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Original Article

Cx43 and Cx45 heteromeric gap junction may play an important role in cerebral vasospasm due to experimental subarachnoid hemorrhage

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Abstract: Objective: To explore the change of Cx43/Cx45 heteromeric gap junction after the model of subarachnoid hemorrhage (SAH) in rabbits, which will provide the basis to investigate whether gap junction play an important role in the mechanism of cerebral vasospasm (CVS). Methods: 36 New Zealand rabbits were divided into 3 groups: control group (n = 12), SAH-7 d group (n = 12) and SAH-7 d + CBX group. The model of CVS following SAH was established. Digital subtraction angiography was performed to detect the change of the basilar arteries diameter before and after SAH. The interaction of Cx43 and Cx45 protien in basilar arteries tissue at different groups following experimental SAH was examined by using co-immunoprecipitation analysis. Results: The model of SAH in rabbits was successfully established. All 36 rabbits were analyzed. Cerebral angiograms of the BAs on SAH-7 d group showed much more narrowing than control group and SAH-7 d + CBX group ($P < 0.01$). Co-immunoprecipitation showed that the interaction of Cx43 and Cx45 protein was increased significantly compared with that of control and SAH-7 d + CBX group ($P < 0.01$). Conclusions: The above results demonstrated for the first time that the interaction of Cx43 and Cx45 protein was increased after the SAH, in other words, heteromeric gap formation of Cx43 and Cx45 was also increased after the SAH, however, the gap junction blocker CBX inhibited this tendency, which suggest that increased Cx43/Cx45 heteromeric gap junctions might be connected with the development of CVS. In summary, our data demonstrate that Cx43/Cx45 heteromeric gap junctions may play an important role in the pathogenesis of CVS after SAH.

Keywords: SAH, cerebral vasospasm, Cx43, Cx45, heteromeric gap junction

Introduction

Subarachnoid hemorrhage (SAH) is one of the common but severe cerebrovascular accidents. Cerebral vasospasm (CVS) is the constantly hot study within scholars, but the mechanism is still unknown, and the therapeutic method is ineffective. The research of the mechanism and therapeutic method to CVS is the tough problem in the world.

Gap junction (GJ), a direct communication pathway through the transmembrane channels which allow the movement electric charge and small signaling molecules directly from one cell to another, participates in the pathologic processes of cerebrovascular diseases and may play an important role [1-5]. Previously, our studies have indicated that GJ could play a role in the pathogenesis of CVS after SAH, and GJ blockers attenuated CVS contrarily [6-8]. In the vessel wall connexins, the individual structural units including connexins 40, 43, 45, and 37 [9] assemble GJs and are involved in information transfer in maintenance and modulation of vascular tone [10].

Gap junction plays an important role in coordinating vasomotion. Gap junction remodeling has been described in many diseases [11-14]. We hypothesize that gap junctional proteins reconstructed after CVS. The model of SAH was established on rabbits and then the alteration of protein for Cx43, Cx45 from the tissue of basilar arteries was determined after cerebral vasospasm. The main objective was designed

to explore the change of interaction of connexin 43 (Cx43) and connexin 45 (Cx45) protein after the model of subarachnoid hemorrhage (SAH) in rabbits, which will provide the basis to study the mechanism of cerebral vasospasm (CVS).

Materials and methods

Materials

Rabbit anti-Cx43/Cx45 antibody were purchased from Zymed (USA); Rabbit anti-GAPDH antibody were purchased from Chemicon (USA); Secondary antibodies were purchased from Jackson (USA); Protein A-garose beads were purchased from Santa Cruz (USA); Carbenoxolone (CBX) were purchased from Sigma (USA); Hyper film were purchased from Amersham-Pharmacia (USA); and Nonionic contrast medium (OMNIPAQUE 350 mg/mL) were purchased from Denmark. All other reagents were indigenous. The digital subtraction angiography system was purchased from Simens (Germany), and Microcatheter was purchased from Terumo (Japan). New Zealand White rabbits were provided by the Department of Laboratory Animal Science of Medical College of Nanchang University. All experimental procedures were carried out according to the guidelines of the Chinese Science & Technology Council for experimental animal care.

Induction of experimental SAH

The rabbit double-hemorrhage model of SAH was used [15]. All animals subjected to SAH were anesthetized by pentobarbital (20 mg/kg i.v.). On both day 1 and day 2, a 25-gauge needle was aseptically inserted into the cisterna magna of an anesthetized rabbit, and 0.5 mL/kg of fresh non-heparinized autologous blood taken from the ear artery was injected after withdrawal of 1 mL of cerebrospinal fluid (CSF). The head was tilted nose-down by 30 degrees for 10 minutes after injection of blood.

Digital subtraction angiography

After anesthesia was induced, the rabbits were placed in a supine position, and a 5-F catheter (Terumo Corp.) was inserted selectively into the aortic arch through a femoral artery by the Seldinger method, as previously described [16]. Angiograms of the BA were obtained by injection of 5 ml Omnipaque contrast medium for 2 seconds at a pressure of 50 psi. The speed of digital image acquisition was 6 frames/second. The angiograms obtained were transferred to an analytic processing system, and the diameter of the BA was measured at 5 points (at the midpoint of the BA, at 1 mm central and peripheral from the midpoint, and at 2 mm central and peripheral from the midpoint), as described previously [17]. The mean diameter at these 5 points was then determined. All angiograms were obtained by one investigator and the diameters of the BAs were measured by a colleague working in a blinded fashion.

Co-immunoprecipitation

In a separate group of animals without angiography, co-immunoprecipitation was used to analyze the expression change of Cx43 and Cx45 protein in basilar artery tissues after SAH. 24 male New Zealand White rabbits weighing 2.5 to 3.5 kg were randomly assigned to 5 groups: normal group (n = 8), SAH-7 day group (n = 8); SAH-7 day + CBX group (n = 8), the procedure of inducing SAH was the same as described above.

Rabbits were euthanatized by an overdose of pentobarbital. The BA was removed, frozen in liquid nitrogen, and stored at -85°C until use. The basilar arteries were immunoprecipitated with either anti-Cx43 or anti-Cx45 antibodies in the presence of 10 mM Hepes (pH 7.2) at 4°C overnight or then protein A-agarose beads were added for another 2 h. The beads were washed three times with wash solution (10 mM Hepes, pH 7.2/0.5% 8-POE) plus 1% bovine serum albumin (BSA) and twice with wash solution without BSA. The immunoprecipitated samples were isolated from beads by boiling in SDS sample buffer for 5 min. Immunoprecipitation of sucrose gradient fractions from bovine samples was performed with antibodies covalently conjugated to agarose beads. Anti-Cx43 antibody or Anti-Cx45 antibody were conjugated to protein A-agarose through a chemical cross linker, dimethyl pimelimidate, as described [18]. Fractions from sucrose sedimentation gradient were immunoprecipitated with the above conjugated antibodies in the presence of 10 mM Hepes, pH 7.2/0.5% 8-POE for overnight at 4°C and the beads were washed with wash solution.

Western blotting for expression of connexin43/connexin45 protein in basilar artery

Samples were quenched by addition of gel loading buffer, and aliquots were loaded onto

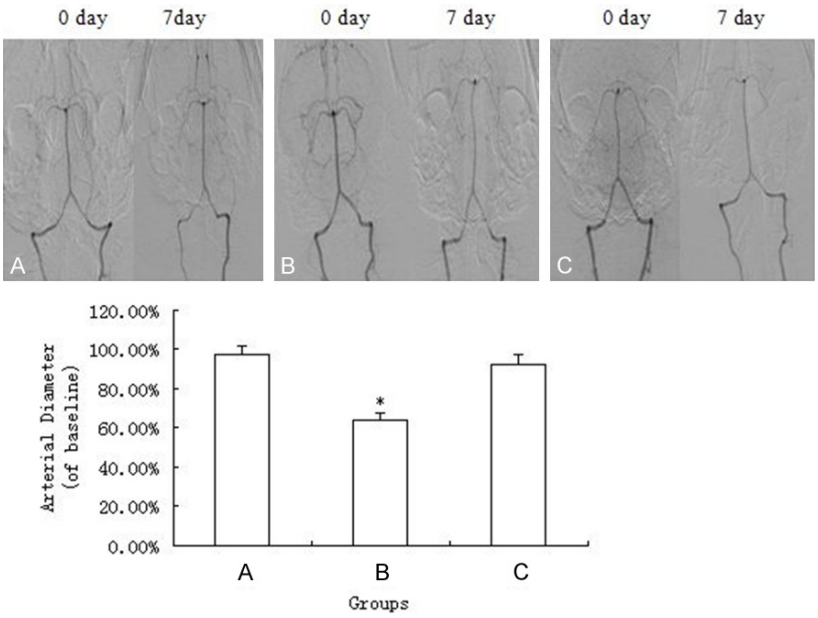

Figure 1. DSA analysis of the rabbit basilar artery diameter in different groups. Representative angiograms of rabbit BA on Days 0 (baseline) and 7 (upper row), and bar graph showing results of angiographic measurements (lower). Asterisks denote probability values, 0.05 (compared with control groups). In the SAH-7 d group, cerebral angiograms on Day 7 showed severe narrowing of the Bas in comparison with Day 0. In contrast, arterial narrowing in both the control group and SAH-7 d + CBX groups on Day 7 was no statistical significance. The group represented in each panel is as follows: A: Control group; B: SAH-7 d group; C: SAH-7 d + CBX group.

10% polyacrylamide sodium dodecyl sulfate gel. After separation, proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight in tris-buffered saline Tween (TBST) containing 5% milk powder, and were subsequently incubated overnight with the polyclonal rabbit anti-Cx43 antibody or anti-Cx45 antibodies (diluted 1:1000) or with anti-GAPDH antibody (diluted 1:1000), in saturation buffer. After extensive washes in TBST, membranes were probed for 2 h with the horseradish peroxide (HRP)-conjugated goat anti-rabbit secondary antibody (diluted 1:2000 in saturation buffer). The time of incubation in ECL detection reagents and exposure to Hyperfilm 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 Image J software.

Statistical analysis

The percentage of the BA diameter on models relative to day 0 (baseline) was calculated for each rabbit, and the results were presented as the mean ± SEM. All of the operated animals were analysed. A Student's t-test was used for analysis of the statistical difference between two means. Statistical significance was accepted when $P < 0.05$.

Results

Arterial diameter on angiography

Figure 1 showed that in normal group, animals there were no differences in the diameter of the BA (97.2% ± 1.5%), the diameter in the SAH-7 day group showed severe narrowing of the BAs (64.2% ± 6.3%, $P < 0.01$ versus normal group, n = 6), but SAH-7 day + CBX group was no statistical significance than normal group (92.4% ± 3.7%, $P > 0.05$ versus normal group, n = 6) (**Figure 1**).

Cx45 antibody co-immunoprecipitated Cx43 protein in basilar artery

Polyclonal anti-Cx43 and anti-Cx45 antibodies have been previously characterized and affinity-purified (32). These antibodies were used to detect possible physical interactions between these two connexins using immunoprecipitation methods (29). Our data demonstrate that the anti-Cx45 antibody can coimmunoprecipitate Cx43 from these detergent-solubilized Basilar arteries. Then the expression of Cx43 protien coimmunoprecipitated by anti-Cx45 antibody in basilar arteries tissue at different groups following experimental SAH was examined by using Western blotting analysis, and the relative values was shown in **Figure 2** when the optical density (OD) of Cx43 protein was compared to OD of GAPDH. In normal group (n = 6), the total protein level of Cx43 showed a slight expression (36.2% ± 3.1%). In SAH-7 day groups, the total protein level of Cx43 was

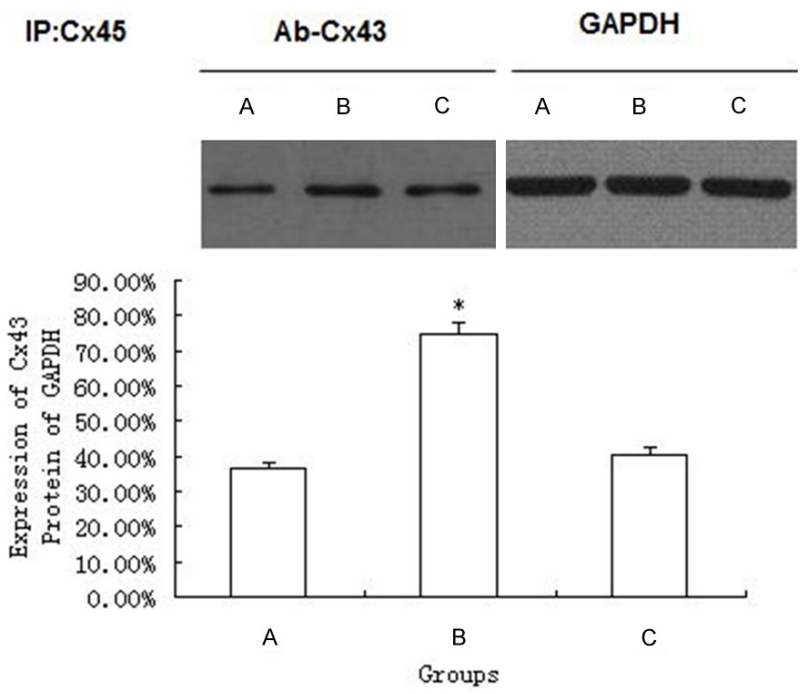

Figure 2. Anti-Cx45 antibody coimmunoprecipitated Cx43. Cx43 protein level co-immunoprecipitated by Anti-Cx45 antibody in each group. In the SAH-7 d group, Cx43 protein level on Day 7 showed high expression in comparison with Day 0. In contrast, the Cx43 protein expression level in both the control group and SAH-7 d + CBX groups on Day 7 was no statistical significance to Day 0. *P < 0.01 versus control group. A: Control group; B: SAH-7 d group; C: SAH-7 d + CBX group.

increased (74.3% ± 4.6%, P < 0.01 versus normal group, n = 6). But looks lower in SAH-7 day + CBX group (40.3% ± 3.5%, *P* > 0.05 versus normal group, n = 6). These results indicated that solubilized gap junctional channels of Basilar arteries contained both Cx45 and Cx43. Than the Cx45 and Cx43 protein can form heteromeric gap junction in normal rabbit basilar arteries tissue. But in SAH-7 day groups the Cx45 and Cx43 protein form heteromeric gap junctions seems increased, however, this increased tendency was inhibited by the gap junction blocker CBX.

Cx43 antibody co-immunoprecipitated Cx45 protein in basilar artery

Our data demonstrates that the anti-Cx43 antibodies can coimmunoprecipitate Cx45 from these detergent-solubilized Basilar arteries. In normal group (n = 6), the total protein level of Cx45 showed a slight expression (47.3% ± 1.8%). In SAH-7 day groups, the total protein level of Cx45 was increased (82.5% ± 5.4%, P < 0.01 versus normal group, n = 6). Also looks lower in SAH-7 day + CBX group (52.6% ± 4.8%, *P* > 0.05 versus normal group, n = 6). These results also indicated that solubilized gap junctional channels of Basilar arteries contained both Cx43 and Cx45. These Cx45 and Cx43 heteromeric gap junctions in SAH-7 day groups much more than normal groups and SAH-7 day + CBX group (**Figure 3**).

Discussion

Cerebral vasospasm after SAH is a major complication following the rupture of intracranial aneurysms [19]. Despite extensive clinical and experimental studies, the pathogenesis of cerebral vasospasm is still controversial and poorly understood. Numerous substances have been implicated in the causes of this phenomenon, but none of these investigations has determined the predominant pathophysiological mechanisms. The cause of vasospasm is presently considered to be multifactorial. Many therapeutic approaches have been suggested; however, no effective pharmacological treatment for cerebral vasospasm has been developed [20].

Cerebral vasospasm shows a typical dynamic change according to angiography. Previous reports have indicated that cerebral vasospasm is observed as early as 30 minutes (the acute phase) [21] after SAH, and reaches a peak at 3-7 days (the chronic phase) [22].

Gap junction channels are formed by members of a family of proteins known as connexins [23]. Connexin molecules oligomerize in the trans Golgi [24] into a membrane channel known as a connexon hemichannel [25], which is defined as homomeric when composed of the same connexin or heteromeric when composed of different connexins. Connexons in adjacent cells join head-to-head across a narrow extracellular "gap" to form intercellular channels, which are defined as homotypic when the same connexin

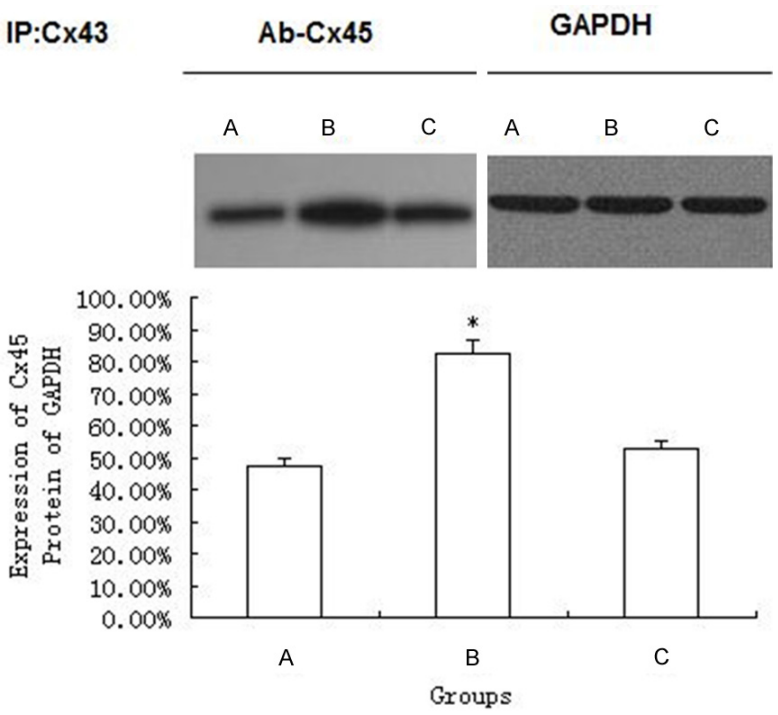

Figure 3. Anti-Cx43 antibody coimmunoprecipitated Cx45. Cx45 protein level coimmunoprecipitated by Anti-Cx43 antibody in each group. In the SAH-7 d group, Cx45 protein level on Day 7 showed high expression in comparison with Day 0. In contrast, the Cx45 protein expression level in both the control group and SAH-7 d + CBX groups on Day 7 was no statistical significance to Day 0. *P < 0.01 versus control group. A: Control group; B: SAH-7 d group; C: SAH-7 d + CBX group.

comprises both connexons, and heterotypic when different connexins comprise each connexon of the pair.

Previously, our studies have indicated that GJ could play a role in the pathogenesis of CVS after SAH, and GJ blockers attenuated CVS contrarily [6-8]. In the vessel wall connexins, the individual structural units including connexins 40, 43, 45, and 37 [9] assemble GJs and are involved in information transfer in maintenance and modulation of vascular tone [10]. Gap junction channels are formed by paired oligomeric membrane hemichannels called connexons, which are composed of proteins of the connexin family. Experiments with transfected cell lines and paired Xenopus oocytes have demonstrated that heterotypic intercellular channels which are formed by two connexons, each composed of a different connexin, can selectively occur. JEAN X. [26] showed by immunoprecipitation that connexons can contain two different connexins forming heteromeric assemblies in vivo.

Our results demonstrate the basilar arteries presence of heteromeric connexons containing Cx43 and Cx45 in vivo, indicating that previously observed selectivity of gap junctional channels is likely to be even more complicated than in vitro models. **Figures 2** and **3** show that Cx45 and Cx43 protein can form heteromeric gap junction in normal rabbit basilar arteries tissue. But in AH-7day groups the Cx45 and Cx43 protein form heteromeric gap junctions seems increased. However, this increased tendency was inhibited by the gap junction blocker CBX. This protien expression trend is in accordance with CVS (**Figure 1**). We hypothesize that an acquisition of new regulatory properties of vascular gap junctions as a result of connexin heteromeric assembly may modify both the extent and the manner of intercellular communication between adjacent cells, such as selectivity and gating of the channels. Sergio Elenes et al. [27] support the idea that new conduction and gating properties of channels become evident when cells form heterotypic channels (such as between Cx45 and Cx43). Agustin et al. [28] show that coexpressed Cx43 and Cx45 can form heteromeric channels. Some characteristics of the heteromeric channels (Lucifer yellow permeability and TPA-induced reduction of neurobiotin transfer) are dominated by one of the connexin components. These properties appear to follow those of the most restrictive component. Differences in the relative expression of Cx43 and Cx45 might lead to changes in the abundances of heteromeric Cx43/Cx45 gap junction channels. Such changes might be accompanied by alterations in conduction and in the intercellular permeability/flux of signaling molecules.

In conclusion, Cx43 and Cx45 protein can form heteromeric gap junction in normal rabbit basilar arteries tissue. These heteromeric gap junctions increase after SAH, and blocking of these

heteromeric gap junctions may relieve cerebral vasospasm after SAH.

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Disclosure of conflict of interest

None.

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