Blockade of Connexin 43 Hemichannels Reduces Neointima Formation After Vascular Injury by Inhibiting Proliferation and Phenotypic Modulation of Smooth Muscle Cells

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Connexins 43 (Cx43) plays a key role in neointimal formation after vascular injury, but the mechanism still needs to be further explored. We hypothesized that the gap junction-dependent function of Cx43 to mediate intercellular communication has a crucial role in the development and progression of vascular diseases. The effect of intercellular communication mediated by Cx43 hemichannels on neointimal formation after vascular injury was investigated. Cx43 was overexpressed or knockdown in rat vascular smooth muscle cell (SMC) by transfection pcDNA-Cx43 plasmid or small interfering RNA (siRNA) against Cx43 (siCx43). SMC proliferation and marker genes expression after Cx43 alteration and blockade of the Cx43 hemichannel were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay and RT-PCR. The effect of carbenoxolone on neointimal formation was investigated in carotid artery injured rat model. We demonstrated that overexpression of Cx43 promoted SMC proliferation, meanwhile, mRNA expression level of smooth muscle α-actin and calponin, which were important markers of SMC in a contractile state, were downregulated in smooth muscle. Knockdown of Cx43 inhibited SMC proliferation but increased SMC marker genes expression level. Carbenoxolone (50 μM) improved SMC contractile differentiation and inhibited its proliferation. Our data showed that carbenoxolone reduced neointimal formation after carotid artery injury. In summary, blockade of intercellular communication via Cx43 hemichannels reduces neointimal formation after vascular injury by inhibiting proliferation and phenotypic modulation of SMCs. Exp Biol Med 234:1192–1200, 2009

Key words: smooth muscle cell; gap junction; connexin; neointimal formation

Introduction

Injury or dysfunction of endothelial cells (ECs) caused by mechanical removal and inflammation induces a cascade of proinflammatory events resulting in the infiltration of monocytic cells and proliferation of smooth muscle cells (SMCs) (1). Phenotypic modulation of vascular SMC is an important step in the development of several pathophysiological processes such as atherosclerosis, restenosis and vascular remodeling (2, 3). During these processes SMCs change from a contractile phenotype to a synthetic phenotype characterized by increased proliferation, migration, increased extracellular matrix production, and decreased expression of SMC marker genes, such as smooth muscle α-actin (SMA), SM22α, calponin (CNN), and myosin heavy chain (SM-MHC). The mechanisms of SMCs phenotypic modulation, migration, proliferation and secreting extracellular matrix still need to be elucidated.

Gap junctions are specific regions in the plasma membrane. They contain intercellular channels which provide pathways for the movement of ions, metabolites

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and secondary messenger molecules between neighboring cells (4, 5). The interchange of signaling molecules between adjacent cells through gap junctions coordinates cellular activity, and is essential for the growth, proliferation, differentiation, tissue homeostasis, tumorigenicity and wound healing (6).

The proteins that comprise gap junction channels are called connexins (Cx) which consist of at least 20 members. Three of them (Cx37, Cx40 and Cx43) have been identified in vascular tissue (4). Cx43 has been demonstrated to be a major Cx expressed in vascular SMCs (7). Besides forming gap junctions to mediate intercellular communication, Cx43 has some gap junction-independent functions such as cell growth, cell differentiation and tumorigenicity (8), which are similar to its gap junction-dependent functions. Cx43 is reported to have a key role in the development and progression of vascular diseases (9–11). The mechanism of Cx in gap junction-dependent or gap junction-independent function is unclear.

Compounds that inhibit intercellular communication have been widely used to probe the role of gap junctional communication in different systems. These compounds, such as heptanol, aoleamide, carbenoxolone and 18a-glycyrrhetinic acid, are nonspecific inhibitors of connexin subtypes (12). Recently, short synthetic peptides that possess sequence homology with conserved domains of the extracellular connexin loops have emerged as connexin-specific inhibitors of gap junction communication in a wide range of cell types (13). For example, the connexin mimetic peptide gap 26, a tridecapeptide containing the SHVR amino acid motif, composed of residue numbers 63–75 of the first extracellular loop of Cx 43 have been used as a connexin-specific inhibitor (14). Connexin-mimetic peptides have also been extensively used to study heterocellular communication between endothelial and smooth muscle cells in the vascular wall (13).

In the present study, we hypothesized that Cx43 acts through a gap junction-dependent function to promote the development and progression of vascular disease. To test this hypothesis, we investigated the effect of a special blocker of the gap junction, carbenoxolone, on the proliferation and phenotypic modulation of vascular SMCs and neointimal formation after vascular injury.

Methods and Materials

Study Approval. All procedures were carried out in accordance with the guidelines of the Animal Research Committee of the Third Military Medical University, Chongqing, China.

Animals. Sprague-Dawley rats (150–180 g) were obtained from the Experimental Animal Center of the Third Military Medical University.

Cell Culture. Cell culture materials were purchased from Gibco BRL (USA). Method to isolate rat SMCs was based on previously described methods (15) with modification. Briefly, the aorta was removed from rats, placed in HBSS buffer with 25 mmol/L HEPES buffer (~4°C). After adipose tissue, adventitia and endothelium were removed, the aorta was cut laterally into 1-mm^2 explants and placed in a flask. The growth medium of SMCs contained low-glucose Dulbecco's modified Eagle's medium (DMEM) with 20% dialyzed fetal bovine serum (FBS), 1% antibiotic/antimycotic. SMCs were used between passage-2 and passage-3.

Cell Staining. SMCs were cultured on a 96-well plate overnight and then fixed in buffered 10% formalin for 15 min at room temperature. Cells were incubated for 30 min in phosphate-buffered saline (PBS) supplemented with 4% fish skin gelatin, 0.3% Triton X100 and 1% serum from the source animal in which the secondary antibodies were made. Cells were incubated with mouse monoclonal anti-smooth muscle α-actin (A-2547, Sigma-Aldrich, USA) antibody in supplemented PBS at 4°C overnight, washed with PBS, and then incubated with goat anti-mouse IgG labeled with rhodamine red for smooth muscle α-actin in PBS for 1 h at 37°C. Samples were washed with PBS and imaged with a fluorescence microscope (Leica, Germany).

Cell Transfection. Small interfering RNA (siRNA) targeting Cx43 (siCx43) was purchased from Santa Cruz Biotechnology, USA. It contained three target specific sequences. pcDNA3.0-Cx43 plasmid was previously constructed in our laboratory, and contained full-length rat Cx43 cDNA (the pcDNA3.0 vector was obtained from Invitrogen, USA). About 10 pmol of siRNAs or 1.0 μg of pcDNA3.0-Cx43 was transfected into SMC cultures using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Quantification of Cx43 mRNA expression was evaluated 24 h post-transfection using the reverse transcription polymerase chain reaction (RT-PCR).

Fluorescence Recovery After Photobleaching (FRAP). FRAP was done as previously described (16). Cells were loaded with the Ca^{2+}-insensitive dye 6-carboxy-fluorescein diacetate (10 mM, Sigma-Aldrich, USA) for 5 min at room temperature. FRAP was measured using a confocal microscope (Leica, Germany). The dye was excited at 488 nm and its emission recorded at 570 nm. A neutral density filter was used to minimize photobleaching. Before bleaching, polygons were drawn around the cells chosen for bleaching, and two pre-bleach images scanned. The cells chosen for bleaching were then exposed to 50 scans with the laser at 95% intensity, and the recovery of fluorescence in the bleached cells measured every 10 s over 5 min. The reduction in fluorescence in a square region of interest far from bleached cells was measured as a reference to correct for bleaching caused by the excitation light. After correction for background bleaching, recovery of fluorescence in the bleached cell at 3 min was compared with that of the pre-bleach scan, and percentage recovery calculated.

3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay. SMCs proliferation was determined by MTT assay. Briefly, SMCs were

cultured in 96-well plates (5×10^3 cells per well) supplemented with carbenoxolone (Sigma-Aldrich, USA) at 50 μM (17, 18) or Cx43 mimetic peptides, gap 26 peptide (Cambridge, UK) at 150 μM (14) for 0 h, 24 h, 48 h and 72 h. Then, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for another 4 h. Media were discarded by aspiration and dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was added (200 μL per well) and shaken for 10 min. Optical density (OD) values were measured at 490 nm.

RT-PCR. Cells were harvested and total RNA was isolated as recommended by the manufacturer (TRIzol Reagent, Invitrogen, USA). First-strand cDNA was synthesized in a 20-μL reaction mixture containing 1.0 μg of total RNA, 1 mmol/L of each dNTP, 200 U Mo-MuLV reverse transcriptase, and 100 pmol of random hexamer oligonucleotides. To detect gene transcripts, 1.0 μg of cDNA was used for PCR amplification. Sense and antisense primers were designed by Primer Premier 5.0. The following pairs of primers were used for PCR amplification: smooth muscle α-actin (α-SMA): 5′-ACTGGGACGACATGGAAAAG-3′ and 5′-CATCTCCAGAGTCCAGCACA-3′ (expected size, 240 bp); CNN: 5′-ATGGGCACCAATAAGTTGC-3′ and 5′-GACCTGGCTCAAAGATCTGC-3′ (expected size, 196 bp); Cx43: 5′-GCGGCTTGCTGAGAAC-3′ and 5′-AGTGGTGGCGTGGTAA-3′ (expected size, 308 bp); and for internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-GCAAGTTCAACGGCACAG-3′ and 5′-TACTCAGCACCAGCATCACC-3′ (expected size, 121 bp). The amplification mixture (50 μL) contained Taq polymerase, primer sets, dNTP and cDNA. After PCR amplification (annealing temperature, 57°C; number of cycles, 30), 10.0 μL of each PCR product was analyzed on 2% agarose gels. Gel images were captured using a densitograph ultraviolet image analyzer (Bio-Rad, USA) and the densities were analyzed by Quantity one v4.4 densitometry software (Bio-Rad). Relative expression levels were calculated by comparing with GAPDH from the same cDNA.

Carotid Artery Injury. Carotid artery injury was induced as described previously (19, 20). Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg body weight, Sigma). The bifurcation of the left carotid artery was exposed via a midline incision of the ventral side of the neck. Two ligatures were placed proximally and distally around the external carotid artery. The distal ligature was then tied off. After temporary occlusion of the internal and common carotid artery, a transverse arteriotomy was carried out between the ligatures of the external carotid artery to introduce a curved flexible wire (0.13 mm in diameter) that was slightly curved (30°) at the tip, and which completely filled out the vessel. The wire was passed along the common carotid artery in a rotating manner three times. After wire removal, the proximal ligature of the external carotid artery was tied off. Normal blood flow was reassured, and the skin closed with single sutures using 6/0 silk. Postoperatively, carbenoxolone (3 mg/kg, i.p.) (21) was administered; 2 mL saline was given to the control group. Carotid arteries were harvested after 14 days (19, 20). Injury was induced by the same flexible wire.

Morphometry. Three serial cryosections (5 μm thickness, 300 μm apart) were taken from the middle portion of the dilated segment and analyzed for total intimal cells. Cell number was counted from 4-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA)-colored nuclei. Morphometric analysis of areas was done on these sections after visualization of arterial elastic laminas with Evan's blue (0.1%) staining and fluorescent light. Sections were photographed, digitalized, and analyzed with Qwin2.8 software (Leica, Germany).

Immunofluorescence Staining. Serial cryosections (5 μm) were obtained from injured carotid arteries and fixed with cold acetone for 3 min. Slides were rinsed with PBS for 3 min and then incubated in 0.3% Triton X100 for 10 min at room temperature. After rinsing with PBS for 15 min, slides were incubated with 10% rabbit normal serum for 30 min at room temperature and then incubated goat anti-Cx43 in supplemented PBS at 4°C overnight, washed with PBS, and then incubated with rabbit anti-goat IgG labeled with FITC (Santa Cruz, USA) for 1 h at 37°C. Goat IgG was used to replace anti-Cx43 as a negative control. Samples were washed with PBS and imaged with a fluorescence microscope (Leica, Germany).

Western Blot Analysis. Western blotting was performed as previously described (9). Briefly, carotid arteries were washed with cold PBS, and lysed in harvesting buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM p-nitrophenyl phosphate, 1 mM EGTA, 50 mM sodium fluoride, 50 μM sodium orthovanadate and 5 mM benzamidine. Samples were scraped, homogenized on ice and centrifuged at 4000 *g* at 4°C for 5 min. Supernatants were collected and subjected to high-speed (12,000 rpm) centrifugation. Supernatants were stored at −80°C until use. Aliquots of each extract were analyzed by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were incubated with mouse anti-α-SMA monoclonal antibody, and developed with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were then stripped using 0.2 M NaOH solution for 30 min at room temperature, and reprobed for total proteins. Band densities were normalized to the untreated controls and presented as the percentage change.

Statistical Analysis. Values are given as mean ± SEM. A Student's *t* test was used to compare differences between groups with SPSS 10.0 for windows software (SPSS, USA). $P < 0.05$ was considered statistically significant.

Results

Cx43 Expression Regulates SMC Proliferation. To determine effect of Cx43 expression on SMC proliferation, we observed SMC proliferation by MTT assay after downregulating or upregulating Cx43 expression. Firstly, we upregulated the Cx43 expression in SMC by transfecting pcDNA3.0-Cx43 plasmid into cells. A blank pcDNA3.0 was transfected as a control. RT-PCR analysis showed that cells transfected with pcDNA3.0-Cx43 exhibited >200% increase of Cx43 compared with vehicle cells (Fig. 1A). Proliferation of pcDNA-Cx43-transfected cells was increased compared with vehicle cells 72 h after transfection ($P < 0.01$), suggesting that overexpression of Cx43 can promote cell proliferation (Fig. 1B).

To further test the role of Cx43 expression on SMC proliferation, siCx43 was transfected in SMCs. Cells transfected with a non-targeting sequence siRNA (si-Control) served as a control. RT-PCR analysis showed that siCx43 could downregulate Cx43 expression to 30% compared with control (Fig. 1A). Proliferation of SMCs decreased after downregulating Cx43 expression by siCx43 after 72 h ($P < 0.01$) (Fig. 1C).

Effect of Carbenoxolone on SMCs Proliferation. SMC proliferation is an important event during the restenosis process. To further confirm the role of Cx43 on SMC proliferation, we investigated the effect of carbenoxolone and gap 26 peptide on SMC proliferation by MTT assay. Cell proliferation decreased compared with control SMCs after carbenoxolone (50 μM) treatment ($P < 0.05$). The effect of Cx43 mimetic peptide, gap26 peptide on SMC proliferation was also investigated. Gap 26 peptide inhibited SMC proliferation at 150 μM ($P < 0.05$), which was consistent with carbenoxolone (Fig. 1D).

Cx43 and Carbenoxolone Modulates the Expressions of SMC Gene Makers. In the present study, we investigated the effect of Cx43 on the expression of α-SMA and CNN mRNA in SMCs by up- and down-regulating Cx43 expression. Firstly, Cx43 was overexpressed in SMC by transfecting pcDNA-Cx43 plasmid. RT-PCR analysis was used to evaluate the level change of SMC gene maker α-SMA and CNN mRNA. Results demonstrated that α-SMA and CNN mRNA levels increased compared with vehicle and normal cells after overexpressing Cx43. Then, we downregulated Cx43 expression by siCx43; α-SMA and CNN mRNA expression levels decreased after Cx43 knockdown.

SMCs were treated with carbenoxolone (50 μM) or gap 26 peptide (150 μM) for 3 days, and CNN and α-SMA mRNA expressions were examined to evaluate the phenotypic modulation of SMCs in vitro. Expressions of CNN and α-SMA mRNA in SMCs increased after treating SMCs with carbenoxolone. There was a significant difference compared with that in control SMCs. Similar effect was observed when treating cells with gap 26 peptide (Fig. 2).

Functional Gap Junctions. Intercellular transfer of hydrophilic dyes (e.g., Lucifer yellow, carboxyfluorescein) after scrape-loading or photobleaching (as in the FRAP protocol) indicates functional gap junctional intercellular communication (GJIC) (22). FRAP was used to measure functional GJIC between adjacent SMCs. In FRAP experiments, fluorescence recovery in the bleached cells reached 83 ± 14.26% within 3 min after photobleaching. FRAP was also done in the presence of the gap junction blocker carbenoxolone and gap 26 peptide. Pretreatment with carbenoxolone (50 μM) or gap 26 peptide (150 μM) in the extracellular solution for 60 min reduced fluorescence recovery by 49 ± 6.72% and 40 ± 5.16%, respectively. These results showed dye-coupling via gap junctions, and hence indicate functional GJIC in SMCs. Both carbenoxolone and gap 26 peptide can effectively inhibit functional GJIC in SMCs (Fig. 3).

Cx43 Expression in the Neointima After Balloon Injury. To evaluate Cx43 expression in the neointima, immunofluorescence staining was carried out on cryosections of the dilated vessels 14 days after balloon distension injury. Results showed that Cx43 expressed in the neointima after vascular injury (Fig. 4A and B).

Effect of Carbenoxolone on Neointimal Formation After Balloon Injury. To evaluate the role of carbenoxolone in neointimal formation, rats with carotid balloon distension injury were given carbenoxolone. Rats were sacrificed 14 days later and carotid arteries were subjected to morphometric analysis. This time point was chosen according to neointimal formation and medial thickening in the control (Fig. 5A and B). Three carotid cross-sections at 300-μm intervals were stained with DAPI/Evan's blue to outline nuclei and elastic laminas. Neointimal formation in rats treated with carbenoxolone was reduced compared with controls (Fig. 5C and D). Total neointimal nuclei were decreased in the carbenoxolone group more than in control animals (Fig. 5E).

α-SMA Expression in Neointima After Balloon Injury. To further investigate the phenotypic transformation of SMCs in neointimal formation, Western blot was performed to evaluate α-SMA expression in the neointima. Western blot also revealed that α-SMA expression in injured vessels from carbenoxolone-treated animals increased significantly compared with control animals (Fig. 6).

Discussion

SMCs migration and proliferation, and the synthesis of extracellular matrix by SMCs, are key events underlying atherosclerotic disease and the healing response to vascular injury following balloon angioplasty, stent implantation and atherectomy (23, 24). Growing evidence has confirmed that the transition of arterial smooth muscle cells from a differentiated contractile phenotype to a synthetic phenotype is widely considered to be an initial step in these pathological processes (25, 26). Contractile phenotype

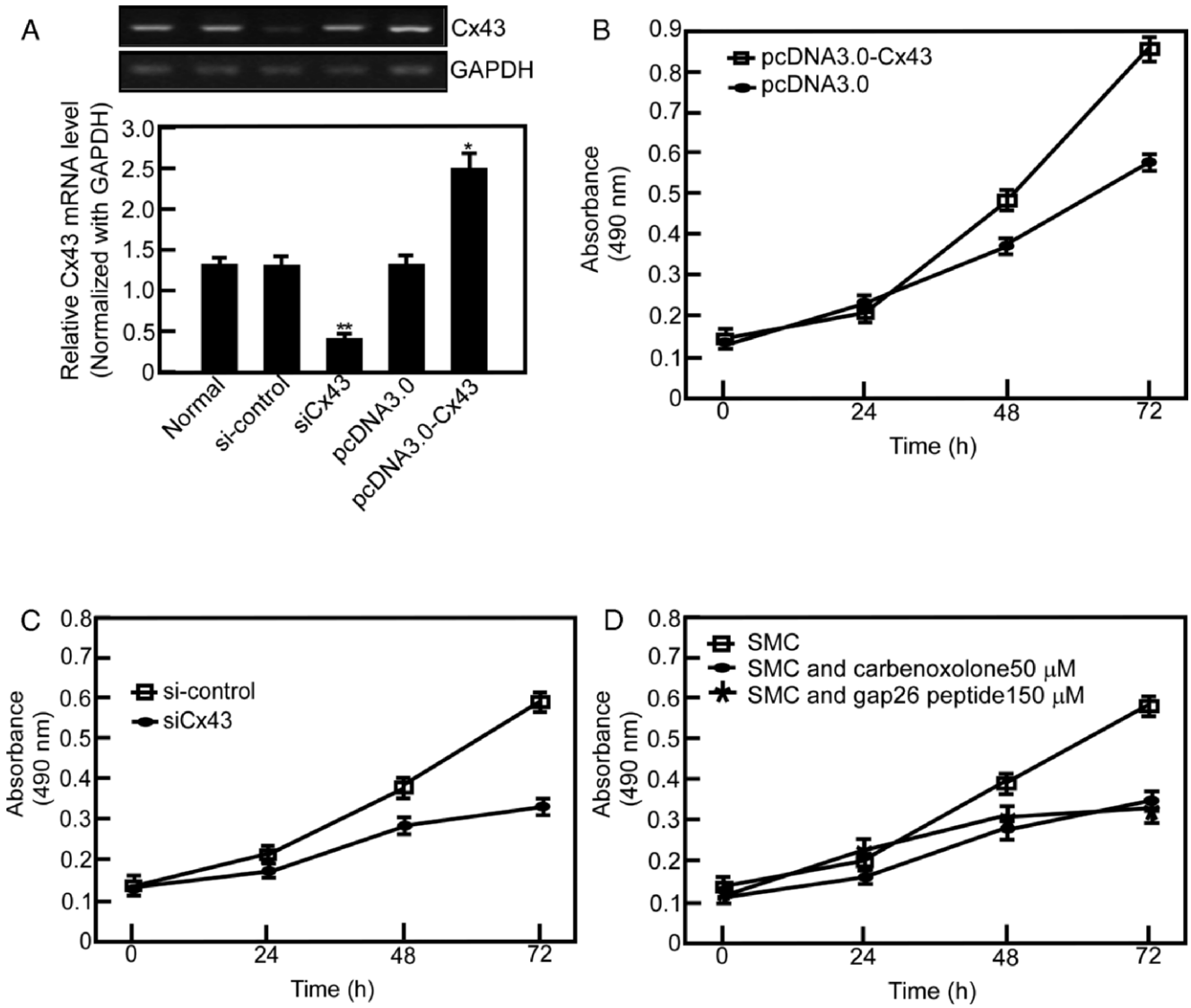

Figure 1. Cx43 mRNA expression affects SMC proliferation. A: RT-PCR analysis for Cx43 mRNA expression after transfecting pcDNA-43 plasmid or siCx43 to SMC. Transfection of pcDNA-Cx43 upregulated expression of Cx43 mRNA compared with normal and vehicle cells ($n = 5$, * $P < 0.01$). Transfection of siCx43 downregulated expression of Cx43 mRNA compared with normal and si-Control cells ($n = 5$, ** $P < 0.01$). B: Overexpression of Cx43 promotes cell proliferation. C: Proliferation of SMCs decreased after downregulating Cx43 expression by siCx43. D: Cell proliferation decreased compared with control SMCs after carbenoxolone (50 μM) treatment. Gap 26 peptide inhibited SMC proliferation at 150 μM.

SMCs expressing protein markers, such as α-SMA, smooth muscle myosin heavy chain, h1-CNN and SM22α actin, indicate higher contractility and lower migration and proliferation, whereas synthetic phenotype SMCs are prone to proliferate, migrate and synthesize extracellular matrix (ECM) components (27, 28). Most of SMCs in the tunica media of arteries and veins are quiescent or in the contractile state. The contractile SMCs will transform to a synthetic phenotype after both physiological and pathophysiological stimuli, followed by migrating, proliferating, and finally leading to atherosclerosis or neointimal formation (29). Such phenotypic transformation is initiated and maintained by interactions between SMCs and other cells or constituents of the arterial wall and blood. Interactions involving extracellular signaling mechanisms, such as those mediated by growth factors and cytokines (30–32), have been extensively studied.

Recent studies have suggested that Cx43 was upregulated in the early stage of atherosclerosis or neointimal formation after vascular injury (33, 34). Some studies found that low expression of Cx43 would limit neointimal formation after vascular injury (20, 35). However, the role of Cx43 in neointimal formation is poorly understood. In the present study, we observed that upregulation of Cx43 expression would decrease the contractile phenotype SMCs marker genes such as α-SMA and CNN mRNA expression, and promote SMCs proliferation. We also observed the decreased Cx43 expression would result in increased

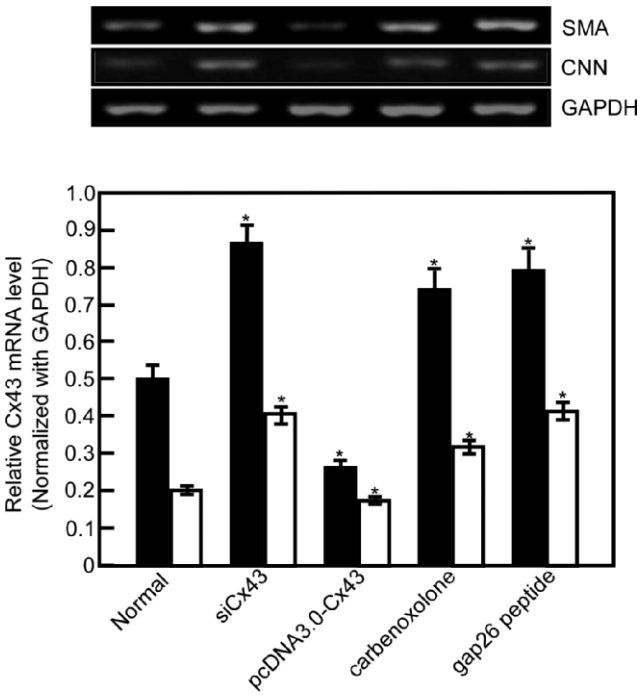

Figure 2. Cx43 and carbenoxolone modulates expressions of SMC gene makers. SMCs were transfected with pcDNA-Cx43 plasmid or siCx43. SMCs were also treated with 50 μM carbenoxolone or 150-μM gap 26 peptide. Total RNA was harvested after 3 days. Expression of SMC marker genes (α-SMA and CNN) was examined by RT-PCR analysis ($n = 4$, * $P < 0.01$ vs. normal).

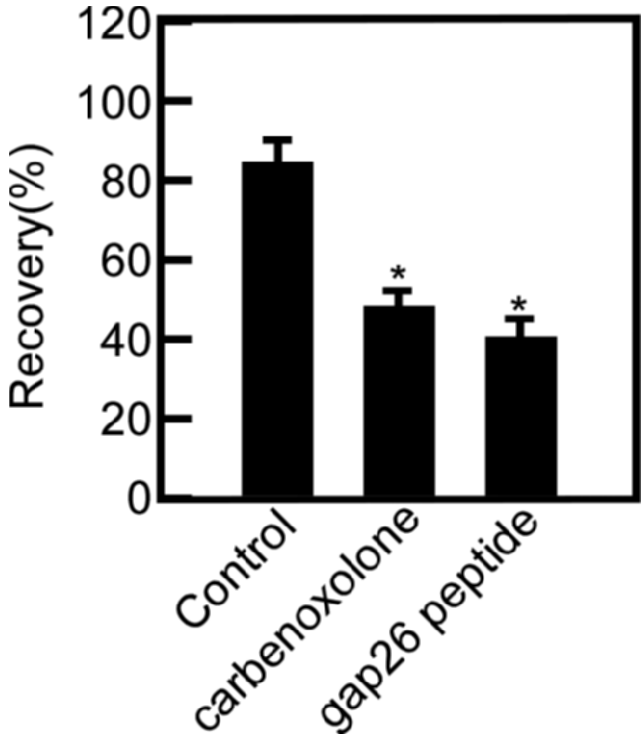

Figure 3. Effect of gap-junction blockers on intercellular communication determined by PRAP. Carbenoxolone (50 μM) and gap 26 peptide (150 μM) significantly reduced percentage recovery of fluorescence after bleaching ($n = 5$, $P < 0.01$ vs. control group).

α-SMA and CNN expression, and inhibit SMCs proliferation. These findings suggested that Cx43 played a vital role in the proliferation and the phenotypic modulation of SMCs, and it would promote SMCs to switch from a contractile to a synthetic state. These findings are in agreement with a previous study that reported Cx43 levels, though having common correlates, are not exclusively linked to the cell phenotype or the state of growth (36).

Connexins have been known to be the protein "building blocks" of gap junctions which mediate cell–cell communication and allow ions, small metabolites, and second messengers such as 3′–5′ cyclic adenosine monophosphate (cAMP), inositol 1,4,5-triphosphate and Ca^{2+} to translocate from cell to cell (7). This exchange is termed "direct intercellular communication", which is essential for various physiological and pathophysiological functions (e.g., cell growth, proliferation and differentiation, tissue homeostasis). Recent evidences demonstrated that, besides forming gap junction channels, Cx43 protein possesses gap junction-independent functions such as cell growth, differentiation, tumorigenicity, injury repair and apoptosis (8). The role of Cx43 in proliferation and phenotypic modulation of SMCs needs to be further elucidated. We found that carbenoxolone, which could significantly inhibit intercellular communication between SMCs examined by FRAP, increased the expression of CNN and α-SMA in SMCs and inhibited SMC proliferation. But, the other pharmacological effects of carbenoxolone besides the role of gap-junction blocker may also contribute to inducing the phenotypic modulation of SMCs. We therefore investigated the effect of gap 26 peptide on the phenotypic modulation and the proliferation of SMCs. Gap 26 peptide is a Cx43 extracellular loop mimetic peptide which can specifically block intercellular communication via the gap junction to inhibit the gap junction-dependent function of Cx43, and not affect Cx43 expression (12). Our data showed that gap 26 peptide could also increase the expression of CNN and α-SMA mRNA in SMCs and inhibit SMCs proliferation. The result is consistent with the role of carbenoxolone. It was suggested that the other pharmacological effect of carbenoxolone besides the role of gap-junction blocker did not participate in the phenotypic modulation of SMCs. These results indicated that the direct intercellular communication via the gap junction, i.e., gap junction-dependent functions of Cx 43, played an important part in the phenotypic modulation of SMCs.

SMC is one major cellular component of neointimal formation after vascular injury (37, 38). In order to explore the roe of the gap junction-dependent functions of Cx 43 on neointimal formation, we investigated the effect of carbenoxolone on neointimal formation in vivo in the present experiment. Results demonstrated that the α-SMA protein level in carbenoxolone treated group in injured carotid artery was markedly higher than in control group, which suggested that the percentage of contractile phenotype

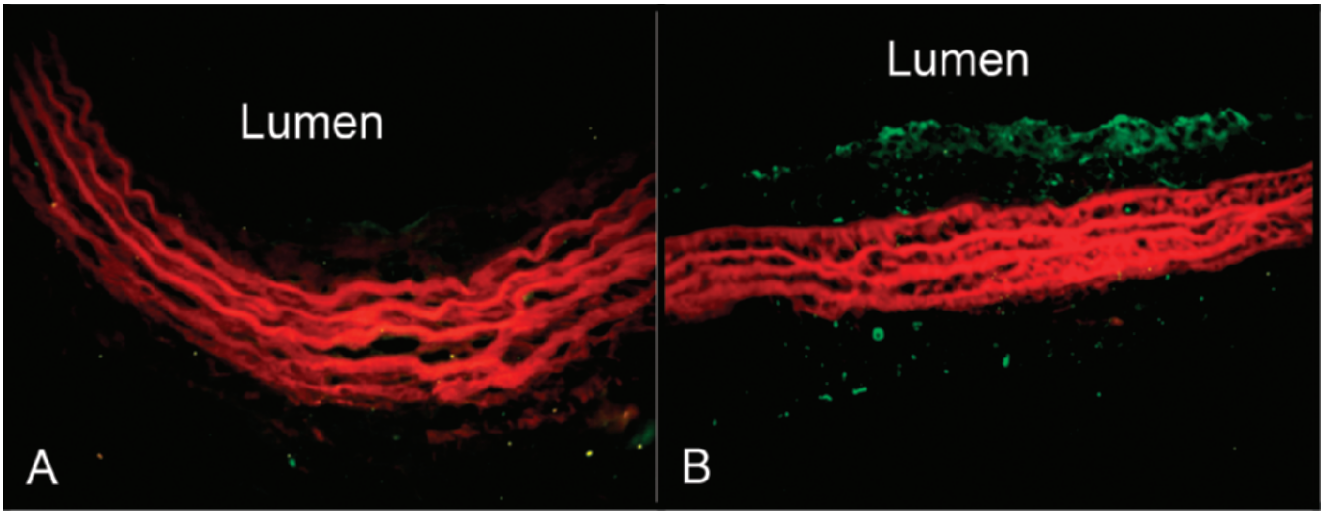

Figure 4. Expression of Cx43 protein in the neointima. A: Negative control of immunofluorescence staining for Cx34 in the neointima (×200). B: Positive immunofluorescence staining for Cx43 in the neointima (×200).

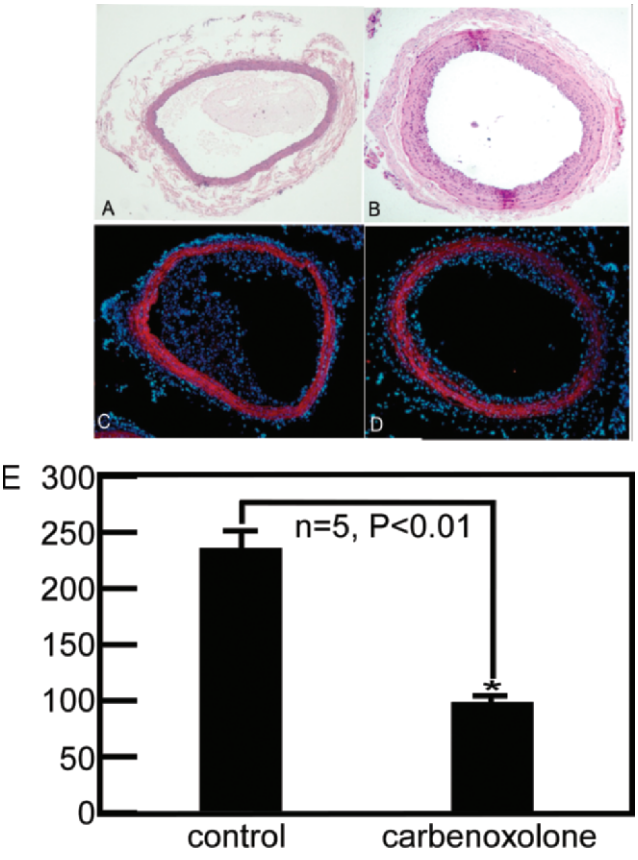

Figure 5. Carbenoxolone reduces neointimal formation after carotid artery injury. A: Normal vessel stained with hematoxylin and eosin (magnification ×40). B: Significant neointimal formation and medial thickening was found 14 days after injury (magnification ×40). C: Neointimal formation was detected by double staining with DAPI (blue) and Evan's blue (red) 14 days after injury. D: Neointimal formation was detected by double staining with DAPI (blue) and Evan's blue (red) 14 days after injury and carbenoxolone treatment (magnification ×40). E: Counts of total neointimal nuclei were significantly lower in the carbenoxolone group than in control rats.

SMCs was significantly increased after carbenoxolone treatment compared with control group and meant that carbenoxolone inhibited the phenotypic modulation of SMCs in vivo. Our data also suggested that blockade of the intercellular communication mediated by Cx43 with carbenoxolone in vivo significantly reduced the neointimal formation after vascular injury. In the past decades, many strategies (such as drug eluting stent and intravascular radiotherapy) had been developed to inhibit SMCs migra-

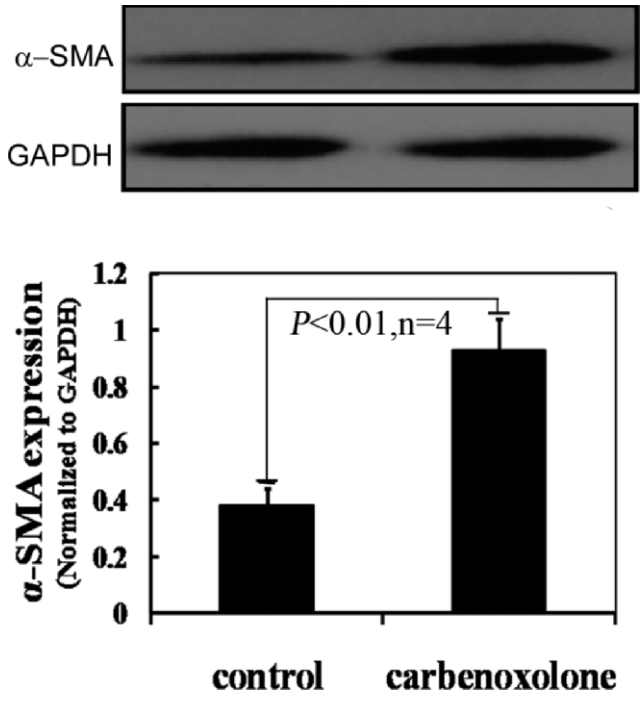

Figure 6. Western blot analysis for the expression of α-SMA in the neointima. Result revealed that α-SMA expression in injured vessels from carbenoxolone-treated animals increased significantly compared with control animals.

tion and proliferation to reduce neointimal formation after vascular balloon injury. Although these methods effectively reduce neointimal formation, they also result in serious complications such as late in-stent thrombosis and reocclusion (39, 40). The present finding that blockade of Cx43 hemichannels in SMCs could effectively reduce neointimal formation would be a potential therapeutic method for neointimal formation.

Taken together, the present study provides evidence that the blockade of Cx43 hemichannels reduces the neointimal formation after vascular injury by inhibiting the proliferation and the phenotypic modulation of SMCs. On the other hand, results also demonstrated that the gap junction-dependent function of connexins, mediating intercellular communication, played a key role in neointimal formation. Thus, the development of novel strategies which target the intercellular communication mediated by Cx43 may be of important clinical benefit to confine restenosis after PCI.

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