Effect of ramipril on the regulation of the expression of connexins 40 and 43 in a rabbit model of arterial balloon injury

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Abstract. Gap junctions (GJs) between the cells play a pivotal role in the transformation and proliferation processes of vascular smooth muscle cells (VSMCs). However, the expression of the component proteins of GJs, connexins 40 and 43 (Cx40 and Cx43), are inconsistent in numerous cases. The aim of this study was to determine whether Cx40 and Cx43 play different roles in the renin-angiotensin system (RAS) involved in the remodeling of GJs in VSMCs under pathological conditions. A total of 28 male New Zealand white rabbits were divided medially into four groups: control, sham injury, injury and injury plus ramipril (0.5 mg/kg/day in the diet for two weeks). The animals were used to set up the rabbit model of arterial balloon injury. Transmission electron microscopy, western blotting, immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) were performed on four samples of ballooned iliac arteries. Larger and more abundant GJs appeared in neointimal VSMCs and there were smaller and fewer GJs following ramipril treatment. mRNA and protein expression levels and level of immunostaining of Cx40 and Cx43 were consistently increased following injury. Although ramipril reduced the change in the levels of Cx43, no significant changes in Cx40 immunostaining, protein or mRNA levels were observed in the ramipril treatments. Ramipril may inhibit neointimal formation and downregulate the expression of Cx43 protein and mRNA, but the drug had no significant effect on the Cx40 protein and mRNA levels, suggesting that it was not Cx40 but Cx43 in GJs that contributes to the process of angiotensin II (Ang II)-converting enzyme inhibitors inhibiting the proliferation of VSMCs in balloon injury.

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Introduction

Gap junctions (GJs) are formed in the cardiovascular system by connexins 40, 37, 43 and 45 (Cx40, Cx37, Cx43 and Cx45, respectively). These low resistance channels allow the transfer of small molecules and ions between cells. The longitudinal coupling of endothelial and vascular smooth muscle cells (VSMCs) via GJs allows the spread of signals of membrane potential along the vascular wall which elicits a coordinated dilation of the arteriole over a considerable distance (1). The significance of GJs in the pathogenesis of a vascular response to injury and disease has been demonstrated *in vitro* and *in vivo*. A previous study reported that a reduction in the expression of the GJ protein Cx43 in mice causes the dysfunction of the proliferation and migration of smooth muscle cells (SMCs) and an inflammatory response resulting in the restriction of intimal thickening in severe vascular injury (2). During the migration and proliferation of VSMCs, Cx43 plays a significant role in intercellular signal transduction. Although Cx40 is homologous to the major vascular Cx43, the proteins have significantly different functions under certain conditions. These functions include ascorbic acid inhibiting the ability of a connexin-mimetic peptide targeted against Cx40 to attenuate the transmission of endothelial hyperpolarization to subintimal smooth muscle and a peptide targeted against Cx43 to attenuate the spread of subintimal hyperpolarization to subadventitial smooth muscle and the associated mechanical relaxation (3).

In pregnant women, the endothelium-derived hyperpolarizing factor-mediated vasorelaxation of subcutaneous resistance arteries is associated with Cx43 but not Cx40 (4). Lipoprotein-derived phospholipid oxidation products promote the upregulation of Cx43 in VSMCs and endothelial cells (ECs), which causes a decrease of Cx40 in ECs and an elevation of Cx40 in VSMCs (5). Downregulation of the Cx40 protein expression and the resulting inhibition of GJ intercellular communication contribute to coronary vascular dysfunction in diabetes, an effect not observed with Cx43 (6). Extracellular calcium may upregulate the expression of the cardiac GJ protein Cx40 but not Cx43 (7). α-adrenoceptor stimulation may affect Cx43 expression, but not Cx40 (8). These functional differences between Cx40 and Cx43 support the conclusion that not all connexin isoforms are regulated by the same mechanisms, even if they involve the same molecular

partners (9). Since electron microscopy has confirmed the predominant role of Cx40 at the myoendothelial junction in VSMCs (10), an increasing number of studies attribute significance to the role of Cx40 in EC and VSMC GJs with regard to the pathological change of vascular remodeling.

Angiotensin II (Ang II), a powerful vasoconstrictor, may activate the mitosis of VSMCs, leading to the multiplication of VSMCs, proliferation of fibroblasts, deposition of collagen and arterial sclerosis (11). A previous study showed that Cx40 hemichannels and extracellular ATP are key molecular elements of the glomerular endothelial calcium wave (12). Cx40, and probably intercellular communication via Cx40-dependent GJs, not only mediates the calcium-dependent inhibitory effects of Ang II and intrarenal pressure of renin secretion and synthesis (1), but also affects renal autoregulation (13) and the regulation of blood pressure (14). Although these new findings enable a better understanding of the correlations between alteration in Cx40 expression and the renin-angiotensin system (RAS), the role of connexins, particularly Cx40, in the migration and proliferation of VSMCs has not been extensively studied. The aim of this study was to prove that the alteration of Cx40 and Cx43 proteins in arteries submitted to balloon injury is associated with RAS and to reveal the roles of Cx40 and Cx43 in the migration and proliferation of VSMCs. The effects of ramipril, the Ang II-converting enzyme inhibitor (ACEI), on Cx40 and Cx43 expression, the structural changes of the GJ and the mechanism involving a change in the Cx40 and Cx43 of ACEIs in VSMCs are also reported in this study.

Materials and methods

Animals and balloon injury. A total of 28 male New Zealand white rabbits from the animal center of Zhejiang University (Hangzhou, China), weighing 2.5-3.0 kg, were randomly divided into four groups (n=7 in each): control, sham injury, injury and injury plus ramipril (Novartis Pharmaceuticals, Basel, Switzerland). The rabbits in the injury and drug intervention groups were subjected to the surgical procedures for balloon injury and the sham-operated rabbits were subjected only to the separation of the femoral artery.

Experimental procedures complied with the ethics and regulations of Zhejiang University to minimize the number of animals used, as well as pain experienced. Ethyl urethane (20%; 5 ml/kg, i.v.) was introduced for anesthesia. The right femoral arteries were separated and exposed and a 2.5 F coronary artery balloon catheter was inserted from a small incision under the guidance of fluoroscopic viewing. An aerocyst of ~3 mm in diameter and 20 mm in length was aerated and inflated at 8 times the atmospheric pressure and pulled along the whole iliac artery, until the end of the aortic bifurcation, and was then deflated and retracted 20 mm. The same procedure was repeated three times to ensure an overall endothelial denudation within the same arterial segments. Following the withdrawal of catheters, the femoral artery was ligated and a layered suture incision was performed. Heparin sulfate (1000 units) was administered intramuscularly (i.m.) to prevent thrombosis.

Following surgery, the rabbits were placed in the animal care unit for three days and benzylpenicillin sodium (400,000 U/day, i.m.) was administered consecutively. If one rabbit died, another was recruited and underwent the same surgical process. Over the three-day care period, the rabbits were submitted to the same experimental conditions for two weeks. Concomitantly, ramipril (0.5 mg/kg/day) was added to the daily diet in the injury plus ramipril group. When these procedures were finished, the animals were sacrificed with an overdosed i.v. injection of pentobarbital sodium (180 mg/kg) and the ballooned iliac arteries were isolated and cut into four segments for analyses using transmission electron microscopy, immunohistochemistry, western blotting and reverse transcription-polymerase chain reaction (RT-PCR).

Pathological analysis and immunohistochemistry. The pathological segments and connexin expression were visualised through hematoxylin and eosin (H&E) staining and immunohistochemistry as described previously (15). Balloon-injured tissues were pretreated overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and sliced to 5 μm for streptavidin peroxidase staining examination. The sections were incubated overnight at 4˚C with anti-Cx40 monoclonal antibody (1:100; Alpha Diagnostics, San Antonio, TX, USA) or anti-Cx43 monoclonal antibody (1:100; Chemicon, Temecula, CA, USA). Following three more rinses of PBS, the sections were developed with peroxidase-labeled reagent (Histofine simple-stain kit, Nichivei, Japan) for 20 min. Following dehydration with graded alcohols and cleaning in xylene, the location of Cx40 and Cx43 was visualized using 3,3'-diaminobenzidine.

Transmission electron microscopy. Ballooned segments were fixed with 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) for 3 h and washed three times with the buffer. The specimens were then treated with cacodylate-buffered 2% osmium tetroxide, dehydrated in a graded ethanol series and embedded in an epoxy resin. Thin sections were then collected and stained with uranyl acetate, contrasted with lead citrate and imaged under a Philips TECNA10 electron microscope (FEI Co., Hillsboro, OR, USA). The PC image analysis software (Foster Findlay Associates, Newcastle upon Tyne, UK) was used to measure the size of the GJs.

Western blot analysis. Western blotting was carried out in accordance with the relevant literature (16). Protein extracts were lysed in a lysis buffer (0.05% Igepal, 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% deoxycholic acid, 0.1% SDS and 1% Triton X-100). The whole-cell lysates were centrifuged (10,000 rpm) at 4˚C for 30 min and the supernatants were preserved for protein quantification. The aliquots of total proteins containing Cx40 (2 mg) and Cx43 (2.8 mg) were separated on 12.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were then soaked in PBS containing 0.1% Tween and 5% non-fat dried milk for 30 min. The membranes were developed overnight with anti-Cx43 (BD Transduction Laboratories™, BD Biosciences, San Jose, CA, USA, 1:1,000), anti-Cx40 monoclonal antibody (Alpha Diagnostic International, San Antonio, TX, USA 1:1,000) or anti-actin antibody (PharMingen, San Jose, CA, USA, 1:500). After serial washes, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000) or anti-mouse IgG (1:500, Chemicon) for 1 h.

Specific signals were visualized with enhanced chemiluminescence and exposed to Hyperfilm (Amersham-Pharmacia, Buckinghamshire, UK). The relative expression level of the protein was represented as the optical density ratio of connexins to actin using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA).

RT-PCR. Samples of 2.5 μg total RNA were extracted using TRIzol reagent (Gibco-BRL, Rockville, MA, USA) and reverse-transcribed to produce cDNA using the typical protocol (17). Briefly, the levels of Cx40 and Cx43 mRNA expression were determined through a semi-quantitative PCR using glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) as a reference. RT-PCR primers were designed (Gene Runner, Hastings Software) as follows: GAPDH-1, 5'-GCG CCT GGT CAC CAG GGC TGC TT-3' and GAPDH-2, 5'-TGC CGA AGT GGT CGT GGA TGA CCT-3'; Cx40-1, 5'-ATG CAC ACT GTG CGC ATG CAG GA-3' and Cx40-2, 5'-CAG GTG GTA GAG TTA GCC AG-3'; and Cx43-1, 5'-CAT CTT CAT GCT GGT GGT GT-3' and Cx43-2, 5'-TAG TTC GCC CAG TTT TGC TC-3'. The size of the products, including Cx40 (399 bp), Cx43 (283 bp) and GAPDH (465 bp) was scaled to match and incorporate each target gene. The PCR mixture (specific primers, reaction buffer, reverse transcriptase and dNTP) was submitted to 28 (Cx40) or 30 (Cx43) amplified cycles. PCR cycling was performed as follows: preincubation for 2 min at 37˚C, denaturation for 5 min at 94˚C, annealing for 45 sec at 57˚C, elongation for 10 min at 72˚C and 45˚C for 15 sec. Gel electrophoresis using 2% agarose was performed to confirm the mRNA expression of the RT-PCR products (10 ml). The amplicon specimens were quantified by measuring the optical density (OD) at 260 nm (Bio-Rad Laboratories).

Statistical analysis. Data were shown as the mean ± SD. Multi-group comparisons of data sets were analyzed through one-way ANOVA and Student-Newman-Keuls t-tests with SPSS 15.0 software (Chicago, IL, USA). $2\alpha=0.05$ was considered to indicate a statistically significant result.

Results

Pathological alterations in the arterial wall. Following balloon injury, the arterial wall became uneven and the neointima began to develop in comparison to the control condition. Following treatment with ramipril, however, the neointima area was markedly reduced (Fig. 1).

Distribution of Cx40 and Cx43 in the arterial wall. Balloon injury led to copious immunolabeled Cx40 and Cx43 staining in arteries, located mainly in the neointima and media. Ramipril markedly reduced the immunostaining of Cx43 but had a lesser effect on the immunolabeled Cx40 in the neointima area (Fig. 2).

Structural changes of the GJs in the arterial wall. Balloon injury precipitated the remodeling of GJs in the neointima consisting of SMCs by increasing the size and volume of GJs. This increase contributes to the proliferation of VSMCs, which may be inhibited by ramipril.

Expression of Cx40 and Cx43 protein and mRNA. The levels of protein and mRNA expression of Cx40 and Cx43 were significantly increased in the rabbit iliac arterial wall subjected to balloon injury. Ramipril markedly inhibited the elevated Cx43 protein and mRNA expression in balloon-injured arteries, but did not cause significant changes in the Cx40 protein and mRNA expression.

Discussion

GJs are one of the most ubiquitous and ancient forms of intercellular connection involved in signal exchange for the maintenance of homeostasis and cell growth regulation in multicellular organisms (18). Over the past two decades, a number of studies have described a role for GJ intercellular communication in the proliferation and differentiation of numerous types of cells, including SMCs (19). We investigated the effect of ramipril, the ACEI, on the expression of Cx40 and Cx43 in a rabbit model of balloon injury. Immunohistochemical analysis and electron microscopy revealed that ramipril inhibits balloon injury-induced neointimal formation, Cx43 expression in neointima and structural changes of GJs between VSMCs. Further studies using RT-PCR and western blot analysis indicated that ramipril inhibits the increase of Cx43 protein and mRNA expression in balloon-injured arteries but not Cx40. These results provide a better understanding of the mechanism of GJs involved in the regulation of VSMC proliferation.

In normal conditions, complete ECs are crucial for the maintenance of the physiology of the blood vessel. The ligation of ECs and completeness of the endothelium are regulated by Cx40 and Cx43. Following balloon injury, various cytokines and growth factors are synthesized and secreted by local platelets, SMCs, inflammatory cells and ECs to promote the migration and proliferation of SMCs in arterial media. During the migration and proliferation of arterial SMCs, the cells transform from a contractile to a synthetic phenotype. Previously, it was shown that Cx43 expression is enhanced in the cultured synthetic phenotype VSMCs compared with its contractile counterparts (11). Moreover, Cx43 expression was maintained at a relatively high level during the proliferation and migration of VSMCs in the neointima following balloon injury. The present study, in agreement with previous findings, demonstrates a significant upregulation of Cx40 and Cx43 protein and mRNA expression in proliferated VSMCs following balloon injury in rabbit iliac artery. Thus far the exact mechanisms of upregulation of Cx40 and Cx43 under this pathological condition have not been elucidated, but it is evident that growth factors in the blood circulation in the synthetic state contribute to the regulation of connexin expression in cultured VSMCs.

Since Cx40 and Cx43 are capable of being homomeric (made from six identical connexins) or heteromeric, and the full GJ channel may be homotypic (consisting of two identical connexons) or heterotypic, great diversity is possible. The permeability and regulatory properties may be different between heteromeric and homomeric channels and the regulation of certain signals for passing through its GJ channels may be provided by the heteromeric channels. It has been reported that Cx40 and Cx43 exist in heterotypic channels,

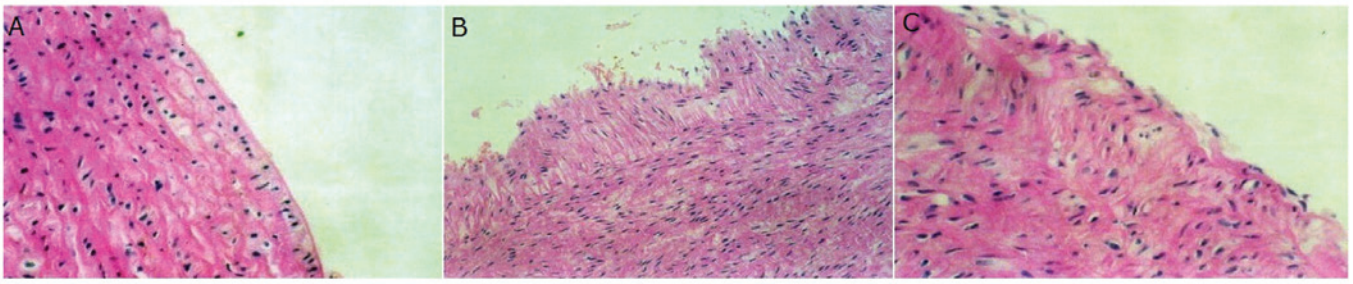

Figure 1. Pathological images of the iliac arterial wall in three experimental groups. (A) The normal cell nuclei are regularly arranged throughout the clear and intact elastic lamina in control rabbits. (B) In balloon-injured rabbits, the incomplete internal elastic lamina and formation of neointima was accompanied by SMCs. (C) Neointima decreases markedly following ramipril treatment. (H&E staining, x200). SMC, smooth muscle cell; H&E, hematoxylin and eosin.

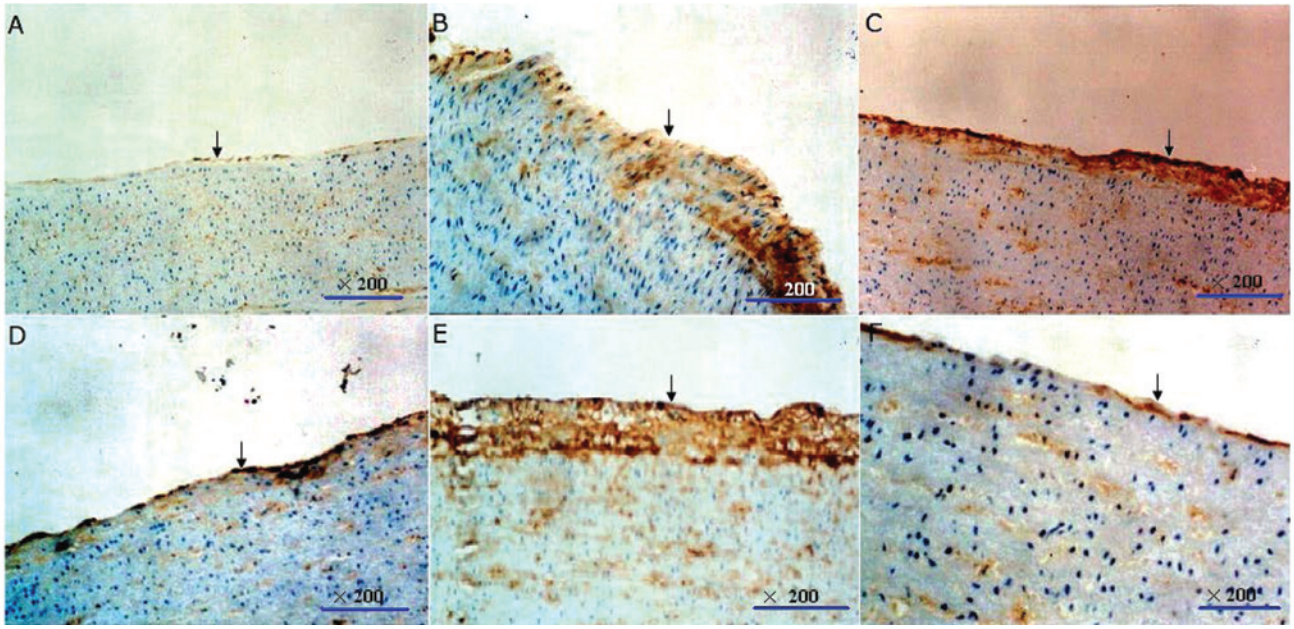

Figure 2. Localization of Cx40 and Cx43 in the rabbit iliac arteries among three experimental groups (x200). There was more abundant staining (brown spots, arrow) of (B) Cx40 and (E) Cx43 in the balloon-injured arterial neointima than the respective controls (A and D). (F) Following ramipril treatment, there was a significant reduction in Cx43 immune expression, whereas (C) the decline of Cx40 was less clear. Cx40, connexin 40; Cx43, connexin 43.

but the functional channels may be formed only via particular heterotypic pairings. For instance, the Cx40 protein is highly restricted in its ability to form heterotypic channels and is only functional when interacting with Cx37, but not Cx43. Additionally, functional Cx40/Cx43 heterotypic junctions have been largely disputed and a number of studies have clearly shown that Cx40 and Cx43 are not compatible to form heterotypic junctions (20). In our study, transmission electron microscopy revealed that larger and more abundant GJs appeared among VSMCs of the neointima in balloon-injured arteries, while smaller and fewer GJs presented between the medial VSMCs of the arteries from healthy or ramipril-treated rabbits, indicating that intercellular communication via GJs plays a crucial role in the migration and proliferation of VSMCs in the balloon-injured condition. These results show that an increasing Cx40 and Cx43 expression in the neointima of injured arteries may result in the enhancement of direct intercellular electromechanical and biochemical signaling, which is associated closely with VSMC proliferation. The ACEI ramipril is able to prevent the proliferation of VSMCs by inhibiting the expression of Cx43 mRNA and protein.

Our study revealed that ramipril was not able to alter the expression of Cx40 mRNA and protein in contrast to Cx43, indicating that the expression of these connexins are regulated differently. Moreover, Cx43 is the major connexin expressed in the working myocardium of the heart and SMCs of the blood vessels, whereas Cx40 is predominantly expressed in the atrium, atrioventricular node, AV bundle and ECs. In contrast to the distribution combining the incompatibility of Cx40 and Cx43, hemichannels may partially determine that direct signal communications are performed mainly through homotypic Cx40 and Cx43 GJs, which promotes the increase of VSMC growth and differentiation in pathological conditions. These findings suggest that intercellular communication is spatially regulated by the selective expression of different connexins. Thus, the upregulation in the expression of Cx40 mRNA and protein in proliferated VSMCs following balloon injury may not be due to Ang II. More experimental studies are required

to clarify why it is not Cx40 but Cx43 GJs that are responsible for the suppression of ACEI on the proliferation of VSMCs following balloon injury.

In conclusion, this study has shown that different roles were performed by Cx40 and Cx43 GJs involved in the suppression of ACEI on the proliferation of VSMCs under pathological conditions.

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