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Effects of angiotensin II on connexin 43 of VSMCs in arteriosclerosis

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Abstract: Objective: To observe the effect of angiotensin II (Ang II) on expression of gap junction channel protein connexin 43 (Cx43) in the proliferation process of vascular smooth muscle cells (VSMCs) during the early stage of arteriosclerosis. Methods: Thirty-two adult male rabbits were randomly divided into 4 groups. Rabbits in Group A were fed common diet while others in Groups B, C, and D were fed high-cholesterol diet. Losartan (10 mg/(kg·d)) and ramipril (0.5 mg/(kg·d)) were added in the diet of Groups C and D, respectively. The animals were sacrificed after 8 weeks and abdominal aortas were removed and dissected. The expression of Cx43 was assayed using RT-PCR and Western Blotting analysis. Results: Cx43 was increased markedly in both protein and mRNA level in Groups B, C, and D fed high-cholesterol diet compared with that in control group (P<0.01). Cx43 level in losartan or ramipril treated groups was higher than that in control group (P<0.01, P<0.05), but lower than that in high-cholesterol diet groups (P<0.05, P<0.01). Conclusion: Cx43 level was upregulated in VSMCs during early atherosclerosis. Losartan and ramipril can inhibit the expression of Cx43.

Key words: Atherosclerosis, Connexin, mRNA, Losartan, Ramipril
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INTRODUCTION

Transformation, proliferation and migration of vascular smooth muscle cells (VSMCs) into endomembrane of arteries are the pathological manifestations of atherosclerosis. Gap junction (GJ) and connexin (Cx) situated between endothelial cells play an important role in this process. Currently, connexin between VSMCs that have been recognized at protein molecular level includes Cx40, Cx43, and Cx37 (Li and Simardm, 1999). It showed that the occurrence of arteriosclerosis is closely related to the changes in function and that quantity of GJ and Cx43 is highly expressed at the thickened areas of the endomembrane during the early stage of arteriosclerosis (Kwak *et al*., 2003). Angiotensin II (Ang II), which is a powerful vasoconstrictor, can stimulate and cause VSMC to karyokinesis leading to multiplication of VSMC, proliferation of fibroblasts, deposition of collagen, and finally result in arteriosclerosis (Schmidt-Ott *et al*., 2000). Some researches also demonstrated that Ang II could activate the expression of Cx43 in the myocardial cells of rats in vitro (Dodge *et al*., 1998). The present study aimed at exploring the change of expression of Cx43 in the proliferation process of VSMCs during early stage of arteriosclerosis due to high-cholesterol diet, and finding out whether this change could be regulated by angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor 1 (AT_1) antagonists.

MATERIALS AND METHODS

Animals

Thirty-two male New Zealand white rabbits, weighing 2.5 to 3.0 kg, were provided by the Animal Center of School of Medicine, Zhejiang University.

Main reagents

Losartan (American Merck Pharma); Ramipril (Beijing Novartis Pharma); rabbit-anti-rat mono-

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clonal antibody for Cx43 (Zymed Laboratories); goat-anti-rabbit secondary antibody (Horse-radish obtained by peroxidase method technique, Beijing Zhongshan Biotechnology Ltd.); ECL (electrochemiluminescence) agent (Santa Cruz); nitrocellulosic filterable membrane (Life Science).

Establishment of animal models

Thirty-two rabbits were randomly housed in four groups of eight. Rabbits in Group A were fed common diet while rabbits in Groups B, C, and D were fed high-cholesterol (1.5% cholesterol) diet. Losartan (10 mg/(kg·d)) and ramipril (0.5 mg/(kg·d)) were added into the diet for Groups C and D, respectively. All animals were killed after 8 weeks by injection of air into the ear vein, and then the iliac arteries were carefully removed and dissected. Afterward plaque free segments of arteries were immediately collected in a lyophile apparatus preserved in nitrogen canister and stored at −70 °C for later use in Western Blotting and reverse transcription-polymerase chain reaction (RT-PCR).

Evaluation of cholesterol level in blood plasma

Five millilitres of blood were collected from the ear vein of each rabbit at two time points: namely, right before drug treatment was administered and before the rabbits were killed. Cholesterol concentrations of these plasma samples were measured by an automated analyzer (Type 7170, Hitachi Corp., Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissues using Trizol reagent (Life Technologies, Gaithersburg, MD). Samples of 2.5 μg total RNA were reverse transcribed into cDNA in 50 μl reaction mixtures using 200 U of recombinant M-MLV (moloney-murine leukemia virus) reverse transcriptase (Superscript II, GIBCO-BRL) and oligo dT_{15} as primer. Reverse transcription (RT) was carried out for 60 min at 42 °C followed by an inactivation step at 94 °C for 10 min. The RT products were stored at −70 °C for later use. The amount of RNA was determined by measuring the specific absorption at 260 nm. Oligonucleotide primers used were (written in the 5′ to 3′ direction): (1) glyceraldehyde phosphated dehydrogenase GAPDH-1, GCGCCTGGTCACCAGGGCTG CTT, and GAPDH-2, TGCCGAAGTGGTCGTG GATGACCT; (2) Cx43-1, CATCTTCATGCTGGTG GTGT, and Cx43-2, TAGTTCGCCCAGTTTTGCTC. Computer-assisted primer selection (Gene Runner, Hastings Software) was conducted to determine the primer for Cx43. The PCR fragments were 283 bp for Cx43, and 465 bp for GAPDH, which match the predicted size for each of these genes. Expression of Cx43 was determined by semiquantitative PCR using GAPDH as an internal standard. The samples were subjected to 30 (Cx43) cycles of amplification as follows: the samples were initially heated to 94 °C for 5 min to ensure complete denaturation of DNA (45 s for subsequent cycles) followed by 45 s at 51 °C (Cx43) to anneal the primers, and then 1 min at 72 °C for extension of the annealed primer. The PCR reaction was concluded by a 10-min elongation phase, again at 72 °C. The PCR products (10 μl) were visualized on 2% agarose gel. Electronic images were captured by using a solid-state black and white video camera (Cohu Electronic, Irvine, CA), and the intensity of the bands was determined using Kodak Digital Science 1D 2.0 imaging software.

Western Blotting

Protein extracts were prepared in a lysis buffer and then repeatedly aspirated (20 times) through a 23-gauge needle. Crude extracts were centrifuged at 4 °C for 30 min at 10000×g and supernatants were submitted for protein quantification. Samples (4 μg of total proteins for Cx43) were quenched by addition of gel loading buffer and aliquots were loaded onto 12.5% polyacrylamide sodium dodecyl sulfate gel. After separation, proteins were transferred to nitrocellulose membranes. Membranes were saturated for 30 min in PBS containing 5% nonfat dried milk and 0.1% Tween and were subsequently incubated overnight with the monoclonal anti-Cx43 antibody (Zymed Laboratories Inc.) diluted 1:1000 or with anti-actin antibody (PharMingen, San Jose, CA) diluted 1:500 in saturation buffer. After extensive washes in PBS containing 0.1% Tween, membranes were probed for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA) diluted in saturation buffer (antirabbit: 1:1000; anti-mouse: 1:500). The time of incubation in ECL detection reagents and

exposure to Hyperfilm (Amersham-Pharmacia, Buckinghamshire, UK) were identical for all experimental conditions. The intensity of the bands after Western Blotting was determined by laser scanning of the films followed by quantitative densitometric analysis using Kodak Digital Science 1D 2.0 Image software.

Statistical analysis

Data were analyzed using SPSS 11.0 softwares with all results were expressed in mean±standard deviation. Variance ANOVA was applied for multiple group comparisons. *P* value less than 0.05 was considered as statistically significant.

RESULTS

Body weight of rabbits and serum level of total cholesterol

The changes of the body weight of rabbits and the serum level of total cholesterol before the experiment and before the rabbits were sacrificed are shown in Table 1, respectively. No difference in body weight and cholesterol level was found among the groups at the beginnings ($P>0.05$). After eight weeks, the serum levels of cholesterol in high-cholesterol group, losartan group, and ramipril group were significantly higher compared with control ($P<0.01$). Although the serum levels of cholesterol in losartan group and ramipril group were lower than that in high-cholesterol group, no difference was found statistically significant in this case ($P>0.05$). Body weight of the rabbits in all groups increased compared with the beginnings, but no significant difference in weight among the four groups was detected ($P>0.05$).

Evaluation of mRNA

The relative values when the optical density (OD) of Cx43 mRNA of four groups were compared to OD of GAPDH mRNA are shown in Table 2. The expression of Cx43 mRNA in high-cholesterol group was significantly higher compared with control ($P<0.01$). The expression of Cx43 mRNA in losartan group and ramipril group were also higher than that in control group ($P<0.05$), but significantly lower than that in high-cholesterol group ($P<0.01$). The expression of Cx43 mRNA in the samples of four different groups is shown in Fig.1.

Connexin 43 assay

The relative values when the OD of Cx43 protein

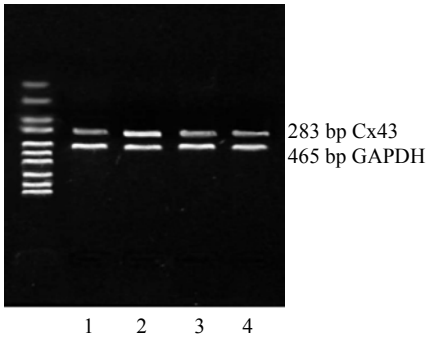

Fig.1 Expression of Cx43 mRNA in four groups (RT-PCR)
1: Control; 2: High-cholesterol group; 3: Losartan group; 4: Ramipril group

Table 1 Body weight of the rabbits and concentration of the total cholesterol in serum

Group	*N*	Total cholesterol in serum (mmol/L)		Body weight (kg)	
		Before experiment	Before sacrificed	Before experiment	Before sacrificed
Control	8	1.35±0.11	1.37±0.18	2.79±0.21	3.01±0.21
High-cholesterol group	8	1.34±0.10	1.68±0.13**	2.81±0.17	3.06±0.20
Losartan group	8	1.36±0.18	1.56±0.15**	2.82±0.20	3.02±0.16
Ramipril group	8	1.33±0.12	1.59±0.14	2.82±0.23	3.05±0.19

** $P<0.01$ vs control; Losartan group and ramipril group vs high-cholesterol

Table 2 Relative value when the OD of Cx43 mRNA was compared to OD of GAPDH mRNA

	N	Control	High-cholesterol group	Losartan group	Ramipril group
Cx43	8	0.46±0.12	0.97±0.13**	0.69±0.13*##	0.60±0.14*##

One way ANOVA, vs control: * $P<0.05$, ** $P<0.01$; vs high-cholesterol group: ## $P<0.01$

of four groups was compared to OD of actin are shown in Table 3. High-cholesterol group had significantly higher expression of Cx43 protein than control group (P<0.01). Expression of Cx43 protein in losartan group and ramipril group were higher compared with control (P<0.01), but significantly lower compared with high-cholesterol group (P<0.05). The expression of Cx43 protein in the samples of four different groups is shown in Fig.2.

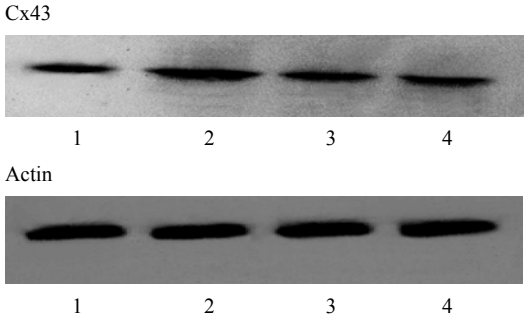

Fig.2 Expression of Cx43 protein in four groups (Western Blotting)
1: Control; 2: High-cholesterol group; 3: Ramipril group; 4: Losartan group

DISCUSSION

The expression of Cx43 mRNA and protein were both markedly increased in the proliferation of VSMC during early arteriosclerosis in the rabbit model in the present study. The results indicated that ACEI ramipril and AT_1 antagonist losartan could not reduce the cholesterol level in the serum of high-cholesterol diet group, but could inhibit the expression of Cx43 mRNA and the proliferation of VSMC, suggesting that the effects of ramipril and losartan on arteriosclerosis were not related to the decrease of cholesterol.

Gap junction is the structural basis of intercellular communication between two adjacent cells and serves as channel for direct intercellular exchange of ions, small molecular metabolites, and as secondary messengers (Sandow *et al*., 2002). Connexins comprise the major component of GJ complex and are also the determinant for permeability and conductivity of such channels (Lin *et al*., 2003). There are nearly 20 members of connexins reported in mammalian cells, including Cx43, Cx40, Cx45, and Cx37 which are expressed in specialized tissues of the mammalian cardiovascular system. The most ubiquitous connexin in the mammalian tissues, is Cx43, which is expressed in a variety of epithelial and other cell types in addition to cardiovascular tissues (Beyer, 1993). Transformation, proliferation, migration to endomembrane of arteries, and lipid phagocytosis of VSMCs are steps in atherosclerotic plaque formation. It was formed that after the transformation of VSMC from differentiated contractile state to the activated synthetic state, the expression of Cx43 in VSMC increased sixfold and GJ diameter was enlarged (Inoguchi *et al*., 1995). Study showed that the expression of Cx43 at the thickened site of the endomembrane during early arteriosclerosis was highly elevated (Blackburn *et al*., 1995). Another study showed that Cx43 level is upregulated during the proliferation process of VSMC after the aorta was subjected to balloon injury or the animal was fed high-cholesterol diet (Polacek *et al*., 1997). Besides, Cx43 also plays an important role in the intercellular communication in proliferating and migrating VSMC. For the present, researchers suggested that the occurrence of arteriosclerosis is closely related to the changes in function and quantity of GJ. Arterial endothelium interacted with leukocyte in the blood circulation via GJ to induce chemotaxis of leukocyte and further secretion of cytokines and biochemical substances from the leukocyte, and finally mediated arteriosclerotic plaque formation (Kwak *et al*., 2003).

Although what triggers the upregulation of Cx43 is not known, it is evident that cells at the synthetic state express growth factor receptors (Saltis *et al*., 1995) and that connexin expression is modulated by growth factors in cultured smooth muscle cell (SMC) (Mensink *et al*., 1995). For example, thrombin is a potent simulator of Cx43 expression in cultured arterial SMC (Mensink *et al*., 1996) and is a candidate for mediating the corresponding effect in vivo. Some recent studies revealed that the upregulation of Cx43

Table 3 Relative value when the OD of Cx43 protein was compared to OD of actin

	N	Control	High-cholesterol group	Losartan group	Ramipril group
Cx43	8	0.69±0.10	1.14±0.08**	0.91±0.12$^{**\#}$	0.83±0.09$^{*\#\#}$

One way ANOVA, vs control: $^{*}P$<0.05, $^{**}P$<0.01; vs high-cholesterol group: $^{\#}P$<0.05, $^{\#\#}P$<0.01

may relate to angiotensin II (Ang II).

Ang II is an effective vasoactive peptide and the main effector substance in renin-angiotensin system (RAS). However, it was noted that many tissues such as vascular wall, heart, and central nervous system can also produce Ang II in addition to the RAS system in peripheral blood circulation. Angiotensin II (Ang II) is a powerful vasoconstrictor, and also activates VSMCs mitosis, leading to VSMCs fibroblasts proliferation, and collagen deposition, and finally results in arterial sclerosis (Schmidt-Ott *et al*., 2000). It was shown that angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor 1 (AT_1) antagonists could prevent SMC from the proliferation and migration induced by Ang II (Bokkala *et al*., 2001). Other studies showed that Ang II could upregulate the expression of Cx43 in WB (whole blood) rat hepatic epithelial cells, and subsequently resulted in the changes of cellular metabolic conditions and secondary messengers (Li *et al*., 1999). Drug research discovered that angiotensin receptor 1 (AT_1) antagonist losartan could inhibit the highly elevated expression of Cx43 due to administration of Ang II to cultured neonatal rat ventricular myocytes for 24 h (Dodge *et al*., 1998). Study also showed that AT_1 antagonist could inhibit the upregulation of Cx43 in rat ventricular myocytes induced by periodic mechanical stretching (Shyu *et al*., 2001), suggesting that the tissues RAS system plays an important role in the remodelling of GJ in ventricular myocytes under pathological conditions (Emdad *et al*., 2001). Polontchouk *et al*.(2002) reported that after administrating Ang II to neonatal rat myocardial cells for 24 h, Cx43 increased by approximately 50% and led to multiplicative electric-coupling.

In conclusion, our results indicated that the expression of Cx43 induced by Ang II in the VSMCs proliferation process during early stage of arteriosclerosis due to high-cholesterol diet being upregulated, and such increase could have strengthened the GJ ligation, and enhanced the intercellular direct signal transmission, and finally resulted in activation of smooth muscle cells and early proliferation of the cells. Treatment with ACEIs and AT_1 antagonists may not be able to reduce the elevated cholesterol level in the serum due to high-cholesterol diet, but could inhibit the expression of Cx43 mRNA and proliferation of VSMCs.

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