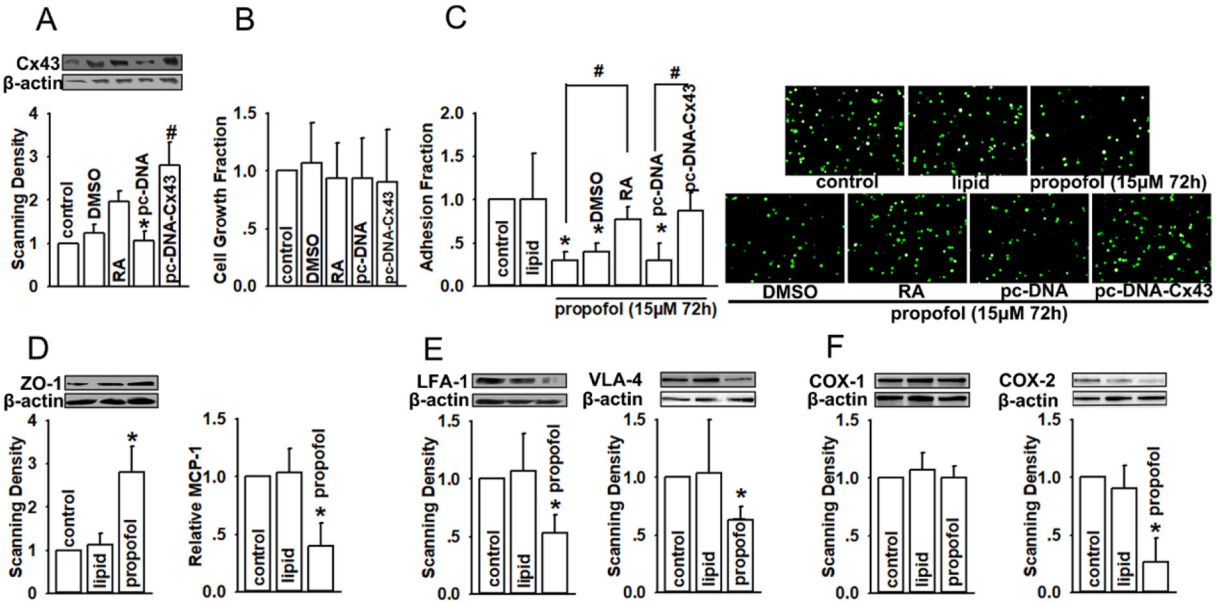

Fig. 5. Cx43 enhancement reversed U937-HUVEC adhesion decrease caused by propofol.
(A-B) Cx43 enhancer, RA (10 μM, 24 h) and pc-DNA-Cx43 increase Cx43 expression in U937 monocytes without cytotoxicity (n = 4 or 3, *P < 0.05 vs control; #P < 0.05 vs pc-DNA). Corresponding solvent of RA was DMSO. Cell survival was determined with CCK8. (C) When U937 monocytes were pretreated with RA (10 μM, 24 h) or pc-DNA-Cx43, U937-HUVEC adhesion declined caused by propofol (15 μM, 72 h) was reversed (n = 5, *P < 0.05 vs control; #P < 0.05). Corresponding solvent of propofol was lipid and corresponding solvent of RA was DMSO. U937 monocytes were labeled with calcein-acetoxymethyl ester. (D) The change of ZO-1 expression and MCP-1 when U937 monocytes were pretreated with propofol (n = 5, *P < 0.05 vs control). Corresponding solvent of propofol was lipid. (E) The change of LFA-1 and VLA-4 expression when U937 monocytes were pretreated with propofol (n = 4, *P < 0.05 vs control). Corresponding solvent of propofol was lipid. (F) The change of COX-1 and COX-2 expression when U937 monocytes were pretreated with propofol (n = 4, *P < 0.05 vs control). Corresponding solvent of propofol was lipid.

molecules and inhibition of Cx43 expression might be an effective strategy to prevent cell adhesion.

Propofol is a commonly used anesthetic in clinical anesthesia and intensive care unit sedation. Its protective effects on various organs has been confirmed: Xia et al. has reported that propofol could reduce post-ischemic oxidative stress and improved myocardial I/R injury of patients [38]. Our previous study also showed that propofol could attenuate ROS transfer between the neighboring cells and protect against liver transplantation-induced AKI [15]. Although Shi et al. reported that propofol could inhibit COX-2 expression and exert neuroprotection against subarachnoid hemorrhage-induced early brain injury in rats [39], its function on monocyte-endothelial adhesion had never been

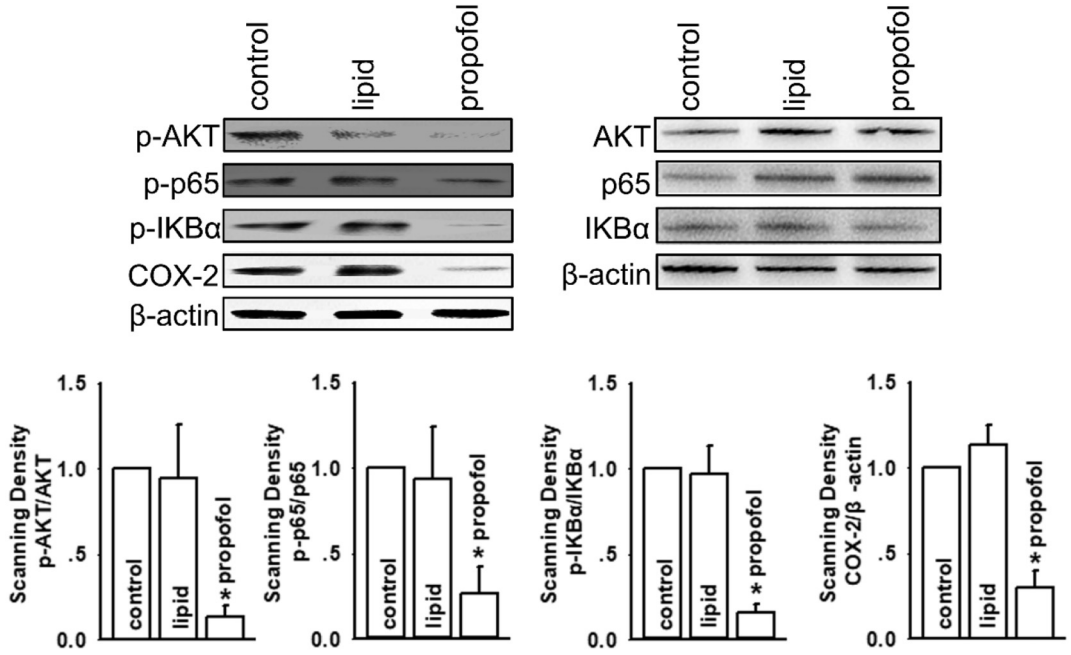

Fig. 6. Propofol inhibited the activity of PI3K/AKT/NF-κB signaling pathways.
The change of p-AKT/AKT, p-p65/p65, p-IKBα/ IKBα and COX-2 expression in U937 monocytes were pretreated with propofol (n = 4, *P < 0.05 vs control). Corresponding solvent of propofol was lipid.

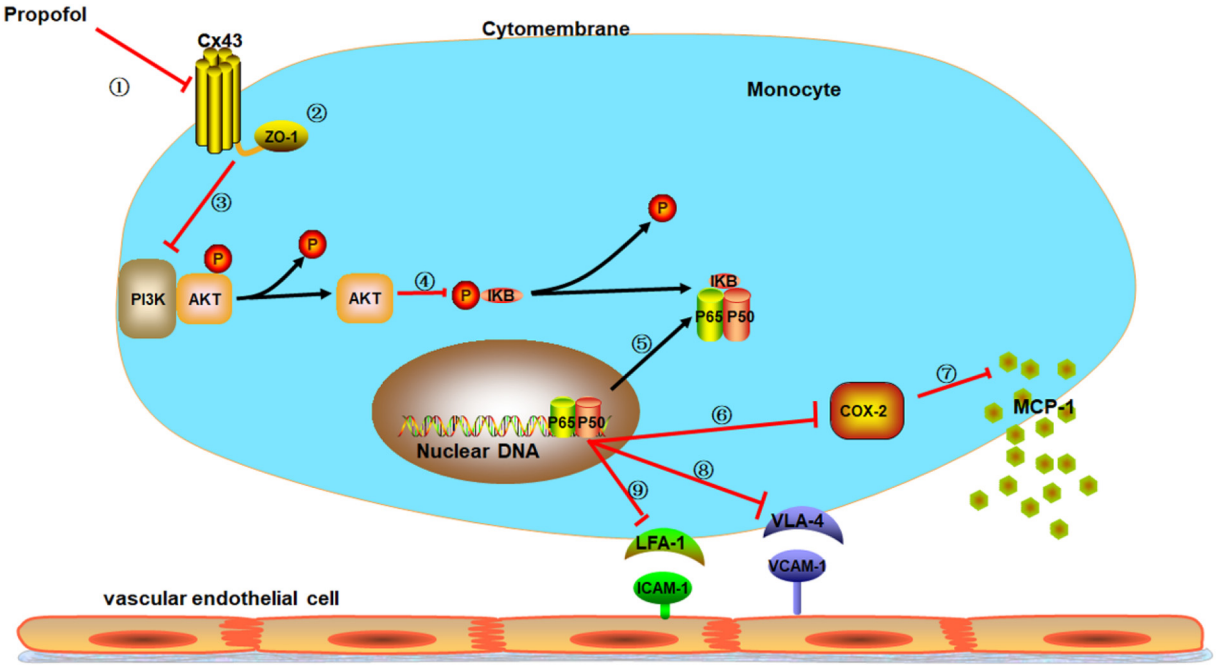

Fig. 7. Proposed signaling pathway for the ability of propofol to alleviate cell adhesion via inhibiting PI3K/AKT/NF-κB signaling pathways.
① propofol inhibited Cx43 expression in U937 monocytes; ② ZO-1 directly linked to the carboxyl-terminal domain of Cx43 also depressed as Cx43 was down-regulated; ③PI3K inhibition induced by Cx43 down-regulation lead to the dephosphorylation of AKT; ④ IKBα dephosphorylation; ⑤ NF-κB was removed from the nucleus and bound to dephosphorylated IKBα; ⑥ the inactivation of NF-κB reduced COX-2 expression; ⑦ COX-2 expression down-regulation attenuated MCP-1 release; ⑧⑨the inactivation of NF-κB also reduced LFA-1 and VLA-4 expression.

explored. In our present study, we firstly clarified propofol application could effectively attenuate cell adhesion via inhibition Cx43 mediating PI3K/AKT/NF-κB signaling pathways. From results we got in our investigation, we could summarized in short (Fig. 7): ① propofol inhibited Cx43 expression in U937 monocytes; ② ZO-1 directly linked to the carboxyl-terminal domain of Cx43 also changed as Cx43 was down-regulated (Supplemental Fig. 3 showed that Cx43 complexes with ZO-1); ③ PI3K inhibition lead to the dephosphorylation of AKT; ④ IKBα dephosphorylation; ⑤ NF-κB was removed from the nucleus and bound to dephosphorylated IKBα; ⑥ the inactivation of NF-κB reduced COX-2 expression; ⑦ COX-2 expression down-regulation attenuated MCP-1 release; ⑧⑨ the inactivation of NF-κB also reduced LFA-1 and VLA-4 expression.

Propofol sedation is often essential for patients undergoing long term surgery or for long-term bed rest in intensive care unit [15]. Prolonged bed rest could greatly increase the risk of venous thrombosis [40]. According to our research, we found that the rational application of propofol could reduce cell adhesion because propofol could act on several key links of cell adhesion: firstly, propofol increased ZO-1 expression, which was crucial for maintaining the stability of cell morphology and polarity [20]; secondly, propofol application attenuated COX-2/MCP-1 release via inhibition Cx43 and PI3K/AKT/NF-κB signaling pathways. MCP-1 had a strong chemotactic effect on monocytes, which could induce the migration, aggregation, adhesion and activation of monocytes to the injury site of arterial wall [35]; thirdly, propofol reduced expression of LFA-1 and VLA-4 [25]. All of above demonstrated that rational use of propofol might be an effective strategy to prevent against atherosclerosis or other inflammatory vascular diseases.

As the primary initiator of atherosclerosis or other inflammatory vascular diseases, monocyte-endothelial adhesion plays an important role in these pathophysiological processes ([1,41]). The pathogenesis of atherosclerosis is very complex. Different reports, including our previous studies, all show that alternation of Cx43 expression and/or function have different roles in this process. For instance, Cx43 expressed in endothelial cells or monocytes induces monocytes migrating and adhering to the injury site of arterial intima through various mechanisms. With the participation of Cx43, monocytes convert to foam cells in arterial intima, which is the characteristic pathological cells in atherosclerotic plaques. Cx43 expressed at the curved areas or the branch points of large arteries contributes to the formation of atherosclerotic plaques. Cx43 expression reduction could help to keep the stable of atherosclerotic plaques [42,43]. Our present study mainly focuses on monocyte-endothelial adhesion, the initial step of atherosclerosis or other inflammatory vascular diseases. It elaborates that alternation of Cx43 expression in monocytes could modulate cell adhesion, the mechanism of which is relative with Cx43 regulating the activity of PI3K/AKT/NF-κB signaling pathways. Meanwhile, the protective effect of propofol on cell adhesion is also verified. Therefore, we believe that this current study provides new research ideas and theoretical basis for the prevention and treatment of atherosclerosis.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116624.

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