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Down-regulation of Cx43 expression on PIH-HUVEC cells attenuates monocyte–endothelial adhesion

Xianlong Li[a,1], Qian Zhang[a,1], Rui Zhang[b], Nan Cheng[a], Na Guo[a], Yiqian Liu[c], Jun Cai[a,*], Dongdong Yuan[a,*]

[a] Department of Anesthesiology, the Third Affiliated Hospital of Sun Yat-sen University, Tianhe Road, Guangzhou, PR China.
[b] Department of Anesthesiology, Zhongshan Ophthalmic Center of Sun Yat-sen University, Xian lie South Road, Guangzhou, PR China.
[c] Southern Medical University, Sha Tai South Road, Guangzhou, PR China.

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ABSTRACT

Introduction: Pregnancy-induced hypertension (PIH) is the most common serious complication of pregnancy, resulting in significant maternal and fetal morbidity and mortality. Vasospasm is the main pathogenesis of PIH, which leads to the hemodynamic changes and the injury of vascular endothelial cells. However, the underlying mechanism is still unclear. Monocyte–endothelial adhesion is always considered to be one of the most important indicators of vascular endothelial cell injury. Connexin43 (Cx43) plays an important part in monocyte–endothelial adhesion. Thus, we explored effects of Cx43 on cell adhesion in PIH-induced vascular endothelial cells injury.

Methods: We obtained human umbilical vein endothelial cells (HUVECs) from patients with or without PIH. Different methods, such as inhibitors: oleamide and Gap26, or specific siRNA were used to alter Cx43 channels function or protein expression in normal or PIH-HUVECs. U937-HUVECs adhesion, adhesion molecules expression, such as VCAM-1 and ICAM-1, and the activity of PI3K/AKT/NF-κB signaling pathway were determined.

Results: Monocyte–endothelial adhesion on PIH-HUVECs was much more obvious than that on normal HUVECs. Inhibition of Cx43 protein expression could attenuate cell adhesion significantly, however, function of Cx43 channels had no effects on it. Alternation of Cx43 protein expression on PIH-HUVECs mediated VCAM-1 and ICAM-1 expression via regulating the activity of PI3K/AKT/NF-κB signaling pathway.

Conclusions: We firstly reported Cx43 protein expression on PIH-HUVECs was much higher than that on normal HUVECs. Elevation of Cx43 protein expression within the vasculature resulted in PI3K/AKT/NF-κB signaling pathway activation and VCAM-1 and ICAM-1 over-expression, which ultimately lead to monocyte–endothelial adhesion increase.

1. Introduction

PIH is an idiopathic disease prone to occur in late pregnancy, and has become the most common serious complication of pregnancy, resulting in significant maternal and fetal morbidity and mortality [1,2]. PIH includes hypertensive disorder complicating pregnancy and preeclampsia. Especially, preeclampsia is a pregnancy-specific disorder that is always diagnosed by the combined presentation of high blood pressure and proteinuria [3]. If suffering from PIH, especially preeclampsia, puerpera will be under an increased long-term risk of abruptio placentae, cerebrovascular events, and even organ failure. Fetuses are also at greater risk of intrauterine growth retardation, prematurity, and intrauterine death [4,5]. Thus, it is critical to understand the underlying mechanisms of PIH and provide potential target for its treatment.

The main pathogenesis of PIH is vasospasm, which leads to the hemodynamic changes and the injury of vascular endothelial cells [6]. Under normal conditions, circulating monocytes interact minimally with vascular endothelial cells, however, they will easily adhere to the inflamed or damaged vascular endothelial cells [7]. Monocytes

Abbreviations: PIH, Pregnancy-induced hypertension; Cx43, connexin43; HUVEC, human umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; GJ, Gap junction; PKA, protein kinase A; PKC, protein kinase C; TNF-α, tumor necrosis factor; HRP, horseradish peroxidase

* Corresponding authors.
E-mail addresses: gzcaijun@hotmail.com (J. Cai), yuandongdong123@126.com (D. Yuan).
[1] These authors contributed equally to this study.

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adhering to the vascular endothelial cells will result in the activation of inflammation related signaling pathways and the release of cytokines, which leads to the vascular endothelial cell injury further deteriorated, vasospasm aggravated, and ultimately blood pressure further elevated [8,9]. Therefore, we speculate that the increase of cell adhesion is an important step in the worsening of vascular endothelial cell injury.

Connexin, a big family of transmembrane proteins, is usually named according to their own molecular weight, such as Cx37, Cx40 and Cx43. Cx43 expresses in almost all the organs and tissues, especially in cardiovascular system. It is critically involved in normal physiology as well as many cardiovascular pathologies, including atherosclerosis. It has been reported that Cx43 expression is upregulated in human atheromatous plaque and in animal models of atherosclerosis [10]. Neutrophil adhesion to endothelium has been verified to be modulated by ATP release via Cx43 hemichannels [11]. In our previous study, we had demonstrated that Cx43 played an important part in cell adhesion [12,13], but its underlying mechanism and its effects on PIH had never been reported. Thus, in present study, we focused on the protein expression of Cx43 in vascular endothelial cells of PIH patients and its effect on cell adhesion.

Six connexins compose a hemi-channel, and two of which in the neighboring cells dock together and form an extracellular gap, called Gap junction (GJ) [14]. Connexin proteins could play roles in the form of hemi-channels or GJs, allowing small molecules transfer between the intracellular and extracellular environment or between the neighboring cells [15]. From another aspect, connexins themselves also could interact with other proteins directly, exerting totally different biological functions from hemi-channels or GJs. As reported, carboxyl-terminal domain of Cx43 could interact with Src, protein kinase A (PKA) or protein kinase C (PKC), exerting corresponding biological effects [12]. PKA and PKC are both important protein kinases, which play an important role in phosphorylation of some gene regulatory proteins and activation of specific gene transcription, such as the signaling pathways of PI3K/AKT and NF-κB. Phosphorylation is the main active form of PI3K/AKT and NF-κB, and more importantly, these proteins could be phosphorylated by PKA and PKC [16–18]. As far as we know, both the activity of PI3K/AKT and NF-κB signaling pathways could influence monocyte-endothelial adhesion through regulating important adhesion molecular expression, such as VCAM-1 or ICAM-1 [19,20]. Thus, we speculate that effects of Cx43 protein expression on cell adhesion might be through modulating the activity of PI3K/AKT/NF-κB signaling pathway.

In our present investigation, we reported for the first time that the protein expression of Cx43 on HUVECs from pregnant women with PIH was significantly elevated, which was closely related to the increase of cell adhesion. Effects of Cx43 on cell adhesion was relative with regulating the activity of PI3K/AKT/NF-κB signaling pathway and its downstream VCAM-1 and ICAM-1 protein expression, but not dependent on the function of hemi-channels or GJs. Through our study, we believed that elevated Cx43 protein expression played an important role in the process of vascular endothelial cell injury induced by PIH. This viewpoint has never been reported before. The new mechanistic insight about of vascular endothelial cell injury of PIH obtained from the present study would provide a new basis for developing valid therapies to combat PIH, which would be beneficial for the puerpera suffering from PIH.

2. Materials and methods

2.1. Cell line and 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. We obtained HUVECs from patients with or without PIH, among which, 8 patients were with normal pregnancy, and other 8 patients were with severe preeclampsia. All patients are pregnant for the first time. Written informed consent was obtained from all study subjects previous to collection of umbilical cord. The PIH patients were in accordance with the severe preeclampsia diagnostic criteria, and without tumors, blood diseases, essential hypertension, contagious diseases and severe liver and kidney diseases. Primary HUVECs (normal HUVECs or PIH-HUVECs) were isolated from human umbilical vein as described and used between 3 and 5 passage [12,21]. The cord was severed from the placenta soon after birth, placed in PBS (Invitrogen, Carlsbad, CA, USA). The cord was inspected, and all areas with clamp marks were cut off. The umbilical vein was cannulated with a blunt 14gauge needle, and the needle was secured by clamping the cord over the needle with hemostatic forceps. The vein was perfused with 100 ml of PBS to wash out the blood and allowed to drain. The other end of the umbilical vein was clamped shut with hemostatic forceps. Then, 0.2% collagenase (Sigma-Aldrich, St. Louis, MO, USA) was infused into the umbilical vein. The umbilical cord, suspended by its ends, was placed in a water bath containing PBS and incubated at 37 °C for 20 min. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with human endothelial SFM. The cells were sedimented at 250 g for 10 min and washed twice with human endothelial SFM. The cells were resuspended by human endothelial SFM (Invitrogen), containing 20% fetal bovine serum (Invitrogen), 150 μg/ml endothelial cell growth supplement (Becton, Dickinson and Company, Frankin Lakes, NJ, USA), 100 μg/ml heparin (Sigma-Aldrich), and 100 U/ml penicillin-streptomycin (Invitrogen).

U937 monocytes were obtained from American Type Culture Collection (Manassas, VA, USA). were cultured in RPMI1640 medium (Invitrogen), supplemented with 20% fetal bovine serum (Invitrogen) and 100 U/ml penicillin-streptomycin (Invitrogen).

Both HUVECs and U937cells were cultured at 37 °C in a 5% CO_2 incubator at 90% humidity (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Cell treatments

HUVECs were pretreated with inhibitors of Cx43, oleamide (50 μM, 1 h or 24 h, Sigma-Aldrich), Gap26 (VCYDKSFPISHVR, 300 μM, 1 h, GenScript, Nanjing, China) or Gap26 scrambled peptide sequence (Gap26-SPS, VCYDQAFPISHIR, 300 μM, 1 h, GenScript). Gap26 corresponds to residues 63–75 of Cx43, which is a specific gap junction and hemichannel blocker). LY294002 (50 μM, for 24 h, Sigma-Aldrich) was used to inhibit the activity of PI3K/AKT signaling pathway, and Bay11–7082 (10 μM, for 24 h, Sigma-Aldrich) was used to inhibit NF-κB. Corresponding solvents of oleamide, LY294002 and Bay11–7082 were all DMSO (Sigma-Aldrich). After pretreatment with oleamide, Gap26, Gap26-SPS, LY294002 and Bay11–7082, HUVECs were used to test cell adhesion or relative protein expression. In order to coincident with cell adhesion assays, in all experiments, HUVECs were pretreated with recombinant mouse tumor necrosis factor (TNF-α, 20 ng/mL, Peprotech, Rocky Hill, NJ, USA) for 12 h.

2.3. U937-HUVECs adhesion assays

Before adhesion assays, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h (supplemental Fig. 1 showed that the pretreatment with TNF-α, 20 ng/mL for 12 h had no cytotoxicity on both normal HUVECs and PIH-HUVECs). U937 monocytes were labeled with 5 μmol/L calcein-acetoxymethyl ester (Invitrogen) and cultured in the incubator for 30 min. The labeled cells were washed twice with PBS (Invitrogen) and resuspended in the medium without serum. The culture of HUVECs was removed. The resuspension of labeled U937 cells were added onto confluent monolayers of HUVECs. Then, the plates were put back into the incubator. After 1 h, the plates were rinsed twice with medium without serum. Adherent U937 monocytes were left on the confluent monolayers of HUVECs. We counted the adherent U937

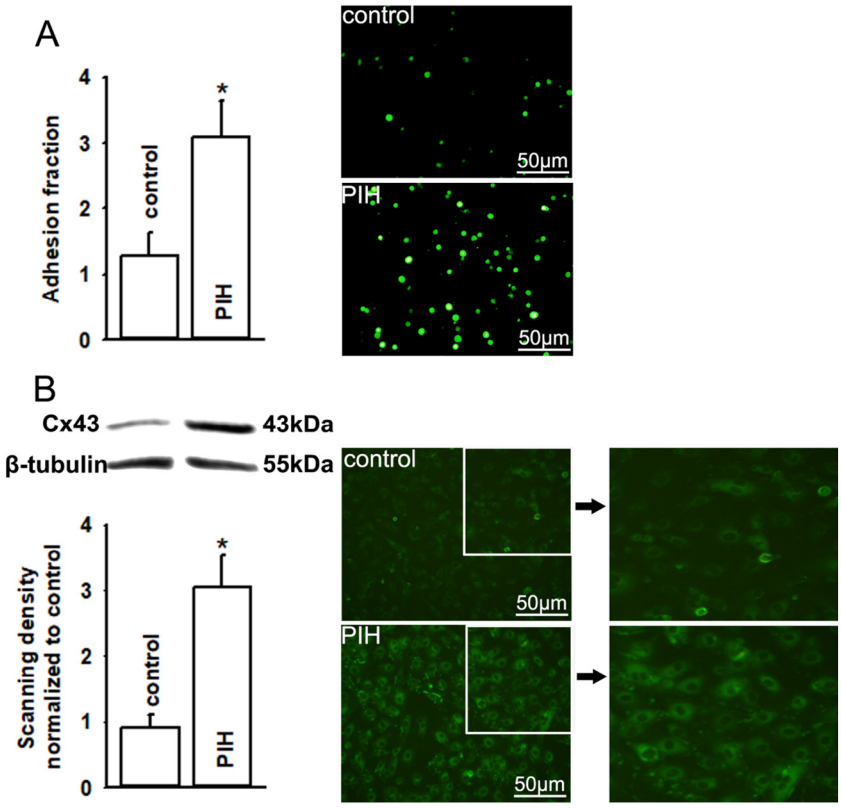

Fig. 1. U937 monocytes adhering to PIH-HUVECs was increased as Cx43 protein expression elevation on PIH-HUVECs. (A) U937 monocytes adhesion on PIH-HUVECs (as PIH) was increased compared to that on normal HUVECs (as control). U937 monocytes were labeled with calcein-AM (Data are presented as mean ± SE; $n = 8$, $^*P < 0.05$ vs control). (B) Cx43 protein expression on PIH-HUVECs (as PIH) was also increased compared to that on normal HUVECs (as control). Cx43 protein expression was determined with western blotting and immunofluorescence. (Data are presented as mean ± SE; $n = 8$, $^*P < 0.05$ vs control; image, 200×). In all experiments, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h before detections.

monocytes, labeled with calcein-acetoxymethyl ester with a fluorescence microscope (Olympus IX71, Tokyo, Japan). We divided each dish into eight quadrants according to its diameter. For each quadrant, one 200× visual field in the middle of the quadrant was chosen for analysis. The average number was considered to be the number of adherent U937 monocytes [12].

2.4. Protein detection

Cx43 was tested with western blotting and immunofluorescence. In western blotting, Cx43 expression was tested with anti-Cx43 (1:4000, mouse monoclonal Cx43 antibody raised against human; cat. no. C8093; Sigma-Aldrich) and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4000, goat polyclonal antibody raised against mouse IgG; cat. no. M6898; Sigma-Aldrich); in immunofluorescence, the dilution of anti-Cx43 is 1:2000 (mouse monoclonal Cx43 antibody raised against human; cat. no. C8093; Sigma-Aldrich). VCAM-1, ICAM-1, p-AKT, p-IKBα, and p-p65 were tested with western blotting. Both anti-VCAM-1 and ICAM-1 (mouse monoclonal Cx43 antibody raised against human; cat. no. of VCAM-1: sc20070; cat. no. of ICAM-1: sc71292; Santa Cruz biotechnology, Santa Cruz, CA, USA) were at 1:200 dilution and HRP-conjugated secondary antibodies were at 1:1000 dilution (goat polyclonal antibody raised against mouse IgG; cat. no. M6898; Sigma-Aldrich). All of the anti-p-AKT, p-IKBα and p-p65 (anti-p-AKT: rabbit polyclonal antibody raised against human, cat. no. SAB4301414, pSer129, Sigma-Aldrich; anti-AKT: rabbit polyclonal antibody raised against human, cat. no. SAB4500796, Sigma-Aldrich; anti-p-IKBα: mouse monoclonal antibody raised against human, cat. no. sc-8404, pSer32, Santa Cruz; anti-IKBα: rabbit polyclonal antibody raised against human, cat. no. SAB4501994, Sigma-Aldrich; anti-p-p65: rabbit polyclonal antibody raised against human, cat. no. SAB4504488, pSer276, Sigma-Aldrich; anti-p65: rabbit polyclonal antibody raised against human, cat. no. SAB4502607, Sigma-Aldrich) were used at 1:1000 dilution and HRP-conjugated secondary antibodies (donkey polyclonal antibody raised against rabbit IgG; cat. no. AP128P; Sigma-Aldrich) were at 1:1000 dilution. Anti-β-tubulin (mouse monoclonal β-tubulin antibody raised against human; cat. no. SAB4200715, Sigma-Aldrich) was at 1:10000 dilution and HRP-conjugated secondary antibody (1:4000, goat polyclonal antibody raised against mouse IgG; cat. no. M6898; Sigma-Aldrich) was at 1:10000 dilution. Protein band sizes were analyzed with Alpha View software (version number: 2.2.14407, Protein Simple, Santa Clara, CA, USA). In every experiment of western blotting, we normalized one of our controls as the standard, and other gray value of protein bands was compared to the standard.

2.5. "Parachute" dye-coupling assay

"Parachute" dye-coupling assay was used to test the function of GJ function. HUVECs were grown to confluence in 12-well cell culture plates. Donor cells from one well were incubated with a freshly made solution of 10 μg/ml calcein-AM (Sigma-Aldrich) in growth medium for 30 min, at 37 °C and pH 7.4. Calcein-AM is converted intracellularly into the GJ-permeable dye calcein. Unincorporated dye was removed by three consecutive washes with culture medium. Then, the donor cells were trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. The cells were permitted to attach to the monolayer of receiver cells and form GJ for 4 h at 37 °C and pH 7.4. The results were examined with a fluorescence microscope (Olympus IX71). The average number of receiver cells containing calcein around per donor cell was counted and considered as the measure of GJ function [22].

2.6. Extracellular ATP measurements

Cellular ATP release was tested with ATP bioluminescence assay kits (Sigma-Aldrich) according to the manufacturer's instructions. The supernatants of HUVEC cultures were collected on ice. Hundred microliters of supernatants were added to 100 μl ATP assay mix solution. The luminescence was determined by a fluorospectrophotometer (Cary Eclipse, FL0811M005, Bio/Chemi-luminescence mode) in 96-well

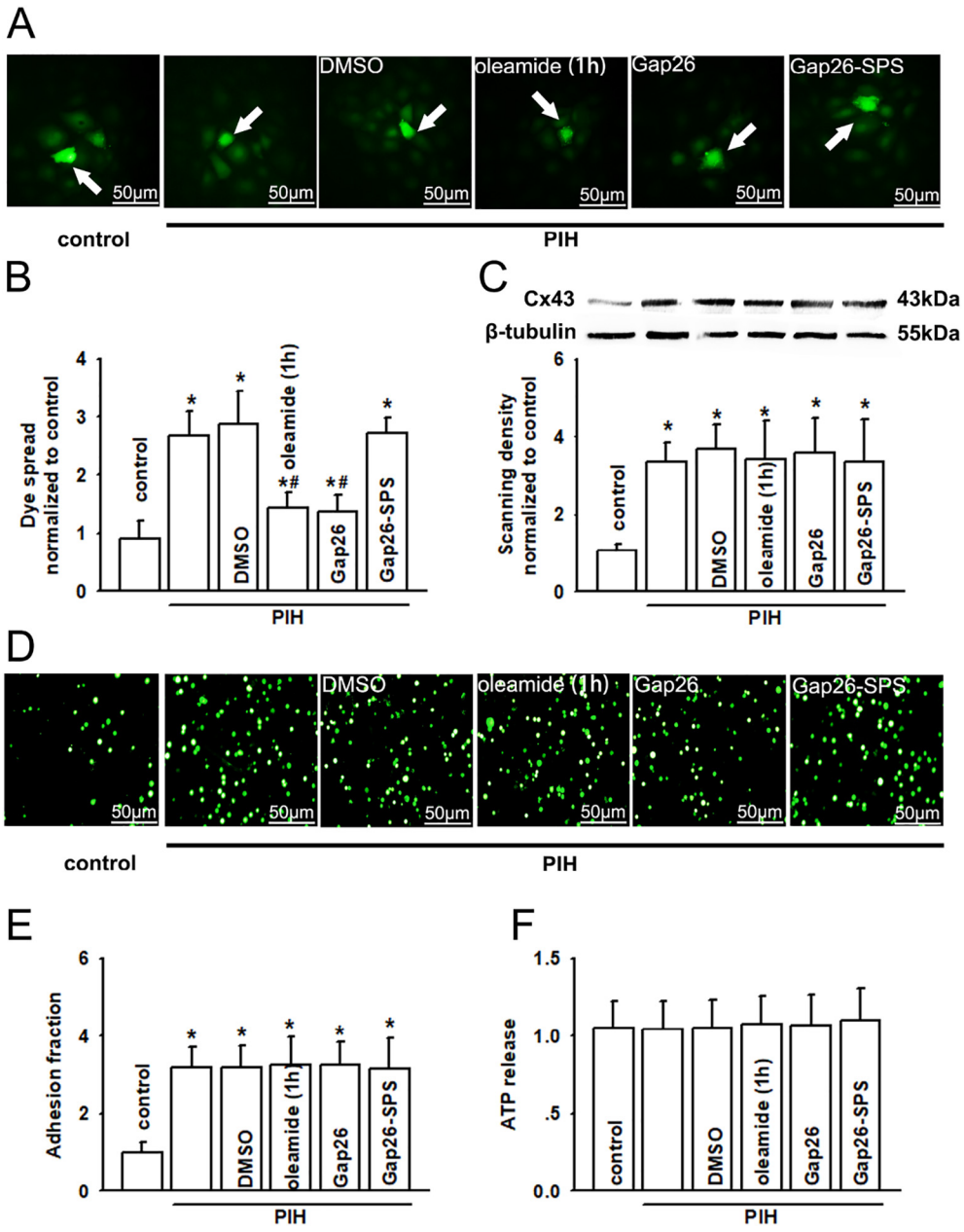

Fig. 2. Alternation of Cx43 channels function on PIH-HUVECs had no effects on cell adhesion.
(A–B) Oleamide and Gap26 inhibited Cx43 channels function. "Parachute" dye-coupling assay was used to tested the function of Cx43 channels. Both oleamide and Gap26 were the inhibitors of Cx43 channels. Oleamide (50 μM) or Gap26 (300 μM) were pretreated for 1 h. Corresponding solvent of oleamide was DMSO. →: Donor cells. The scrambled peptide sequence was showed as Gap26-SPS (300 μM, 1 h). (Data are presented as mean ± SE; $n = 8$, $^*P < 0.05$ vs PIH). (C) Oleamide (50 μM, pretreatment for 1 h) or Gap26 (300 μM, pretreatment for 1 h) pretreatment had no effects on Cx43 protein expression on PIH-HUVECs. Corresponding solvent of oleamide was DMSO. The scrambled peptide sequence was showed as Gap26-SPS (300 μM, 1 h). (Data are presented as mean ± SE; $n = 6$, $^*P < 0.05$ vs PIH). (D-E) Oleamide (50 μM, pretreatment for 1 h) or Gap26 (300 μM, pretreatment for 1 h) pretreatment had no effects on cell adhesion. U937 monocytes were labeled with calcein-AM. Corresponding solvent of oleamide was DMSO. The scrambled peptide sequence was showed as Gap26-SPS (300 μM, 1 h). (Data are presented as mean ± SE; $n = 6$, $^*P < 0.05$ vs PIH). (F) Oleamide (50 μM, pretreatment for 1 h) or Gap26 (300 μM, pretreatment for 1 h) pretreatment had no effects on ATP release from PIH-HUVECs. Corresponding solvent of oleamide was DMSO. The scrambled peptide sequence was showed as Gap26-SPS (300 μM, 1 h). (Data are presented as mean ± SE; $n = 8$, $^*P < 0.05$ vs PIH). In all experiments, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h before detections.

culture plates [12].

2.7. Cx43 knock-down

SiRNAs targeting to the human Cx43 gene (CAGUCUGCCUUUCGU UGUA) and a nonspecific control siRNA (as NC in the figures) were used to transfect HUVECs in the experiments. Transfection into HUVECs was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The knockdown efficiency was assessed by western blotting [15].

2.8. Statistical analysis

Statistical analysis was performed by using SPSS 15.0 software (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

1. Cell adhesion and Cx43 protein expression were both increased when HUVECs were from patients with PIH.

Fig. 1A showed that cell adhesion on PIH-HUVECs was increased compared to that on HUVECs from patients without PIH. And more importantly, Cx43 protein expression was also increased significantly on PIH-HUVECs. Locally enlarged images showed that Cx43 protein expression on cell membranes of PIH-HUVEC cells was also significantly higher than that on normal HUVEC cells (Fig. 1B). The results prompt us that alternation of Cx43 protein expression on HUVECs might be relative with cell adhesion.

2. Function of Cx43 channels on PIH-HUVECs had no effects on cell adhesion.

According to the reports, in different situations, Cx43 protein itself, hemi-channels or GJ all could affect cell adhesion [13]. Thus, we used

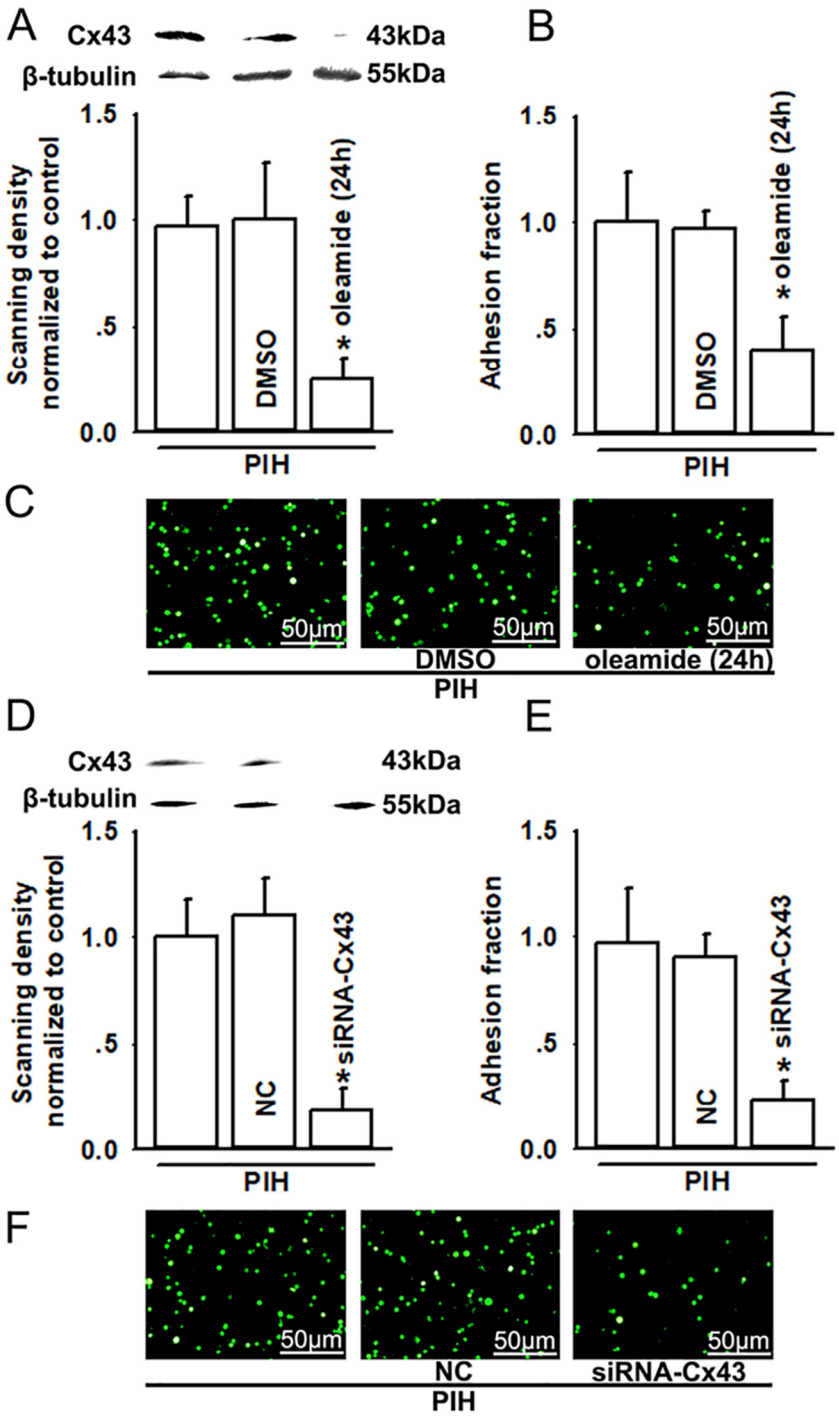

Fig. 3. Inhibition of Cx43 protein expression on PIH-HUVECs attenuated cell adhesion.
(A) Oleamide (50 μM, pretreatment for 24 h) attenuated Cx43 protein expression on PIH-HUVECs. Corresponding solvent of oleamide was DMSO. (Data are presented as mean ± SE; $n = 5$, $^*P < 0.05$ vs PIH). (B–C) Oleamide (50 μM, pretreatment for 24 h) attenuated cell adhesion. Corresponding solvent of oleamide was DMSO. U937 monocytes were labeled with calcein-AM. (Data are presented as mean ± SE; $n = 4$, $^*P < 0.05$ vs PIH). (D) Specific siRNA-Cx43 pretreatment attenuated Cx43 protein expression on PIH-HUVECs. NC: negative control. (Data are presented as mean ± SE; $n = 3$, $^*P < 0.05$ vs PIH). (*E*-F) Specific siRNA-Cx43 pretreatment attenuated cell adhesion. NC: negative control. U937 monocytes were labeled with calcein-AM. (Data are presented as mean ± SE; $n = 3$, $^*P < 0.05$ vs PIH). In all experiments, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h before detections.

oleamide (pretreated 1 h) and Gap26 to inhibit the function of hemichannels or GJ on PIH-HUVECs and observe effects of them on cell adhesion. Fig. 2A and B demonstrated that oleamide (pretreated 1 h) and Gap26 effectively attenuated Cx43 channels function, manifested as the dye transfer was down-regulated obviously (Gap26-SPS, the scrambled peptide sequence had no effects on dye transfer), but had no effects on Cx43 protein expression on PIH-HUVECs and cell adhesion (Fig. 2C). And even more, Cx43 channels inhibition with oleamide (pretreated 1 h) and Gap26 did not influence cell adhesion (Fig. 2D and E) or ATP release (Fig. 2F). ATP release was always considered to be important in cell adhesion [12]. Thus, we concluded that Cx43 channels function on PIH-HUVECs had no relationship with cell adhesion.

3. Inhibition Cx43 protein expression on PIH-HUVECs attenuated cell adhesion.

When PIH-HUVECs were pretreated with oleamide (24 h), Cx43 protein expression was depressed significantly (Fig. 3A), which was consistent with our previous study [12]. As Cx43 protein expression was down-regulated, cell adhesion was also reduced (Fig. 3B and C). These results demonstrated that inhibition Cx43 protein expression on PIH-HUVECs could attenuate cell adhesion. This conclusion was also proved when Cx43 protein expression on PIH-HUVEC s was knocked down with specific siRNA (Fig. 3D to F).

4. Inhibition Cx43 protein expression on PIH-HUVECs could down-

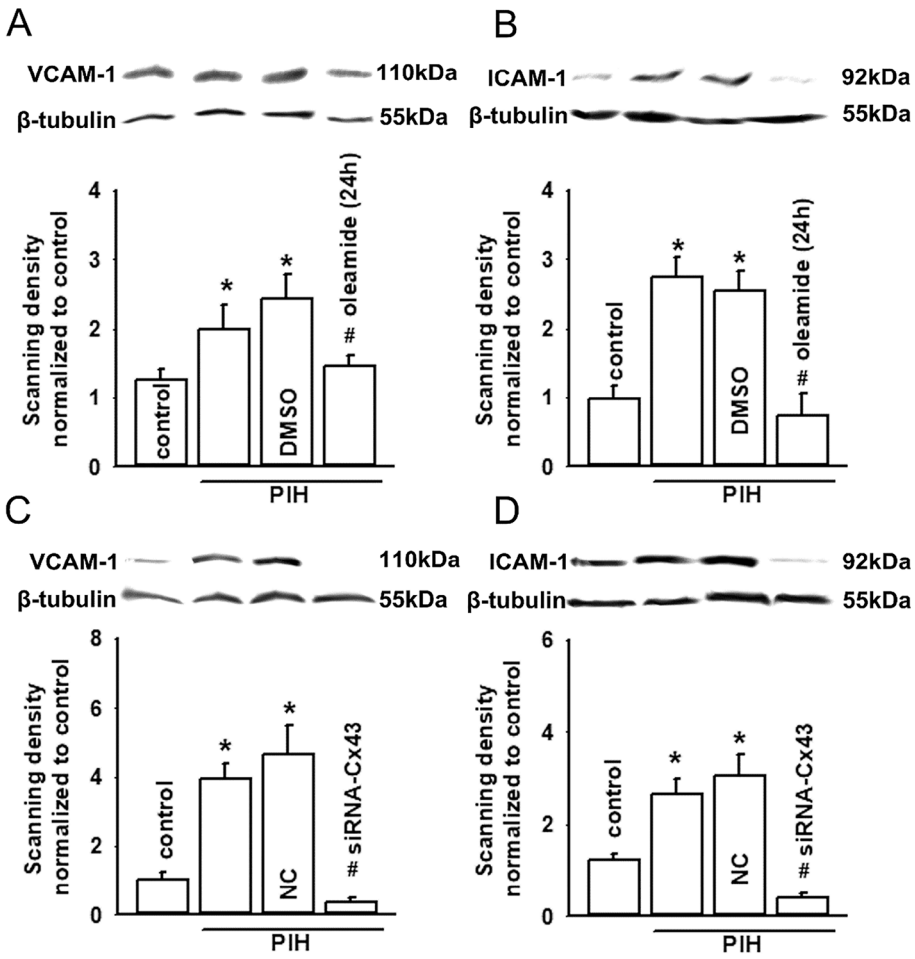

Fig. 4. Inhibition of Cx43 protein expression on PIH-HUVECs attenuated VCAM-1 and ICAM-1 protein expression.
(A–B) Oleamide (50 μM, pretreatment for 24 h) attenuated VCAM-1 and ICAM-1 protein expression on PIH-HUVECs. Corresponding solvent of oleamide was DMSO. (Data are presented as mean ± SE; $n = 5$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). (C–D) Specific siRNA-Cx43 attenuated VCAM-1 and ICAM-1 protein expression on PIH-HUVECs. NC: negative control. (Data are presented as mean ± SE; $n = 5$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). In all experiments, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h before detections.

regulate protein expression of VCAM-1 and ICAM-1.

Cell adhesion alternation was always related to the changes of adhesion molecules expression. Therefore, we examined the effect of changes in Cx43 protein expression on VCAM-1 and ICAM-1 [23,24], two of the most important adhesion molecules. Fig. 4 showed that as Cx43 protein expression on PIH-HUVECs was decreased with oleamide (pretreated 24 h) or specific siRNA, both VCAM-1 and ICAM-1 protein expression were reduced obviously. These results indicated that reduction of cell adhesion mediated by Cx43 protein expression inhibition might be via regulating adhesion molecules expression.

5. Cx43 down-regulation inhibited the activity of PI3K/AKT/NF-κB signaling pathway on PIH-HUVECs.

In order to explore the underlying mechanism, we mainly tested the activity changes of PI3K/AKT/NF-κB signaling pathway, which was always considered to play an important in cell adhesion [25,26]. Fig. 5A showed that the key molecules of PI3K/AKT/NF-κB signaling pathway, including p-AKT, p-IKBα, and p-p65 were all attenuated as Cx43 protein expression was reduced with siRNA-Cx43 (the corresponding total proteins were not affected by siRNA-43), which indicated that Cx43 protein expression on PIH-HUVEC cells could affect the activity of PI3K/AKT/NF-κB signaling pathway. Then, PIH-HUVECs were pretreated with LY294002 (a classic PI3K inhibitor). As p-AKT was decreased by LY294002, the expression of p-IKBα and p-p65 were also down-regulated. However, NF-κB inhibitor, Bay11–7082 had no influence on PI3K/AKT (Fig. 5B). The corresponding total proteins, AKT, IKBα and p65 were not affected by LY294002 and Bay11–7082.

6. Inhibition the activity of PI3K/AKT/NF-κB signaling pathway on PIH-HUVECs attenuated VCAM-1 and ICAM-1 protein expression and cell adhesion.

Fig. 6 showed that both LY294002 and Bay11–7082 could reduce VCAM-1 and ICAM-1 protein expression on PIH-HUVECs (Fig. 6A and B), and even attenuate cell adhesion (Fig. 6C and D). These results mean that the activity of signaling pathway of PI3K/AKT/NF-κB on PIH-HUVECs could regulate cell adhesion via altering VCAM-1 and ICAM-1 protein expression. Combined with the results obtained from Fig. 5 that inhibition of Cx43 protein expression attenuated the activity of PI3K/AKT/NF-κB signaling pathway on PIH-HUVECs, we concluded that alternation of Cx43 protein expression mediated cell adhesion through regulating the activity of PI3K/AKT/NF-κB signaling pathway.

4. Discussion

As far as we know, PIH is a very important cause of maternal and fetal death or neonatal death, which not only causes serious complications during perinatal period, but also increases the incidence of cardiovascular and cerebrovascular diseases, seriously affecting the health and safety of pregnant women [27,28]. The main pathogenesis of PIH is vasospasm, which leads to the injury of vascular endothelial cells and releases a large number of vasoactive substances, aggravating vasospasm and elevating blood pressure [29]. In our present study, we obtained HUVECs from patients with or without PIH and found that Cx43 protein expression was increased obviously on PIH-HUVECs, which might be an important factor of vascular endothelial cell injury. We admitted that there was a limitation about the small sample size in normal HUVECs or in the PIH-HUVECs ($n = 8$ in each group). Although

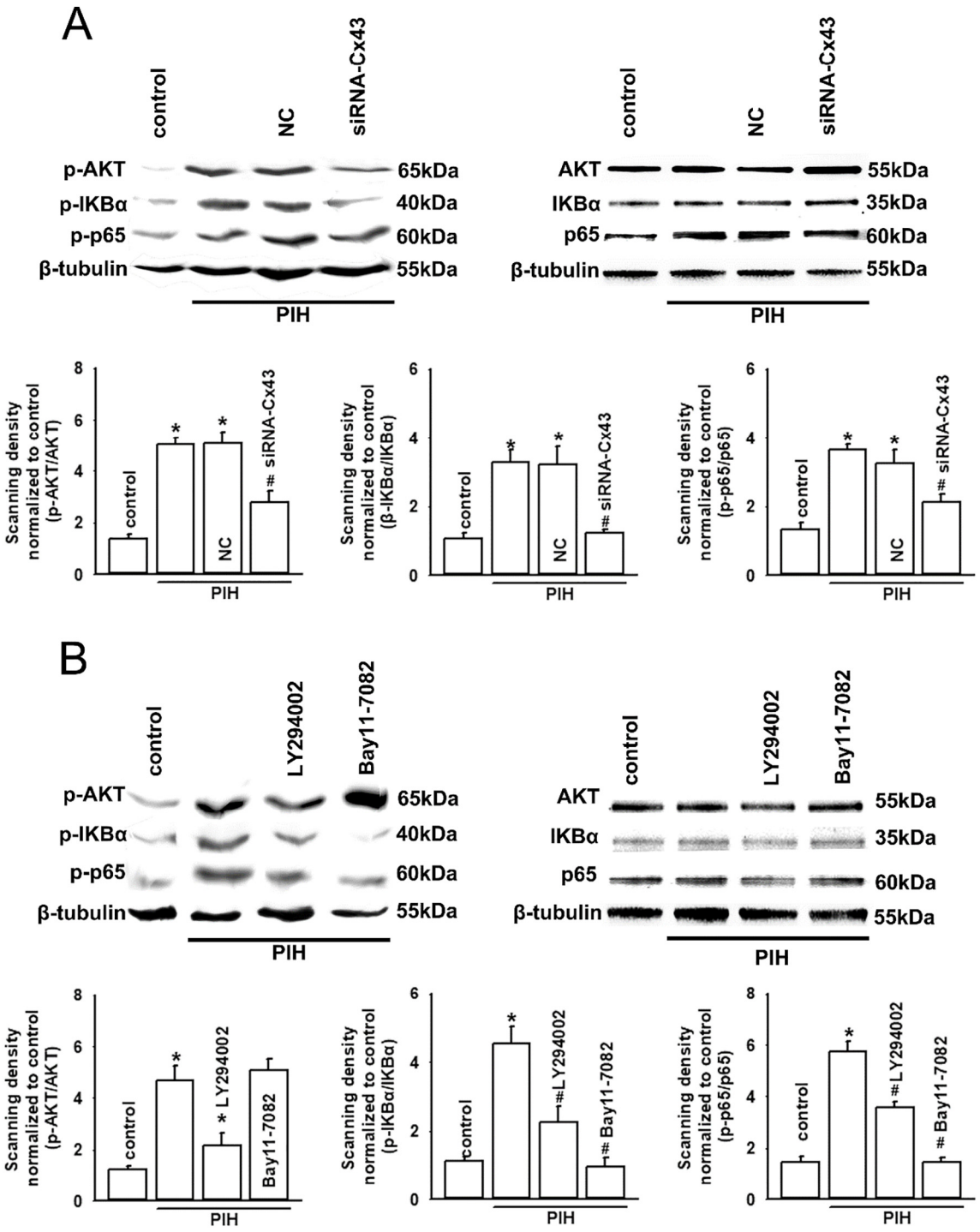

Fig. 5. Inhibition of Cx43 protein expression on PIH-HUVECs attenuated the activity of PI3K/AKT/NF-κB signaling pathway.
(A) P-AKT, p-IKBα and p-p65 protein expression were all attenuated on PIH-HUVECs when Cx43 protein expression was knocked-down by specific siRNA-Cx43. In contrast, the corresponding total proteins were not affected by siRNA-43 (Data are presented as mean ± SE; $n = 5$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). NC: negative control. (B) P-AKT, p-IKBα and p-p65 protein expression were all attenuated on PIH-HUVECs when PIH-HUVECs were pretreated with LY294002 (50 μM, pretreatment for 24 h). (Data are presented as mean ± SE; $n = 5$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). P-IKBα and p-p65 protein expression were both attenuated on PIH-HUVEC s when PIH-HUVECs were pretreated with Bay11–7082 (10 μM, pretreatment for 24 h), but P-AKT protein expression was not affected by Bay11–7082. (Data are presented as mean ± SE; $n = 5$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). In contrast, the corresponding total proteins were not affected by LY294002 and Bay11–7082. Corresponding solvent of LY294002 and Bay11–7082 was DMSO, and DMSO had no effects on P-AKT, p-IKBα and p-p65 protein expression on PIH-HUVECs (Supplemental Fig. 2). In all experiments, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h before detections. Histograms were made according to the ration of phosphorylated protein/total protein.

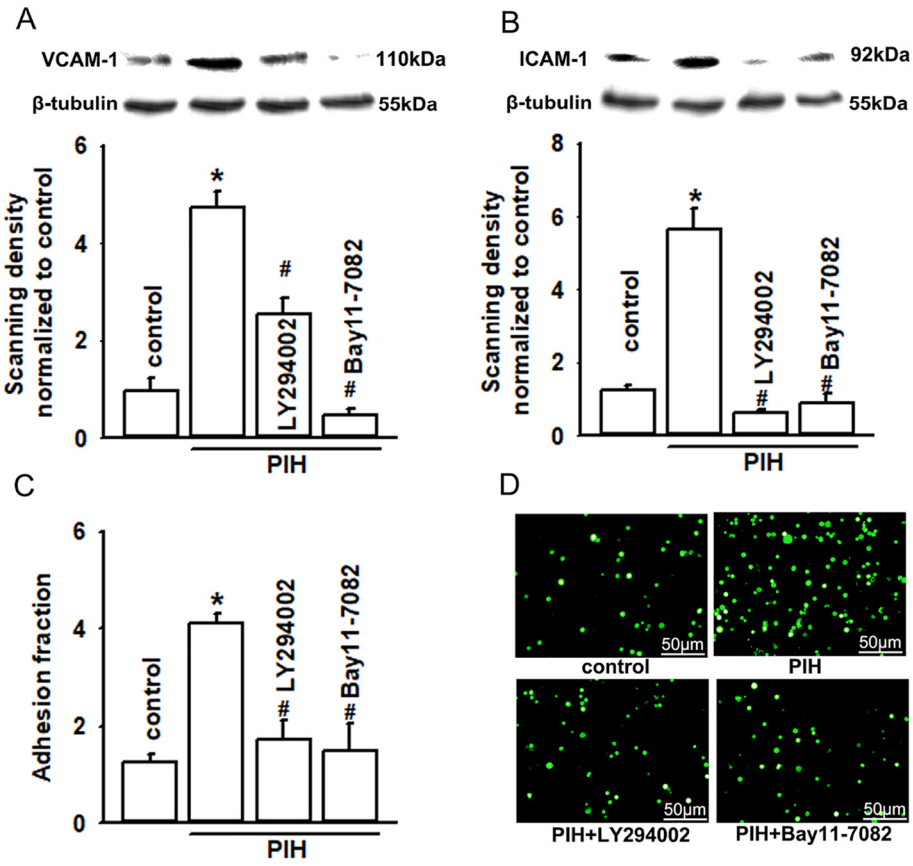

Fig. 6. Inhibition of the activity of PI3K/AKT/NF-κB signaling pathway on PIH-HUVECs attenuated VCAM-1 and ICAM-1 protein expression, and cell adhesion.
(A-B) LY294002 (50 μM, pretreatment for 24 h) and Bay11–7082 (10 μM, pretreatment for 24 h) pretreatment attenuated VCAM-1 and ICAM-1 protein expression on PIH-HUVECs (Data are presented as mean ± SE; $n = 3$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). (C-D) LY294002 (50 μM, pretreatment for 24 h) and Bay11–7082 (50 μM, pretreatment for 24 h) pretreatment attenuated cell adhesion. U937 monocytes were labeled with calcein-AM. (Data are presented as mean ± SE; $n = 3$, $^*P < 0.05$ vs control; #P < 0.05 vs PIH). Corresponding solvent of LY294002 and Bay11-7082 was DMSO, and DMSO had no effects on VCAM-1 and ICAM-1 protein expression on PIH-HUVECs (Fig. 4A and B). In all experiments, HUVECs were pretreated with TNF-α, 20 ng/mL for 12 h before detections.

the sample size is small, we still find the significant difference in Cx43 expression and cell adhesion between the normal HUVECs and the PIH-HUVECs (Fig. 1). We believe that our current study could prompt the importance of increased Cx43 expression in PIH-HUVECs. Certainly, in future study, we consider to increase the number of samples to further confirm our conclusion.

In current study, when we depressed Cx43 protein expression on PIH-HUVECs, U937 monocytes adhering to PIH-HUVECs was attenuated significantly, and even more, Cx43 downregulation effectively inhibited the activity of PI3K/AKT/NF-κB signaling pathway and its downstream VCAM-1 and ICAM-1 protein expression, all of which improved vascular endothelial cell injury. It could be seen that the increased protein expression of Cx43 on PIH-HUVECs played an important role in the pathophysiology of PIH, and Cx43 might also be a potential target for prevention of vascular endothelial cell injury of PIH.

Connexin expressed in almost all human organs and tissues and exerted different pathological and physiological functions according to their characteristics. As reported, there were mainly three different types of connexins expressed on HUVECs, Cx37, Cx40 and Cx43 [30,31]. Our previous study had demonstrated that Cx43 protein expression was higher than that of Cx37 and Cx40 on HUVECs, and even its effect on cell adhesion were specific and distinctive, independent of Cx37 or Cx40 [13]. Thus, we mainly explored influence of Cx43 on cell adhesion in present investigation for PIH.

Cx43 usually existed in HUVECs in three different forms, Cx43 protein itself, hemi-channels and GJs composed of Cx43, all of which could exert different effects on cell adhesion [32]. According to the reports that hemi-channels composed of Cx37 promotes ATP release, which reduced monocyte adhesion to endothelial cells [33], and Cx40-mediated GJ communication contributed to the attenuation of leukocyte adhesion to the endothelium [34]. We used oleamide (pretreated 1 h) and Gap26 (both having no effects on Cx43 protein expression) to inhibit function of hemi-channels or GJs composed of Cx43 on PIH-HUVECs. Results showed that cell adhesion as well as ATP release, had no changes (Fig. 2). These results indicated that on PIH-HUVECs, effects of Cx43 on cell adhesion did not depend on hemi-channels or GJs composed of Cx43.

In our present study, our mainly findings were that Cx43 protein expression was much higher on PIH-HUVECs than that on normal HUVECs, and depression of Cx43 protein expression could attenuate cell adhesion via down-regulation the activity of PI3K/AKT/NF-κB signaling pathway mediating downstream VCAM-1 and ICAM-1 protein expression. We speculated that during the development of PIH, Cx43 protein expression was increased on vascular endothelial cells. The carboxyl-terminal domain of Cx43 also could interact with some special elements of cellular signaling pathways, such as Src, PKC and PKA, which provided the possibility that changes of Cx43 protein expression affecting the activity of other signaling pathways [35,36]. And our results demonstrated that p-AKT, p-IKBα and p-p65 were all decreased along with the down-regulation of Cx43 protein expression on PIH-HUVECs (Fig. 5). These results indicated that both PI3K/AKT and NF-κB signaling pathways participated in this process of Cx43 modulating cell adhesion. Along with the activation of PI3K/AKT/NF-κB signaling pathway and its downstream VCAM-1and ICAM-1 protein expression increase on PIH-HUVECs, cell adhesion was increased significantly, which might lead to changes in hemodynamics and aggravation of vasospasm [37,38]. This vicious cycle would lead to further deterioration of vascular endothelial cell injury. Therefore, we speculated that breaking this vicious cycle would help reduce cell adhesion and alleviate vascular endothelial cell injury, which would be beneficial for the puerpera suffering from PIH. Our findings also supported this hypothesis, for example, inhibition of Cx43 protein expression on PIH-HUVECs, the activity of PI3K/AKT/NF-κB signaling pathway and its downstream VCAM-1and ICAM-1 protein expressions were all reduced, and cell adhesion was also attenuated (Fig. 5 and 6).

We firstly reported that Cx43 protein expression on PIH-HUVECs

was much higher than that on normal HUVECs, which might be involved in hypertension and increased estrogen. It had been confirmed that hypertension could lead to the increase of Cx43 protein expression in cardiovascular system. For example, Cx43 played an important part in hypertension-mediated inflammation and participated in this pathological process in many different ways, such as regulating the homeostasis of T-lymphocyte and the production of cytokines [39]. And our research indicated that inhibition of Cx43 protein expression attenuated the activity of PI3K/AKT/NF-κB signaling pathway mediating VCAM-1and ICAM-1, which could reduce PIH-HUVECs injury and improve hemodynamics indirectly. Another possible factor contributing to the increase of Cx43 protein expression might be the elevation of estrogen during pregnancy [40,41]. Mitchell JA reported that estrogen could initiate transcription of Cx43 gene via up-regulating expression of c-Jun/c-Fos and AP-1 [42]. Thus, we speculated that in the pathogenesis of PIH, elevated blood pressure due to vasospasm and elevated estrogen during pregnancy lead to an abnormal increase of Cx43 protein expression, which mediated vascular endothelial cell injury, manifested as the activation of inflammatory related PI3K/AKT/NF-κB signaling pathway, the increase of VCAM-1 and ICAM-1 protein expression and even the enhancement of cell adhesion.

As far as we know, lots of factors are relative with cell adhesion. In our current study, monocytes stimulation or TNF-α pretreatment are the most likely interfering factors affecting cell adhesion. In our cell adhesion assay, before resuspension of U937 cells was added to the confluent monolayers of HUVECs, the culture of HUVECs containing TNF-α was removed. U937 monocytes were not activated. Thus, effects of monocytes stimulation on cell adhesion could be excluded. Throughout our Supplemental Fig. 3, we could conclude that (1) the increase of Cx43 expression in PIH-HUVECs is not relative with TNF-α; (2) although TNF-α pretreatment could increase the expression of VCAM-1 and ICAM-1 or the PI3K/AKT/NF-κB signaling pathway in normal HUVECs, it had no effects on those protein expression on PIH-HUVECs. Therefore, we believe that the reason that cell adhesion on PIH-HUVECs is much higher than that on normal HUVECs is because of increased Cx43 expression on PIH-HUVECs, but not because of monocytes stimulation or TNF-α pretreatment.

5. Conclusions

From the above analysis, we could find that Cx43 was an important link of vascular endothelial cell injury induced by PIH. When cells were exposed to harmful factors, Cx43 located in the cell membrane might change before the intracellular signaling pathways, thus, we have reasons to believe that abnormal elevation of Cx43 protein expression might be the “motor” of vascular endothelial cell injury during PIH.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

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