Astrocytic Gap Junctions Composed of Connexin 43 Reduce Apoptotic Neuronal Damage in Cerebral Ischemia

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Background and Purpose—Astrocytes may play a vital role in neuroprotection by providing energy substrates to neurons and regulating the concentration of K⁺ and neurotransmitters through gap junctions. Connexin 43 (Cx43) is one of the major gap junction proteins in astrocytes. We have shown that, after focal stroke, heterozygote Cx43 null (Cx43+/−) mice exhibited larger infarction volumes than wild-type (Cx43+/+) mice. We explored the underlying mechanism by which gap junctional intercellular communication influences astrocytic activation and neuroprotection in ischemia.

Methods—Both Cx43+/− and Cx43+/+ mice underwent right side permanent middle cerebral artery occlusion (MCAO). Mice were prepared by transcardial perfusion, and at 24 hours and 4 days after surgery, brains were prepared for immunohistochemistry or Western blot analysis.

Results—Four days after MCAO, Cx43+/− mice showed severe apoptosis in the penumbral lesion compared with Cx43+/+ mice. The level of caspase-3 was significantly higher in the stroke lesion of Cx43+/− mice than in Cx43+/+ mice. Four days after MCAO, Cx43+/− mice showed a significantly larger infarct volume but a smaller area of astrogliosis than did Cx43+/+ mice. The penumbra of Cx43+/− mice showed an increased level of Cx30 compared with Cx43+/+ mice.

Conclusions—Gap junctions may play an important role in astrocytic activation. Reactive astrocytes may reduce neuronal apoptosis under ischemia by regulating extracellular conditions through their gap junction. (Stroke. 2003;34:1987-1993.)

Key Words: apoptosis ■ astrocytes ■ connexin 43 ■ gap junctions ■ stroke

Astrocytes are the major cell type in the brain. Recent studies have revealed that they not only receive signals from neurons but also release neuroactive substances and provide energy substrates to neurons. Moreover, glial cells secrete various neurotrophic factors and cytokines that stimulate and protect neurons against oxidative stress. In the central nervous system, astrocytes establish a glial syncytium through intercellular connection via gap junctions. Connexin 43 (Cx43) is the primary component protein in astrocytic gap junctions. Gap junctional intercellular communication (GJIC) mediates electronic coupling and permits rapid propagation among cell networks. GJIC between astrocytes may regulate the concentration of extracellular K⁺ and distribute neurotransmitters. According to these contexts, astrocytes play an important role in neuronal support in both normal and pathologic conditions.

Stroke lesion increases progressively after ischemia. Neurons in the core lesion die immediately after ischemia; however, some neurons in the penumbra are still viable for several hours. The cells in the penumbra may succumb to apoptosis, which may cause the expansion of the stroke lesion. To rescue the penumbra is critical in considering strategies for ischemic stroke treatment. Under the ischemic condition, the role of astrocytes is still controversial. The wave of spreading depression goes through astrocytic gap junctions and is suspected to cause expansion of stroke volume. Inhibition of astrocytic gap junction permeability potentially restricts the flow of neurotoxic metabolites and may reduce neuronal death. On the other hand, glial cells protect neurons from hypoxic depolarization. Astrocytic GJIC decreases neuronal vulnerability to oxidative stress observed in primary coculture and hippocampal slice culture. Furthermore, blocking gap junctions in astrocytes increases cocultured neuronal death by glutamate cytotoxicity.

Although Cx43 knockout (Cx43−/−) mice, which have been established by Reaume et al, die immediately after birth, heterozygote Cx43 null (Cx43+/−) mice are viable. Cultured astrocytes derived from Cx43+/− mice show reduced Cx43 expression and intercellular Ca²⁺ signaling. Our laboratory has shown that Cx43+/− mice exhibited a significantly larger infarct volume than did wild-type (Cx43+/+) counterparts after middle cerebral artery (MCA) occlusion (MCAO). In this study we explored the underly-
ing mechanism by which astrocytic GJIC influences glial activation and subsequent neuroprotection in ischemia. Cx43+/− mice exhibited increased apoptosis and caspase-3 level compared with Cx43+/+ mice. Our results indicate that reactive astrocytes may reduce neuronal apoptosis in the ischemic penumbra.

Materials and Methods

Cx43+/− Mice

Cx43+/− mice were maintained as a breeding colony in the CD-1 background in the animal facility at the University of Western Ontario.

Surgery

All procedures were approved by Animal Care and Veterinary Services of the University of Western Ontario. Adult male Cx43+/− mice (n=13) and counterpart Cx43+/+ mice (n=13), each weighing 30 to 40 g, were anesthetized with sodium pentobarbital (65 mg/kg IP). Each animal was secured to a heating pad with surgical tape, and the head was held securely in place with a stereotaxic frame. The rectal temperature was maintained at 37.5°C throughout the surgery and after the procedure until the animal regained consciousness. Under anesthesia, the right MCA was exposed according to a procedure described previously. Briefly, an incision was made on the right side of the head from the anterior of the ear to the corner of the eye horizontally and from the corner of the eye to the top of the eye vertically 5 mm. The squamosal bone was exposed by removing the temporal muscle. By using an electronic-powered dental drill, a small burr hole was made approximately 3 mm in diameter on the bone. The overlying dura mater was removed to expose the MCA. The MCA was occluded above and below the rhinal fissure with an overlying bone. The overlying dura mater was removed to expose the MCA. The MCA was occluded above and below the rhinal fissure with an electronic coagulator. After it was confirmed that there was no recanalization, the skin incision was closed with sutures. Sham-operated mice (3 of each genotype) received the same surgical procedure without the incision of the skull and the skin incision was closed with sutures. Sham-operated mice (3 of each genotype) received the same surgical procedure without the incision of the skull and the skin incision was closed with sutures.

Histological Analysis

One or 4 days after surgery, animals were placed under deep anesthesia with an overdose of sodium pentobarbital (130 mg/kg IP) and perfused transcardially first with 10 mmol/L PBS (pH 7.4) and then with 4% paraformaldehyde (pH 7.4) in PBS. The brains were removed and fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose in PBS. Coronal sections were cut with a cryostat microtome at a thickness of 20 μm for measuring the infarct volume. The brain slices were mounted sequentially onto glass microscope slides and stained with thionin to allow measurement of infarct size, areas in each section were measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral hemisphere. The areas of infarction on each slice were summed and multiplied by slice thickness to give the infarction volume.

TUNEL Staining

TUNEL staining was performed according to protocol of the Apoptosis Detection System, Fluorescein (Promega). The stained sections were examined under a photomicroscope (Zeiss Axiophot, Carl Zeiss). TUNEL-positive cells were counted and averaged in a 0.09-mm² area of 6 randomly chosen areas in the ischemic penumbra.

Immunohistochemistry

Brain sections (7 μm in thickness) were mounted on glass slides and blocked with 10% goat serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS for 1 hour. The sections were then incubated with Cx43 (Sigma), connexin 30 (Cx30) (Zymed Laboratory), cleaved caspase-3 (Cell Signaling), and glial fibrillary acidic protein (GFAP) (Sigma) antibodies (dilution was 1:1000, 1:500, 1:100, and 1:1000, respectively) in 1% BSA and 0.3% Triton X-100 in PBS, washed in PBS, and reacted with appropriate secondary antibodies (Alexa Fluor, Molecular Probes Inc) in 1% BSA and 0.3% Triton X-100 in PBS for 1 hour. The sections were observed under a photomicroscope (Zeiss Axiophot).

Western Blot Analysis

For protein extraction, the cerebula were dissected into 4 parts: right hemisphere cortex, hippocampus, left hemisphere cortex, and hippocampus. Each part was homogenized in homogenization buffer (1:1 wt/vol) containing 0.5 mol/L NaCl, 5 mmol/L Tris (pH 8.0), 1% Nonidet P-40, 10% glycerol, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail tablets (Complete, Roche) on ice. Homogenates were subjected to vortexing for 1 hour at 4°C and sonicated 2 times for 20 seconds on ice. Protein concentrations were determined with the bicinchoninic acid protein assay regent (PIERCE). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Twenty-five micrograms of each homogenate was loaded into each lane. For Western blotting, the gel was transferred to nitrocellulose filter (0.22-μm pore, Bio-Rad) and blocked in 5% (wt/vol) nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline (pH 7.6) for 1 hour. After incubation with Cx43, Cx30, GFAP, and β-actin (Sigma) antibodies (dilution was 1:8000, 1:1000, 1:10 000, and 1:20 000, respectively) in Tris-buffered saline with 5% nonfat dry milk and 0.1% Tween 20, membranes were washed and incubated with a goat anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (Cedarlane, Hornby). The immunoreaction was detected with a chemiluminescence kit (SuperSignal West Pico, PIERCE). To confirm a consistent protein loading for each lane, membranes were stained for β-actin. After the x-ray films (X-Omat, Kodak) were scanned (ScanJet 6300C, Hewlett-Packard), the signal intensities of the bands were analyzed with Scion Image software (Scion Corp).

Statistical Analysis

All data are presented as mean±1 SEM values for each group. In Western blot experiments, mean values in the 2 groups were compared by paired (2-tailed) t test. To compare mean values in 2 separate groups for infarct volume, we used an unpaired t test. Values of P<0.05 were considered significant.

Results

Representative images of thionin-stained sections are shown in Figure 1A. Cx43+/− mice showed a larger infarction area than Cx43+/+ mice. We quantified these differences of infarction volume and found that the volume in Cx43+/− mice was significantly larger than that in Cx43+/+ mice (Figure 1B; 10.7±1.59 and 6.1±1.90 mm³, respectively). These results support our previous findings. To explore the nature of differences of infarction volume between Cx43+/+ and Cx43+/− mice, we examined the apoptotic reaction in the penumbra. Representative TUNEL stainings are shown in Figure 2A and 2B. The Cx43+/− penumbra showed an increase in apoptosis compared with Cx43+/+ at 4 days after MCAO. Although there was no significant difference in the number of TUNEL-positive cells between Cx43+/+ and Cx43+/− mice at 24 hours after MCAO (5.5±0.57 cells per 0.09 mm² and 6.8±2.68 cells per
0.09 mm$^2$, respectively), the TUNEL-positive cells were significantly increased in Cx43$^+/−$ mice at 4 days after MCAO (Figure 2C; Cx43$^+/+$, 4.8±0.45 cells per 0.09 mm$^2$; Cx43$^+/−$, 11.2±2.12 cells per 0.09 mm$^2$). To determine which cells were undergoing apoptosis, we stained sections with GFAP and caspase-3 antibodies (Figure 3A and 3B). As seen in the representative images, caspase-3 reactivity was located primarily on the GFAP-negative cells. Moreover, caspase-3 staining was stronger in Cx43$^+/−$ than in Cx43$^+/+$ mice.

Figure 1. Representative thionin staining of brain sections (A) shows larger infarction area in Cx43$^+/−$ than in Cx43$^+/+$ mice (see arrows delineating lesion). The average of total infarction volume (n=5 each) was significantly larger in Cx43$^+/−$ than in Cx43$^+/+$ mice at 4 days after MCAO (B). *$P<0.05$. Bar=1 mm.

Figure 2. TUNEL-positive cells appear in green fluorescence, and all nuclei are visible with propidium iodide red fluorescence at 4 days after MCAO in Cx43$^+/+$ (A) and Cx43$^+/−$ (B) penumbra. Apoptosis in the area surrounding the ischemic core was predominant in Cx43$^+/−$ mice. In the cell-counting analysis (C), the number of apoptotic cells was not significantly different between Cx43$^+/+$ and Cx43$^+/−$ mice at 1 day after MCAO. Although the level of apoptosis was unchanged in Cx43$^+/+$ mice at 4 days compared with 1 day after MCAO, the number of apoptotic cells was significantly increased in Cx43$^+/−$ mice at 4 days after MCAO. **$P<0.01$. Bar=100 μm.
GFAP-immunofluorescent staining of the cortex taken from mice at 4 days after MCAO is shown in Figure 4A and 4B. We used GFAP antibody in reduced dilution. Therefore, only activated astrocytes with increased levels of GFAP should be recognized.21 The area of astrogliosis was smaller in Cx43+/−/H11002 than in Cx43+/+ mice. We measured the width of the astrogliosis in 6 different cortical lesion areas in each mouse (n=5 in each group) and observed that the area was significantly smaller in Cx43+/−/H11002 than in Cx43+/+ mice (Figure 4E; 499.8±18.80 and 678.7±23.65 μm, respectively). We counted the number of GFAP-positive astrocytes in a certain cortical area to determine its density; however, there was no significant difference between Cx43+/+ and Cx43+/− mice. As seen in representative images in the penumbra, GFAP-positive astrocytes were observed to exhibit longer processes in Cx43+/− than in Cx43+/+ mice (Figure 4C and 4D). Ten independent GFAP-positive astrocytes were taken from different cortical lesions to measure the length of the processes. Three different processes were chosen in each astrocyte. The Cx43+/− astrocytes had significantly longer processes than Cx43+/+ astrocytes (Figure 4F; 30.1±1.13 and 23.3±0.86 μm, respectively).

The Cx43 level was significantly reduced in the contralateral cortex as well as in the penumbra in Cx43+/− mice compared with Cx43+/+ mice, as expected (Figure 5A and 5B; 0.68±0.21 and 0.73±0.20 in Cx43+/− and 1.73±0.47 and 1.73±0.41 in Cx43+/+ mice, respectively). We measured the levels of Cx30, also a major astrocytic gap junction protein, by Western blot to examine whether it was altered (Figure 5A and 5C). Although the Cx30 level was the same between contralateral cortex and stroke lesion in Cx43+/+ mice (1.18±0.11 and 1.17±0.09, respectively), the Cx30 level was significantly increased in the stroke lesion compared with the contralateral cortex in Cx43+/− mice (1.47±0.13 and 1.18±0.09, respectively). From the results of GFAP-immunofluorescent staining, astrogliosis in the cortical lesion was significantly reduced in Cx43+/−/H11002 mice. However, by Western blot analysis, the increased ratio of GFAP level in the stroke versus contralateral cortex was significantly higher in Cx43+/− than in Cx43+/+ mice (Figure 5A and 5D; 2.03±0.16 and 3.12±1.33, respectively).

Discussion
Our results support the previous report that Cx43+/− mice showed a significantly larger stroke volume than Cx43+/+ mice after right MCAO.18 Thus, we explored the possibility that the reduction of astrocytic GJIC influences the propagation of stroke lesion. Decreased gliosis and increased apoptosis, even 4 days after MCAO, in Cx43+/− stroke lesion suggest that astrocytic GJIC plays a critical role in development of the reactive astrocytic network and possibly in removal of cytotoxic factors.
We first examined the differences of apoptosis between the cortical lesion in Cx43+/+ and Cx43+/− mice. It has been shown that neuronal apoptosis in the penumbral lesion occurs several hours after ischemia.8,9 After the blood supply is blocked or significantly reduced, the ischemic core of the lesions develops necrosis, and the cells of the boundary zone trigger the apoptotic process. Massive glutamate release activates downstream mechanisms such as Ca2+ influx.22 Reperfusion after ischemia causes free radical stress, which accelerates glutamate release.23 Our results demonstrated that Cx43+/− mice showed significantly enhanced apoptosis at 4 days after MCAO compared with Cx43+/+ mice. While the number of apoptotic cells becomes maximal at 24 and 48 hours after ischemia,8 the number was still increased at 4 days after MCAO in Cx43+/− lesion. Caspase-3 is one of the key molecules of apoptosis, activated by proapoptotic stimuli such as initiator caspases and cytochrome C.24 In this study caspase-3 was not colocalized with GFAP immunoreactivity. Therefore, apoptosis may be occurring in cells other than reactive astrocytes, most likely neurons. Astrocytes in Cx43+/− brain may not be able to remove apoptosis-stimulating factors as efficiently as astrocytes in the normal network. Subsequently, the amount of caspase-3 protein remained high, and the apoptotic reaction continued for a longer period compared with the wild-type group.

We observed a difference in reaction of astrocytes, ie, gliosis, between Cx43+/+ and Cx43+/− mice. Cerebral ischemia leads to profound glial activation as well as neuronal loss.25,26 Astrocytes exchange inositol 1,4,5-triphosphate, lactate, glutamate, and other smaller molecules through gap junctions and provide energy substrates such as ATP to neurons.27,28 Moreover, in the ischemic condition, astrocytes immediately remove cytotoxic factors, such as Ca2+ and glutamate.28,29 In our results, astrogliosis was significantly reduced in the cortex of Cx43+/− mice compared with Cx43+/+ mice, and reactive astrocytes in Cx43+/− mice had significantly longer processes compared with Cx43+/+ astrocytes. Western blot analysis clearly demonstrated that GFAP levels after MCAO were significantly higher in Cx43+/− than in Cx43+/+ mice. Because the function of Cx43+/− astrocytes is compromised, the formation of gliosis may be impaired. Astrocytes may react by extending their processes longer and searching for contacts to each other. The stronger stimulation in each astrocyte may cause the observed increase in total volume of GFAP-immunoreactive tissue.

**Figure 4.** In cortex with GFAP-fluorescent staining (A, Cx43+/+; B, Cx43+/−), the area where GFAP-positive astrocytes existed was more extensive in Cx43+/+ than in Cx43+/− mice. The average width of the area was significantly smaller in Cx43+/− than in Cx43+/+ mice (E). In penumbra (C, Cx43+/+; D, Cx43+/−), the size of astrocytes in Cx43+/− was large compared with that in Cx43+/+ mice. The average length of the processes of astrocytes was significantly longer in Cx43+/− than in Cx43+/+ mice (F). **P<0.01. Bar=500 μm in A and B and 50 μm in C and D.
Cx43 is also expressed by endothelial, microglial, and macrophage cells as well as by astrocytes. Maintaining the permeability of blood vessels is an important role of the endothelial gap junctions in inflammation. Microglia may communicate through their gap junctions and be able to respond systematically to inflammation. In Cx43/H11001/H11002 mice, these inflammatory responses may also be affected and result in increased neuronal injury.

The level of Cx43 protein was significantly reduced in Cx43/H11001/H11002 mice, whereas the level of Cx30 was significantly upregulated in the ischemic cortex of those mice. However, the increase of Cx30, which is thought to be mainly in astrocytes, could not compensate for the reduction of Cx43, as seen in the greater infarct volume for Cx43+/− mice. Although Cx43 expression is detected from early postnatal days, the Cx30 level becomes stronger in adult brains. Since there is no apparent compensation regarding infarct size, the function of Cx30 may be different from that of Cx43. Connexin 32 (Cx32) and connexin 36 (Cx36) expression was increased in hippocampus after ischemia. Moreover, Cx32 knockout mice exhibited enhanced vulnerability to ischemia, suggesting that Cx32 may play a critical role in neuronal survival. It is becoming increasingly clear that permeability of gap junctions varies depending on the connexin composition. According to our results, Cx43 protein may be critical for the function of astrocytic GJIC after stroke.

In conclusion, since the astrocytic Cx43 protein was reduced in the cortex of Cx43+/− mice, the reactive astrocytes may have extended their processes longer and increased Cx30 protein to compensate for the decrease of Cx43. However, Cx30 may not aid in preventing the damage caused by stroke. Formation of astrogliosis may become insufficient under compromised GJIC. Apoptosis-stimulating factors may not be removed from the penumbra quickly, and apoptosis may be observed strongly even 4 days after MCAO. Those results suggest that astrocytic gap junctions may be a therapeutic target for stroke.

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