Different expression of connexin 43 between culprit arteries and non-culprit arteries and role of angiotensin II on expression of connexin 43 in non-culprit arteries

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Abstract: Introduction and objectives: The purpose of the study was to observe different expression of connexin 43 between culprit arteries and nonculprit arteries in ischemia-reperfusion model and investigate on the mechanism of nonculprit arteries lesions progression. Methods: Rabbit hyperlipidemia ischemia-reperfusion model was established, vascular smooth muscles of culprit arteries and nonculprit arteries were divided into 4 groups: ① culprit arteries control group, ② nonculprit arteries control group, ③ culprit arteries ischemia-reperfusion group, ④ nonculprit arteries ischemia-reperfusion group. Immunohistochemistry analysis of connexin 43 was performed in each group. Smooth muscle cells of nonculprit arteries were divided into 4 groups: ① normal control group, ② hyperlipidemia control group, ③ angiotensin II intervention group, ④ mitogen-activated protein kinase pathway inhibitor pretreatment plus angiotensin II intervention group. Expression of connexin 43 was analysed in each group. Results: Fluorescence immunohistochemistry analysis of connexin 43 showed there was significant difference between culprit arteries ischemia-reperfusion group and nonculprit arteries ischemia-reperfusion group (1723.52±138.64 vs 2136.15±237.82, $P<0.001$). Expression of connexin 43 in angiotensin II intervention group was higher than that in hyperlipidemia control group (1.79±0.31 vs 1.25±0.21, $P<0.05$), expression of connexin 43 in mitogen-activated protein kinase pathway inhibitor pretreatment plus angiotensin II intervention group was lower than that in angiotensin II intervention group [(0.85±0.19 vs 1.79±0.31, $P<0.05$), (0.99±0.13 vs 1.79±0.31, $P<0.05$), (0.81±0.15 vs 1.79±0.31, $P<0.05$) respectively]. Conclusions: Expression of connexin 43 in nonculprit arteries was higher than that in culprit arteries, it may be involved in angiotensin II--mitogen-activated protein kinase pathway. Keywords: Nonculprit arteries progression, connexin 43, angiotensin II, ischemia-reperfusion

Introduction

Although primary percutaneous coronary intervention (PCI) could salvage dying myocardium, reduce cardiovascular event, and improve prognosis in patients with acute myocardial infarction, recent studies showed that nonculprit arteries lesions may progress, and progression of nonculprit arteries lesions may be the important factor that influenced prognosis of patients with acute myocardial infarction after primary PCI [1]. The mechanism of nonculprit arteries progression was unclear. Our recent animal experiment showed that expression of connexin 43 (Cx43) was increased along with smooth muscle cells proliferation in nonculprit arteries, it indicated that expression of Cx43 was the key element of nonculprit arteries lesions progression. A recent research showed that angiotensin II (AgII) up-regulated expression of Cx43 via mitogen-activated protein kinase (MAPK) pathway and induced smooth muscle cells proliferation and migration in saphenous vein smooth muscle cells [2], we supposed that progression of nonculprit arteries may be due to up-regulation of expression of Cx43 in vascular smooth muscles via Ag II-MAPK pathway. Therefore, we planned to establish rabbit myocardium ischemia-reperfusion model, observed different expression of connexin 43 between culprit arteries and non-culprit arteries and investigated the role of AgII--MAPK--Cx43 pathway on progression of nonculprit arteries.

Methods

Hyperlipidemia mode

Male Japanese white rabbits were fed with high fat diet for 80 days (free access to food and...
water, 12 h light/dark cycle) and enrolled those total cholesterol >3 multiples of normal value (1~2 mmol/L) for ischemia-reperfusion model. Hyperlipidemia rabbits were divided into four groups: ① culprit arteries control group (8 rabbits), ② nonculprit arteries control group (8 rabbits), ③ culprit arteries ischemia-reperfusion group (8 rabbits), ④ nonculprit arteries ischemia-reperfusion group (8 rabbits). Ischemia-reperfusion model was established as followed.

**Ischaemia-reperfusion model**

The animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Capital Medical University, and the study protocol was approved by the Institutional Ethics Committee of Capital Medical University. Rabbits possess double-deck pleura, they could survive even if unilateral pneumothorax happened during thoracotomy, so we selected rabbit for ischemia-reperfusion mode. Male Japanese white rabbits, weighing 2.5~3.0 kg were used in this study. The experimental animals were placed on an operating table. Sterile solutions were used to limit the inflammatory reaction to the surgical preparation. They were anaesthetised with pentobarbital sodium (40 mg/kg) administered via the marginal ear vein. Additional doses were given when required (10 mg/kg/h). A thoracotomy was performed at the 4th intercostal space and the pericardium was opened. A 3/0 silk 4th read was placed around left anterior descending coronary artery (LAD). Lead II of the surface electrocardiogram, heart rate and arterial pressure were continuous recorded on a computer (Mac Power PC) using a data acquisition system (MacLab 8 channels, AD Instruments). Heparin (2500 i.u.) was injected intravenously at the beginning of the experiment [3].

**Experimental protocol**

Experimental animals were allowed a 20 min stable period in order to reach a steady state after surgical preparation. Coronary artery occlusion was produced in the ischemia-reperfusion group by clamping the snare around the left anterior descending artery. Myocardial infarction was confirmed by elevation of the ST segment of surface electrocardiogram. The ligation was loosened after 30 min ischemia, chest incision was closed with a three-layer suture, and then experimental animals were feed on ordinary diet for one week. Sterile solutions were used for 3 days to limit the inflammatory reaction to the surgical experiment. Lead II of the surface electrocardiogram was continuous recorded for 72 h. Experimental animals from the control group were submitted to the same surgical and experimental protocol, but coronary artery occlusion was not performed. All experimental animals that survived the experimental protocol were enrolled in this study [3]. Experimental animals were sacrificed to the marginal ear vein air embolism. Thereafter, animals from four groups ① culprit arteries control group (8 rabbits), ② nonculprit arteries control group (8 rabbits), ③ culprit arteries ischemia-reperfusion group (8 rabbits), ④ nonculprit arteries ischemia-reperfusion group (8 rabbits)) were submitted to either of the following protocols for tissue immunohistochemistry analysis and smooth muscular cells separation.

**Reagents**

Rabbit anti Cx43 monoclonal antibody (Cat. no. 35-5000), goat anti-rabbit IgG (H+L) second antibody (Cat. no. 31460), 10% goat serum albumin, rabbit antiβ-actin monoclonal antibody (Cat. no. ZB001) (all from Sigma-Aldrich Company Ltd).

**Coronary arteries staining**

Culprit arteries were LAD, and nonculprit arteries were right coronary arteries (RCA). Coronary artery tissues (LAD and RCA) were collected. tissue blocks were fixed in tissue stationary liquid (Triangle Biomedical Sciences, Durham, NC), and cut into slices of thickness of 6 μm. Tissue slices were infused in methanol and 3% hydrogen peroxide solution for removal of endogenous peroxidase. Tissue slices were stained with HE for observing atherosclerosis lesions.

**Cx43 immunohistochemistry analysis**

Expression of Cx43 of vascular smooth muscles of culprit arteries and non-culprit arteries were quantitative analysed with immunohistochemical method [2], each tissue block was cut into 3 slices of thickness 3 μm, one slice was stained with hematoxylin-eosin for observing

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smooth muscle structure under light microscope. The other two slices were processed with immunohistochemistry method, they were dewaxed and dipped in 0.25% Triton X-100 solution for 30 min, digested by 0.1% trypsinase for 20 min in 37°C, rinsed by phosphate buffer solution (PBS, pH: 7.4, components: KH₂PO₄ 0.2 g, Na₂HPO₄·12H₂O 2.9 g, NaCL 8.0 g, KCL 0.2 g added distilled water to 1000 ml), sealed by 10% goat seralbumin for 20 min, and decaed 1:400 anti Cx43 specific antibody in 4°C for incubation overnight (about 14 h). Next day slices were rewarming in room temperature for 1 h, and rinsed by phosphate buffer solution, then were decaed 1:60 goat anti rabbit IgG antibody and incubation for 1 h, were mounted by 60% buffer glycerine. All process required protection from light. The color of Cx43 Immunohistostaining was yellow brown. Each slice was selected four Immunohistostaining zone, 2 slices were selected for each rabbit, Cx43 was quantitative analysed by MIAS image analysis system (Beijing University of Aeronautics and Astronautics).

Smooth muscle cells separation culture and subgroup

Rabbits (included normal control group (8 rabbits) and hyperlipidemia group (8 rabbits)) were executed, left anterior descending arteries (culprit arteries) and left right coronary arteries (nonculprit arteries) were separated under asepsis condition, and were moved to super clean bench, intima and tunica media were scraped off, smooth muscle cells were digested and separated by collagenase type I, smooth muscle cells were inoculated in a 100 mL culture bottle (concentration: 1×10⁶/mL), cultivated by 20% solcoseryl DMEM/F12 culture medium, serial subcultivated to the fourth generation for reserve. Smooth muscle cells were divided into 4 groups (12 samples in each group): ① normal control group, smooth muscle cells supplied

Figure 1. Histological changes of culprit arteries and non-culprit arteries. A. Culprit arteries control group; B. Nonculprit arteries control group; C. Culprit arteries ischemia-reperfusion group; D. Nonculprit arteries ischemia-reperfusion group. Note: arrow indicated coronary atherosclerosis plaque.
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Table 1. Thickness of coronary atherosclerosis plaque in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Thickness (μm)</th>
</tr>
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<tbody>
<tr>
<td>Culprit arteries control group</td>
<td>8</td>
<td>41.03±11.41</td>
</tr>
<tr>
<td>Nonculprit arteries control group</td>
<td>8</td>
<td>38.21±10.17</td>
</tr>
<tr>
<td>Culprit arteries ischemia-reperfusion group</td>
<td>8</td>
<td>83.98±16.25**</td>
</tr>
<tr>
<td>Nonculprit arteries ischemia-reperfusion group</td>
<td>8</td>
<td>111.73±23.43***</td>
</tr>
</tbody>
</table>

Note: *compared with culprit artery control group, P>0.05; **compared with culprit artery control group, P<0.001; ***compared with nonculprit artery control group and culprit artery ischemia-reperfusion group, P<0.0001, P<0.001 respectively.

Table 2. Cx43 of culprit arteries and non-culprit arteries immunohistochemistry analysis one week later after ischemia-reperfusion (mean integral optical density)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cx43 quantitative analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culprit arteries control group</td>
<td>8</td>
<td>1315.21±151.47</td>
</tr>
<tr>
<td>Nonculprit arteries control group</td>
<td>8</td>
<td>1302.76±142.31*</td>
</tr>
<tr>
<td>Culprit arteries ischemia-reperfusion group</td>
<td>8</td>
<td>1723.52±138.64**</td>
</tr>
<tr>
<td>Nonculprit arteries ischemia-reperfusion group</td>
<td>8</td>
<td>2136.15±237.82***</td>
</tr>
</tbody>
</table>

Note: *compared with culprit arteries control group, P>0.05; **compared with culprit arteries control group, P<0.001; ***compared with nonculprit arteries control group and culprit arteries ischemia-reperfusion group, P<0.001.

Results

Atherosclerosis plaques were shown in culprit arteries and nonculprit arteries one week later after ischemia-reperfusion (Figure 1). Coronary artery tissues HE staining quantitative analysis showed that atherosclerosis plaque thickness was 41.03±11.41 μm in culprit arteries control group, 38.21±10.1 μm in nonculprit arteries control group (compared with culprit arteries control group, P>0.05), 83.98±16.2 μm in culprit arteries ischemia-reperfusion group (compared with culprit arteries control group, P<0.005; compared with nonculprit arteries ischemia-reperfusion group, P<0.001), 111.73±23.43 μm in nonculprit arteries ischemia-reperfusion group (compared with nonculprit arteries control group and culprit arteries ischemia-reperfusion group, P<0.0001, P<0.001 respectively) (Table 1).
mean integral optical density of Cx43 was 1315.21±151.47 in culprit arteries control group, 1302.76±142.31 in nonculprit arteries control group; mean integral optical density of Cx43 was 1723.52±138.64 in culprit arteries ischemia-reperfusion group, and 2136.15±237.82 in nonculprit arteries ischemia-reperfusion group. It indicated that there was no significant difference between culprit arteries control group and nonculprit arteries control group (P = 0.4084), there was significant difference between culprit arteries ischemia-reperfusion group and nonculprit arteries ischemia-reperfusion group (P<0.001) (Table 2).

Isolating culture and subgroup study of smooth muscle cells showed that Cx43 expression in hyperlipidemia group was significant higher than that in normal control group [1.25±0.21 vs 0.62±0.14, P<0.05], Cx43 expression in Angiotensin II intervention group was significant higher than that in hyperlipidemia group [1.79±0.31 vs 1.25±0.21, P<0.05], Cx43 expression in MARK pathway inhibitor plus an-
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giottensin II intervention group was significant lower than t in angiotensin II intervention group [(0.85±0.19 vs 1.79±0.31, P<0.05), (0.99±0.13 vs 1.79±0.31, P<0.05), (0.81±0.15 vs 1.79±0.31, P<0.05) respectively] (Figure 3; Table 2).

**Discussion**

Nowadays there are few studies about nonculprit arteries progression in patients with acute ST elevation myocardial infarction. We have studied blood flow perfusion and myocardium tissue perfusion of non-culprit arteries in 117 patients with acute myocardial infarction, and found that blood flow perfusion and myocardium tissue perfusion of nonculprit arteries were correlation with those of culprit arteries [4, 5]. Moreover, we have performed a clinical follow-up study for 519 patients with STEMI underwent primary percutaneous coronary intervention, and found that recurrent PCI was mainly due to non-culprit arteries lesions progression. Chronic inflammation and sustained stress

![Figure 3. Effect of MAPK pathway inhibitor pretreatment and Angiotensin II intervention on Cx43 expression of non-culprit arteries. Notes: A. From left to right: normal control group, hyperlipidemia group, Angiotensin II intervention group, PD98059+ Angiotensin II intervention group; B. From left to right: normal control group, hyperlipidemia group, Angiotensin II intervention group, SB203580+ Angiotensin II intervention group; C. Note: from left to right: normal control group, hyperlipidemia group, Angiotensin II intervention group, SP600125+ Angiotensin II intervention group. *Compared with normal control group, P<0.05; **Compared with hyperlipidemia group, P<0.05; #Compared with Angiotensin II intervention group, P<0.05.]
The role of connexins (Cxs) on vascular smooth muscle cell (VSMC) proliferation and migration in atherosclerosis (AS) was more and more attractive. Cx43 was a transmembrane protein, it consists of four transmembrane domains, two extracellular and one intracellular loop. There is increasing evidence that connexins are involved in the development of intimal hyperplasia and restenosis involving mouse and human atherosclerotic lesions. Gap junctions provide an enclosed channel for direct exchange of signaling molecules, including ions and small metabolites. Evidence suggested that Cx43 participated in cell growth, differentiation, and the development of intimal hyperplasia [7]. In early atherosmas, the expression of Cx43 in smooth muscle cells has been observed in the neointimal lesion. Cx43 was found in the endothelium covering the shoulder of the plaques and neointimal smooth muscle cells. Cx43 gene expression was also altered in rabbit arterial wall and plays a key role in neointimal formation after balloon injury. Deglise and colleagues observed coronary arteries samples in patients under went heart transplantation, and found that expression of Cx43 in intima hyperplasia region was 10 fold higher than in the normal vessel in prophase stage of atherosclerosis. Along with atherosclerosis progression, expression of Cx43 was decreased gradually, and in the end stage of atherosclerosis, expression of Cx43 was lower than that in the normal vessel. In the prophase stage of atherosclerosis, inhibiting expression of Cx43 in vessel smooth muscle may inhibit progression of atherosclerosis, it indicated that Cx43 participate in prophase progression of atherosclerosis [8].

As to Cx43 signal transduction pathway, Jia et al. observed that expression of Cx43 in smooth muscle cell of saphenous vein was up-regulation and lead to smooth muscle cell proliferation and migration after pretreated with angiotensin II. Up-regulation of Cx43 expression was depend on AP-1, and was regulated by ERK1/2, p38MAPK and JNK signal transduction pathway [9, 10]. It indicated that Ang II-MAPK-Cx43 pathway may participate in proliferation and migration of smooth muscle cells in vein. However, it was not clear that Ang II-MAPK-Cx43 pathway whether or not participate in proliferation and migration of smooth muscle cells in nonculprit arteries.

In view of above-mentioned study, we supposed that nonculprit arteries progression may be due to increased angiotensin II, and angiotensin II may induce up-regulation of expression of Cx43 by MAPK pathway.

Our study showed that atherosclerosis plaques were shown in nonculprit arteries one week later after ischemia-reperfusion (Figure 1; Table 1). Atherosclerosis plaque thickness in nonculprit arteries ischemia-reperfusion group was more than nonculprit arteries control group and culprit arteries ischemia-reperfusion group. It indicated that nonculprit arteries progress more quickly than culprit arteries (Figure 1; Table 1).

Cx43 of culprit arteries and non-culprit arteries immunohistochemistry analysis showed that mean integral optical density of Cx43 was 1315.21±151.47 in culprit arteries control group; 1302.76±142.31 in nonculprit arteries control group; mean integral optical density of Cx43 was 1723.52±138.64 in culprit arteries ischemia-reperfusion group, and 2136.15±237.82 in nonculprit arteries ischemia-reperfusion group. There was no significant difference between culprit arteries control group and nonculprit arteries control group ($P = 0.4084$), and there was significant difference between culprit arteries ischemia-reperfusion group and nonculprit arteries ischemia-reperfusion group ($P < 0.001$) (Figure 2; Table 2). It indicated that...
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The expression of Cx43 in nonculprit arteries was more than that in culprit arteries after ischemia-reperfusion, moreover, nonculprit arteries lesions progress more quickly than culprit arteries lesions after ischemia-reperfusion. It suggested that Cx43 may be the key element of nonculprit arteries progression. The reason that expression of Cx43 in nonculprit arteries was more than that in culprit arteries after ischemia-reperfusion may be involved in injury-repair mechanism: injury in culprit arteries was more than that in nonculprit arteries, but repair was similar in both culprit arteries and nonculprit arteries.

Isolation culture in vitro for smooth muscle cells in nonculprit arteries showed that expression of Cx43 (Cx43/β-actin photoabsorption ratio) in Hyperlipidemia group was higher than that in the control group [(0.86±0.17 vs 0.45±0.12, \(P<0.05\)), (1.25±0.21 vs 0.62±0.14, \(P<0.05\)), (0.85±0.18 vs 0.39±0.12, \(P<0.05\)) respectively]; expression of Cx43 in Angiotensin II intervention group was higher than that in hyperlipidemia group [(1.26±0.34 vs 0.86±0.17, \(P<0.05\)), (1.79±0.31 vs 1.25±0.21, \(P<0.05\)), (1.28±0.37 vs 0.85±0.18, \(P<0.05\)) respectively]; Cx43 expression in MARK pathway inhibitor plus Angiotensin II intervention group was significant lower than that in Angiotensin II intervention group [(0.85±0.19 vs 1.79±0.31, \(P<0.05\)), (0.99±0.13 vs 1.79±0.31, \(P<0.05\)), (0.81±0.15 vs 1.79±0.31, \(P<0.05\)) respectively] (Table 3). It indicated that, as neuroendocrine was activated (serum catecholamine level was increased) and hemodynamics was altered in patients with acute ST elevation myocardial infarction after primary PCI [6] renin-angiotensin system was activated. Increased angiotensin II may affect signal transduction of MARK pathway via autocrine, paracrine, emiocytosis (or via concomitance microcirculation pathway between culprit artery and nonculprit artery), leaded to Cx43 expression upregulation, smooth muscle cells proliferation, migration, and atherosclerosis lesions progression [11, 12].

Conclusions

We concluded that expression of Cx43 in nonculprit arteries was more than that in culprit arteries after ischemia-reperfusion, moreover, nonculprit arteries lesions progress more quickly than culprit arteries lesions after ischemia-reperfusion. Sympathetic nerve–catecholamine–angiotonin II–Cx43 may participate in nonculprit arteries progression. Angiotensin-converting enzyme inhibitor (ACEI) may inhibit nonculprit arteries progression. It may provide new thinking and new therapy target for nonculprit arteries progression.

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Disclosure of conflict of interest

None.

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