Original Article

Inhibition of ERK or Akt ameliorates intimal hyperplasia via up-regulation of Cx37 and down-regulation of Cx43 in balloon injury rat model

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Background: Connexins (Cxs) are reported to participate in atherosclerosis associated intimal hyperplasia (IH), while their function involved in the balloon injury (BI) induced IH and restenosis is less reported.

Methods: Forty-eight male Sprague-Dawley rats were randomly assigned to not injured (NI) group and BI group, which were further administrated with ERK-inhibitor U0216 and Akt-inhibitor MIK2206. Western blot and RT-PCR were utilized to detect the expression of Cx30, Cx37, Cx40, and Cx43 at 6 hours, 24 hours, 7 days, and 14 days post-surgery. H&E staining and related intima area, media area, and luminal area measurement were applied to indicate neointima formation and IH. ERK and Akt phosphorylation levels and proliferating cell nuclear antigen (PCNA) immunostaining were also detected.

Results: Among the four Cxs detected, Cx37 showed down-regulated, and Cx43 showed up-regulated temporal expression pattern in BI rats with confirmed neointima formation. Up-regulated p-ERK ($P<0.01$) and p-Akt ($P<0.01$) can be detected in BI rats compared with NI rats. Meanwhile, U0216 and MIK2206 can significantly reduce Cx43 expression and increase CX37 expression accompanied with reduced neointima formation and PCNA staining ($P<0.05$ or $P<0.01$) in BI rats.

Conclusions: ERK or Akt inhibition can alleviate BI-induced IH via up-regulation of Cx37 and down-regulation of Cx43.

Keywords: ERK; Akt; Cx37; Cx43; intimal hyperplasia (IH)

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Introduction

Vascular endothelial injury can trigger the development of atherosclerosis, carotid artery stenosis, and percutaneous coronary intervention (PCI) induced restenosis, which could be attributed to the occurrence of intimal hyperplasia (IH) (1,2). When the vascular endothelium is injured, vascular smooth muscle cells (VSMCs) can migrate from the medial to the intimal layer, proliferate, and secrete extracellular matrix to form restenotic lesions (3). Although drug-eluting stents are designed to decrease the incidence of mechanical injury associated stenosis, it remains a bane to high-risk patients with atherosclerosis, hypertension, and diabetes. It is estimated that almost 40–50% of patients with stenosis will endure restenosis within one year after angioplasty intervention at previous atheromatous lesions. Therefore, it is vital to understand the mechanisms underlying the IH process to develop novel approaches to control the restenosis.

Heterocellular communications, such as gap junction, between VSMCs and endothelial cells (ECs) are essential to the function of the vascular wall (4,5). It is further testified that gap junctions can be homotypic or formed from combinations of the 21 connexin isoforms. In the development of atherosclerosis, a progressive change in connexins (Cxs) expression can be observed at the point of contact between EC and VSMC, and altered Cxs expression pattern can be detected in atherosclerotic plaques (6-8). All of these indicate that Cxs take part in the development of atherosclerosis, whether such molecules are also involved in the balloon injury (BI) induced IH and restenosis is less reported.

In this investigation, restenosis after BI is utilized as a model to decipher the relevant mechanism associated with Cxs in the development of IH.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/cdt-20-345).

Methods

Animals

Male Sprague-Dawley rats (250 g) were purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China), which were maintained with a standard 12 hours light-dark cycle. All experimental procedures and animal feeding were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University (#WZYKDX-311), in compliance with institutional guidelines of Wenzhou Medical University for the care and use of animals.

Induction of BI rat model

In this research, 48 rats were randomly assigned into BI group and not injured (NI) group. BI rats were anesthetized with isoflurane, and the left external carotid artery was exposed and temporally blocked with arterial clamps on both proximal and distal sides (9). A balloon catheter (ϕ =1.5 mm, Sprinter Legend™, Medtronic, Inc., MN, USA) was inserted into the lumen and inflated to 6 atm (Dolphin inflation device, Perouse Medical, France), which was further pulled back and forth three times for a short distance to produce endothelial denudation (10). NI rats underwent a sham surgery was utilized as a control. Pain relievers and antibiotics were given subcutaneously, and the rats were monitored routinely during recovery. Rats were sacrificed by cervical dislocation at 6 hours, 24 hours, 7 days, and 14 days post the surgery for further assessments.

Histopathological assessment

After harvest, carotid arteries were fixed with paraformaldehyde (4%) and embedded with paraffin. Serial cross-sections (2 μm) were performed along the entire injured segment, then hematoxylin and eosin (H&E) staining was utilized to detect neointimal hyperplasia. Intima, media, and lumen areas quantification was obtained with NIH-Image J1.51p 22 (National Institutes of Health, Bethesda, MD, USA).

Proliferating cell nuclear antigen (PCNA) was stained in carotid sections. Microwave-heating was applied for antigen retrieval, and nonspecific blocking was performed by 4% normal goat serum plus 1% bovine serum albumin (BSA) in phosphate-buffered saline for 30 min. Then, an anti-rat PCNA rabbit polyclonal antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used to incubate the sections. Biotinylated secondary antibody, avidin:biotinylated enzyme complex, and 3,3'-diaminobenzidine substrate (Zhongshan Goldenbridge Biotechnology, Guangzhou, China) were utilized to develop the signal. Finally, the slides were counterstained with hematoxylin and analyzed.

Treatment

Each group (N=6) BI and NI rats were administrated with U0216 (ERK-inhibitor, 50 mg/kg body weight) or MIK2206 (Akt-inhibitor, 100 mg/kg body weight) via tail vein per day until day 14, which were then sacrificed for assessment. The dose chosen was based on the previous report (11).

Western blots

Carotid tissues were homogenized with IKA disperser in SDS-lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 1% SDS), which was further separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinyl difluoride membrane. After block with 5% non-fat dry milk, the membranes were incubated with the primary antibodies of Cx30, Cx37, Cx40, Cx43, ERK, AKT, phospho (p)-ERK and (p)-AKT (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:1,000 dilution (4 ℃, overnight), which were further incubated in peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) at a 1:1,000 dilution for 1 hour at room temperature. The signal

was developed with an ECL system (GE Healthcare Life Sciences, Chalfont, UK). After intensity normalization to its respective β-actin, the ratio of phosphorylated over total protein expression was calculated with Image J software.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from carotid tissues (n=6 per group) with TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was utilized to reverse-transcribed cDNA from 1 μg RNA. SYBR Green master mix (Roche, Mannheim, Germany) was adopted to indicate the amplification efficiency, and the reaction procedures were as follows: 95 ℃ for 10 min, 40 cycles of 95 ℃ for 15 s, and 60 ℃ for 1 min. Interest genes expressions were normalized to β-actin mRNA expression. Primer sequences were listed: β-actin, forward primer 5'-CCCGCGAGTACAACCTTCT-3', reverse primer 5'-CGTCATCCATGGCGAACT-3'; CX30, 5'-ATCTTCCGCATCCTGGTG-3', reverse primer 5'-AGTGACAGAGTGGCAGGTCA-3'; CX37, forward primer 5'-CGGTCGTCCCCTCTACCT-3', reverse primer 5'-GTCGAGTGCTCCTGGACCT-3'; CX40, forward primer 5'- AAGGCTTAGTTAGCATCACAGAAAG-3', reverse primer 5'-TGGACCCCAGAGTTCAATG-3'; CX43, forward primer 5'-GAGCCTGAACTCTCATTTTTCC-3', reverse primer 5'-CCATGTCTGGGCACCTCT-3'.

Statistical analysis

All experiments were quantitatively analyzed with GraphPad Prism 6, and results were reported as the mean ± SEM and analyzed by Student's *t*-test or one-way ANOVA analysis followed by a Tukey's *post hoc* test. P<0.05 was considered to be statistically significant.

Results

Temporal expression of Cxs

Both BI and NI rats kept healthy after the surgery without significant weight loss during the whole period of this investigation. Every 6 rats of the two groups were sacrificed at 6 hours, 24 hours, 7 days, and 14 days post the surgery for Cx30, Cx37, Cx40, and Cx43 assessments. Western blot results indicated that Cx37 showed decreased expression pattern as time passed and Cx43 showed increased expression trend, while there was no significant difference in Cx30 and Cx40 (*Figure 1A*,*B*) (P<0.01 or P<0.001). In accordance with this, the relative mRNA expression of Cx30, Cx37, Cx40, and Cx43 also showed the same expression trend (*Figure 1C*). All of these indicated that among the four Cxs detected, only Cx37 and Cx43 were differentials expressed during the whole process after BI.

BI-induced neointima formation

According to the results of Cxs expression, neointima formation determined by H&E staining was observed 14 days after BI (6 rats in each group). There was no development of IH could be observed in rats received sham surgery, and significantly increased neointimal formation could be easily identified in the BI group (*Figure 2A*). Quantitative morphometry estimation exhibited notable increases in the intimal area (*Figure 2B*, P<0.01). Nevertheless, there was no significant difference in the media areas between BI group and NI group (*Figure 2C*). The luminal areas were decreased in the BI group compared with the NI group (*Figure 2D*, P<0.01), while a significant increase could be found in intima-to-media (I/M) ratio between the two groups (*Figure 2E*, P<0.01).

Up-regulated ERK and Akt expression

Mitogen-activated protein kinase (MAPK, also known as ERK) pathway and PI3K/Akt pathway are testified to be essential in neointima formation (12,13). In this investigation, total ERK, p-ERK and p-Akt showed up-regulated expression in BI rats (*Figure 3A*), and quantification of the Akt and ERK phosphorylation levels also showed up-regulated expression when compared with NI rats (*Figure 3B*, P<0.01). Administration with Akt-inhibitor MIK2206 could significantly down-regulate the expression of Cx43 (*Figure 3C*,*D*, P<0.01) and up-regulate the expression of Cx37 (*Figure 3C*,*E*, P<0.01). Meanwhile, ERK-inhibitor U0216 could also significantly down-regulate the expression of Cx43 (*Figure 3C*,*D*, P<0.01) and up-regulate the expression of Cx37 (*Figure 3C*,*E*, P<0.001). Taken together, these results indicated that the MAPK and Akt pathway could regulate the expression of Cx37 and Cx43.

ERK and Akt mediated neointima formation and cell proliferation

Neointima formation assayed by H&E staining was utilized

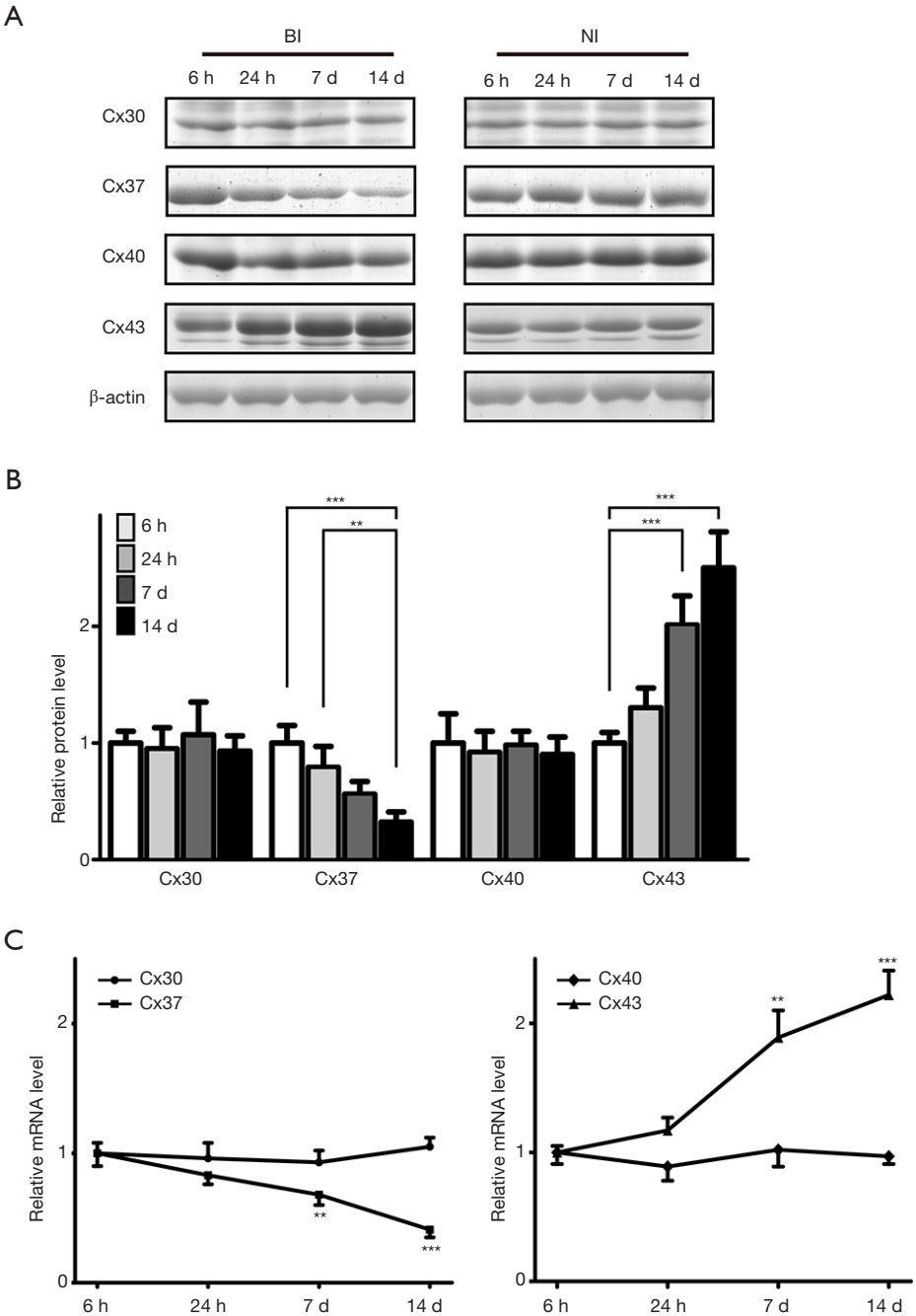

Figure 1 Temporal expression of connexins in the rat carotid artery after balloon injury. 48 male rats were divided into two groups, BI group (n=24) that accepted balloon injury and NI group (n=24) without injury as the control. And every 6 rats of the two groups were sacrificed at 6 hours, 24 hours, 7 days and 14 days after BI surgery for assessments. (A) Western blotting analysis of four kinds of connexins. Protein levels of Cx30, Cx37, Cx40, and Cx43 were analyzed, and β-actin was utilized as a control. (B) Quantification of Western blotting data. Connexin/β actin of BI groups was normalized against that of NI groups. **, P<0.01; ***, P<0.001. (C) Analysis of mRNA levels of the connexins by qRT-PCR. The mRNA levels of connexins were first normalized to β-actin mRNA levels, and the values were further normalized to the value of the respective 6-hour group for comparison. Normalized mRNA levels of Cx30 and Cx37 of BI groups were shown in the left-panel, and those of Cx40 and Cx43 were shown in the right panel. **, P<0.01; ***, P<0.001, versus 6 h groups. Bar graph indicates mean ± SEM. BI, balloon injury; NI, not injured.

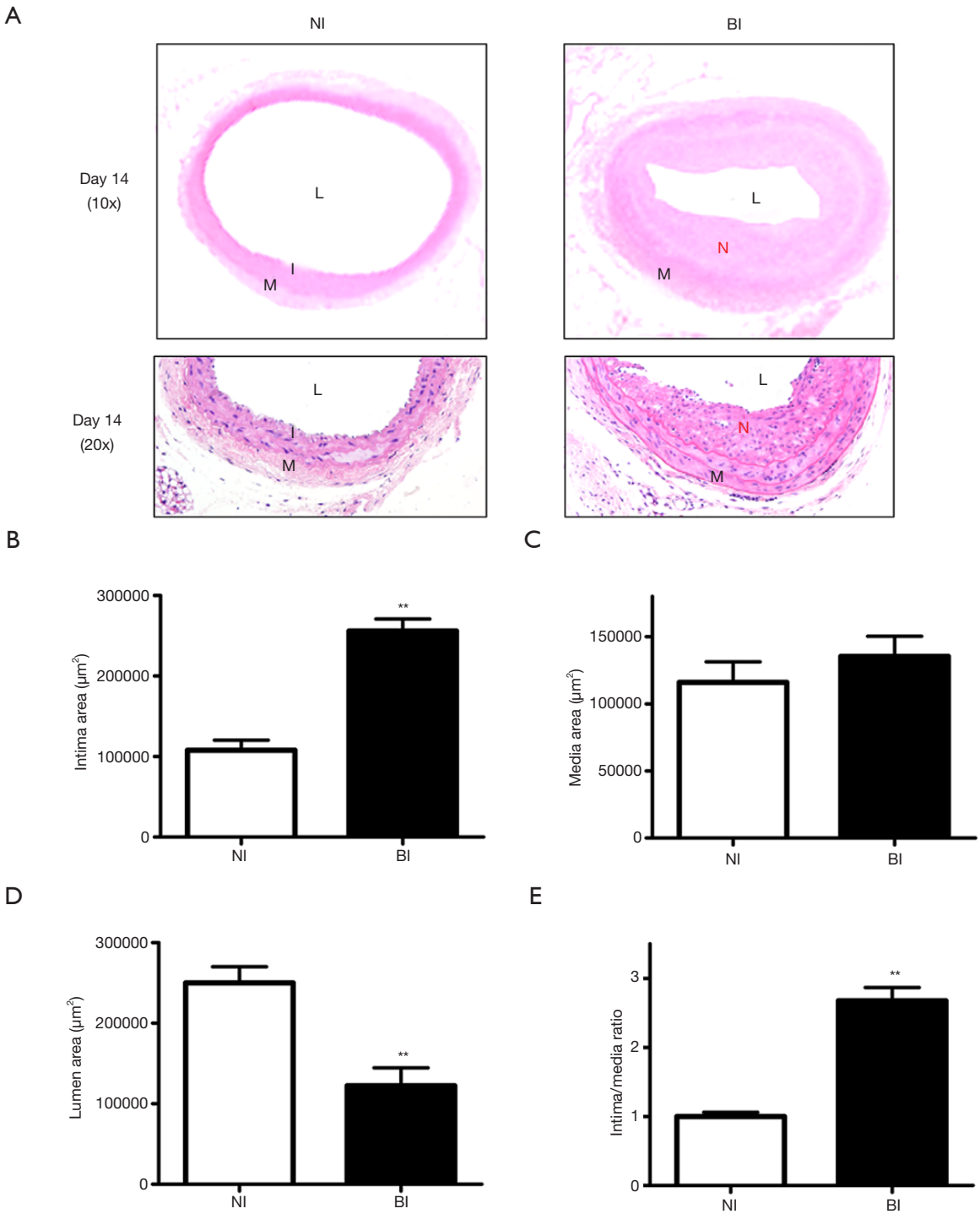

Figure 2 Neointima formation was enhanced after balloon injury. Each group (N=6) of BI and NI rats were sacrificed on day 14 for assessment. (A) Representative H&E staining photomicrographs of cross-sections of the left common carotid arteries. L, lumen; M, media; I, intima; N, neointima. (B) Quantification of the intima area. (C) Quantification of the media area. (D) Quantitative analysis of the luminal area. (E) Quantitation of the intima-to-media (I/M) ratio. **, P<0.01; BI rats versus NI rats. Bar graph indicates mean ± SEM. BI, balloon injury; NI, not injured.

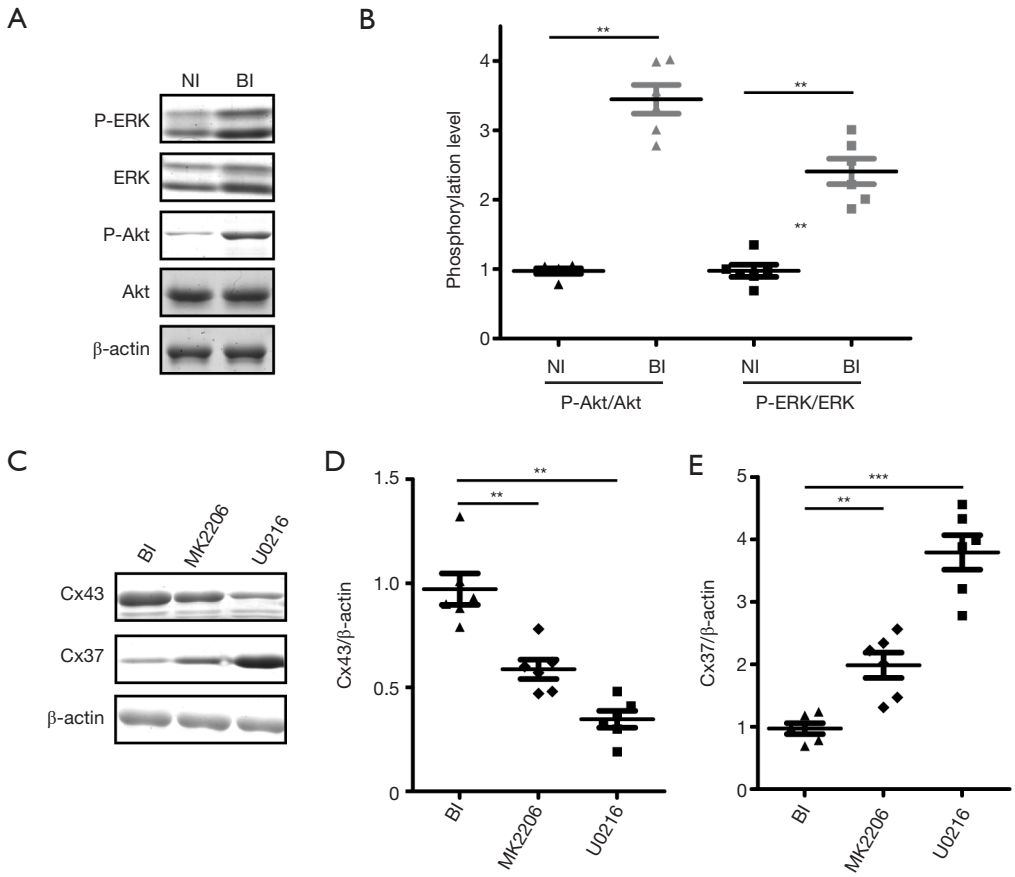

Figure 3 Phosphorylation levels of ERK and Akt correlate to protein levels of Cx37 and Cx43 in the balloon injury rat model. Each group (N=6) of NI, BI, BI treated by MIK2206 or U0216 rats were sacrificed on day 14 for assessment. (A) Western analysis of phosphorylation levels of Akt and ERK. (B) Quantification of the Akt and ERK phosphorylation levels. (C) Western analysis of changes of Cx37 and Cx43 protein levels by treatment of ERK and Akt inhibitor. (D,E) Quantitative analysis of change of Cx37 and Cx43 protein levels. **, P<0.01; ***, P<0.001. Bar graph indicates the mean ± SEM. BI, balloon injury; NI, not injured.

to determine the treatment effect of Akt-inhibitor and ERK inhibitor. When compared with BI rats, MK2206 (P<0.05) and U0126 (P<0.01) could significantly attenuate the neointima formation indicated by the I/M ratio (*Figure 4A,B*). MK2206 and U0126 could remarkably down-regulate PCNA expression in carotid arteries of BI rat (*Figure 4C*), which was further indicated by quantitative analysis of PCNA positive area in the media (*Figure 4D*) and intima area (*Figure 4E*) over the total carotid area. All of these indicated that ERK and Akt mediated balloon-injury induced neointima formation and associated cell proliferation.

Discussion

Of the 21 identified Cxs, vascular ECs primarily express Cx37, Cx40, and Cx43 (14,15), all of which will facilitate the formation of gap junction channels and hemichannels. Gap junction channels, composed of two docked hemichannels oligomerized from six connexin molecules, can directly connect the cytoplasm of adjacent VSMCs and ECs and facilitate the propagation of chemical and electrical signals, which is essential for the function of the blood vessels (16,17).

Alterations in Cxs expression, localization, phosphorylation status, and gap junction properties are thought to contribute to cardiovascular dysregulation (18-20). It is Cx37, not Cx40, that is associated with aging and cardiac disease, which can serve as predictive markers for prognosis of post-stenting complications in patients with atherosclerosis (21). Up-regulation of connexin43 gap junctions between smooth muscle cells after balloon

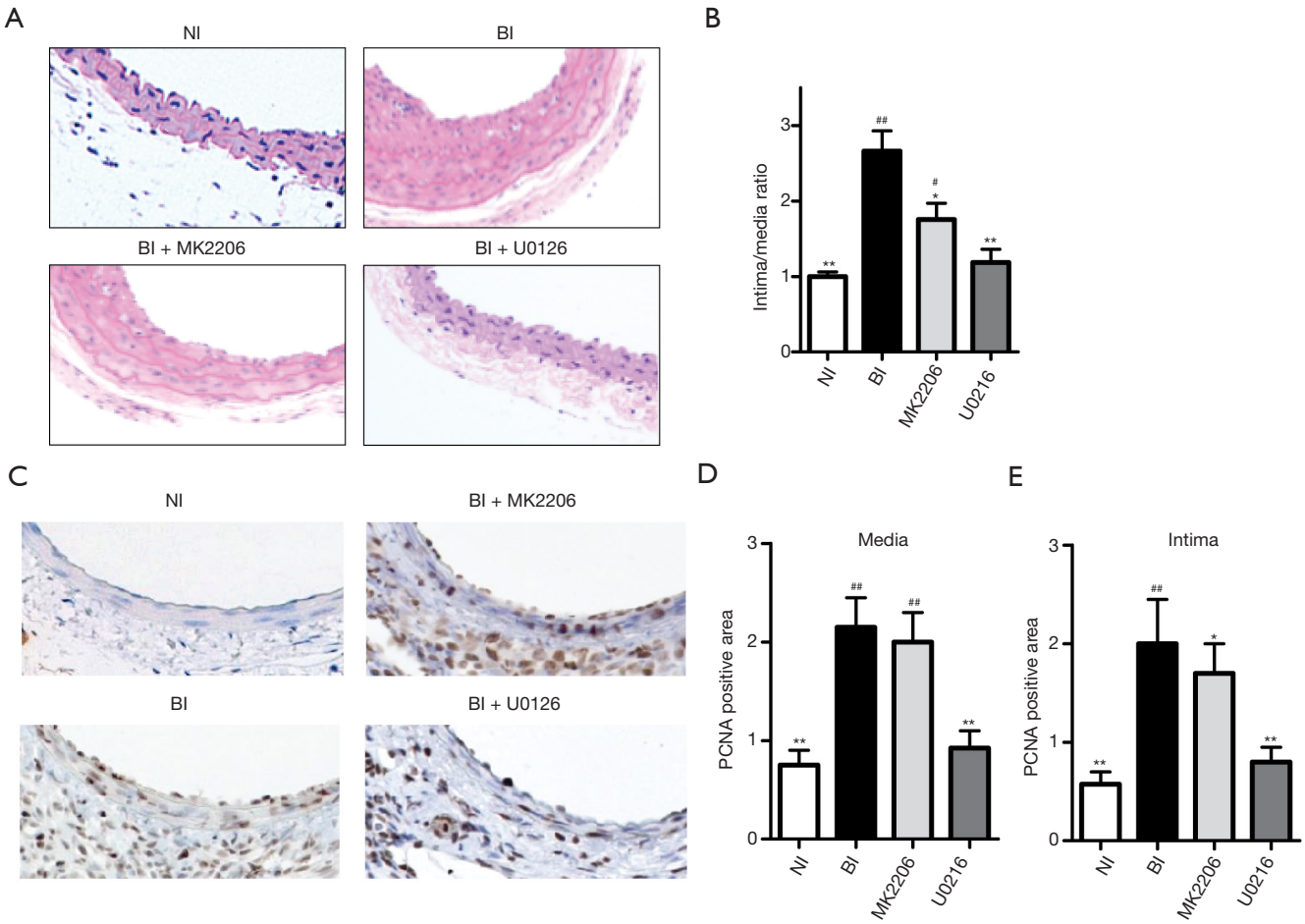

Figure 4 Inhibition of ERK and Akt reduced neointima formation and cell proliferation in the balloon-injured rat model. (A) Representative H&E staining photomicrographs of carotid arteries for NI rats and untreated, MK2206-treated, and U0126-treated BI rats on day 14 surgery. Magnification 200×. (B) Quantification of the intima-to-media ratio. (C) Representative PCNA immunostaining 14 days post carotid ligation in NI, BI, MK2206-treated BI and U0126-treated BI rats. Magnification 200×. (D) Quantitative analysis of the luminal area. (E) Quantitative assessment of PCNA positive area over the total carotid area. *, P<0.05; **, P<0.01, versus BI rats. #, P<0.05; ##, P<0.01, versus NI rats. Bar graph indicates the mean ± SEM. BI, balloon injury; NI, not injured; PCNA, proliferating cell nuclear antigen.

catheter injury in the rat carotid artery is observed (22). In contrast, reduced connexin43 expression could limit neointima formation after balloon distension injury in hypercholesterolemic mice (23). It is worth noting that Cx43 is induced in VSMCs during coronary arteriogenesis (24,25).

Cx43 over-expression in human primary VSMCs can increase P-Akt levels (26). In this investigation, both Cx37 and Cx43, not Cx40 are found to be involved in the BI-induced IH, which is further testified to be regulated by MAPK and Akt pathway. It is testified that MAPK cascade can be activated by mechanical injury-induced growth factors release (27), which can phosphorylate connexin to be involved in the regulation of assembly, disassembly, trafficking, and degradation, as well as the gating of gap junction channels (28,29). While the role of Akt in the process of IH is less discussed. This investigation also demonstrates that ERK-inhibitor U0216 is more effective than Akt-inhibitor MIK2206 to attenuate IH, although detailed mechanism still needs further decipher.

Different expression patterns of Cxs in diverse tissue suggest that there might be molecular movement specificity through gap junctions. It is worth noting that Cx40/Cx43 expression ratio can influence heteromeric/heterotypic gap junction channel properties, such as selectivity for dye movement, electric signaling, and second messenger coupling (30). Besides, phosphorylation of connexin

proteins can regulate the junctional permeability (31,32). Whether co-expression or mutually expression of Cx37 and Cx43 or phosphorylation modulation is involved in BI-induced IH needs further investigation.

Although anti-platelet drugs, drug-eluting stent implantation, anti-thrombotic drugs, and brachytherapy have been utilized to overcome restenosis after PCI, none of them has shown long-term benefit (33,34). The quiescent VSMCs' re-entry into the cell cycle, proliferation, and migration are hallmark biological events in IH process, which is thought to be regulated by the gap junction communication between VSMCs and ECs. So, it is necessary to deepen the research on the regulation of Cxs.

Although the detailed mechanism involved in the BI-induced IH remains unclear, this investigation suggests that ERK and Akt mediated down-regulated Cx37 and up-regulated Cx43 are involved.

Conclusions

ERK-inhibitor U0216 and Akt-inhibitor MIK2206 can ameliorate BI-induced IH via up-regulation of Cx37 and down-regulation of Cx43.

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Footnote

Reporting Checklist: The authors present the study following the ARRIVE reporting checklist. Available at http://dx.doi.org/10.21037/cdt-20-345

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/cdt-20-345). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures and animal feeding were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University (#WZYKDX-311), in compliance with institutional guidelines of Wenzhou Medical University for the care and use of animals.

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