Different Expression Patterns of Bcl-2, Bcl-xl, and Bax Proteins After Sublethal Forebrain Ischemia in C57Black/Crj6 Mouse Striatum

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Background and Purpose—Ischemic injury in neurons can be strongly reduced by a preceding sublethal ischemic episode, of which the mechanism is poorly understood. Although changes in the expression of apoptosis-related proteins (Bcl-2, Bcl-xl, and Bax) have been considered to be crucially important in ischemic injury, the roles these proteins play in ischemic preconditioning induced by sublethal forebrain ischemia have not been elucidated. Therefore, we investigated the transcription and expression of Bcl-2, Bcl-xl, and Bax in striatum of mice subjected to sublethal forebrain ischemia and lethal ischemia, with or without ischemic preconditioning.

Methods—Sublethal forebrain ischemia was induced in C57Black/Crj6 (C57BL/6) mice by 6 minutes of bilateral common carotid artery occlusion. The transcription and expression of Bcl-2 family genes were detected by reverse transcription–polymerase chain reaction, Western blot, and immunofluorescent staining.

Results—No detectable neuronal loss was induced in striatum by 6 minutes of bilateral common carotid artery occlusion. Transcription and expression of Bcl-2 and Bcl-xl were increased after sublethal forebrain ischemia, which attenuated the DNA fragmentation induced by lethal ischemia. The transcription and expression of Bax remained unchanged.

Conclusions—Upregulation of Bcl-2 and Bcl-xl but not Bax may have a role in protective ischemic preconditioning. This result indicates a potential strategy for further ischemic neuronal injury therapies. (Stroke. 2003;34:1803-1808.)

Key Words: cerebral ischemia ■ ischemic preconditioning ■ neuroprotection ■ proto-oncogene proteins c-bcl-2 ■ mice

A brief episode of sublethal ischemia, as well as some other stresses, produces strong protection against subsequent detrimental ischemic insult. This phenomenon has been termed ischemic preconditioning and was first described in the heart and the brain.2,3 A forebrain ischemic preconditioning model developed in our laboratory showed that striatal neuronal injury after 18 minutes of lethal ischemia can be markedly reduced by an episode of 6 minutes of preceding sublethal ischemia.4 However, the mechanism of this protective effect is unclear.

Compelling evidence indicates that apoptosis may occur after transient cerebral ischemia.5,6 Moreover, recent cerebral ischemia studies in rats and gerbils have revealed that dysregulation of Bcl-2 family proteins can exacerbate ischemic neuronal injury and that the interaction between Bcl-2 family members that suppress (such as Bcl-2 and Bcl-xl) and those that promote (such as Bax) apoptosis determines whether cells undergo survival or apoptosis.7,8 In our preliminary experiments, we subjected the animals to bilateral common carotid artery occlusion (BCCAO) for 3, 6, and 8 minutes as ischemic preconditioning at 24, 48, and 72 hours and 7 days before 18 minutes of lethal ischemia. Although no striatal and hippocampal neuronal damage was found after 3 minutes of ischemia, the protection against the subsequent lethal ischemia was weaker. In contrast, 8 minutes of ischemia sometimes caused serious striatal and hippocampal neuronal damage. Interestingly, the protective effect of ischemic preconditioning was more dramatic and stronger at 48 hours after 6 minutes of BCCAO than at the other time points. We thus subjected mice to 6 minutes of BCCAO as the conditioning ischemic episode 48 hours before the 18 minutes of lethal ischemia in this experiment.

To address the question of whether these Bcl-2 family proteins participate in protective ischemic preconditioning induced by sublethal forebrain ischemia, we examined the transcription and expression of Bcl-2, Bcl-xl, and Bax by reverse transcription–polymerase chain reaction (RT-PCR), immunoblot assays, and immunohistochemistry.

Materials and Methods

This study was approved by the Committee on the Guidelines for Animal Experiments at Niigata University.
Animal Model

Experiments were performed in adult male C57Black/Crj6 (C57BL/6) mice weighing 19 to 23 g (aged 8 to 10 weeks). Sublethal forebrain ischemia was induced by BCCAO for 3, 6, and 8 minutes, and lethal ischemia was induced for 18 minutes with the use of microvascular clips. We also subjected some animals to 6 minutes of BCCAO as a conditioning ischemic episode 48 hours before the 18 minutes of lethal ischemia. Exposure of the bilateral common carotid arteries without occlusion was used to produce sham control animals. Animals were killed at variable time points according to the experimental protocols.

RT-PCR and Detection of mRNA Expression

Transcription of Bcl-2, Bcl-xl, and Bax at 6, 12, 24, 48, and 72 hours after 6 minutes of ischemia was analyzed by RT-PCR (n=6 per group). Total RNA of mouse striatum was extracted with the use of TRIzol reagent (Invitrogen, Life Technologies). Total RNA (2 μg) was reverse transcribed at 37°C for 1 hour. The use of oligo(dT) primer and reverse transcriptase (superscript Life Technologies). Primers used in this experiment are listed in the Table. Reverse-transcribed RNA was amplified with Taq DNA polymerase (AmpliTaq, Perkin-Elmer) by PCR for 35 cycles (94°C for 1 minute, 64°C for 1 minute, 72°C for 1 minute). To optimize the experimental condition, 35 cycles were used, which was still in the exponential stage on the basis of analysis of the preliminary experiments. The PCR products were run on 1% agarose gel and stained with ethidium bromide. The intensity of each band was quantified by a Scion image system (Scion Corporation).

Western Blot Analysis

Mice were anesthetized at 4, 24, 48, and 72 hours after reperfusion (n=6 per group). Immediately after decapitation, striatum was dissected, then homogenized and lysed on ice in RIPA buffer containing protease inhibitors. The supernatant was collected with low-molecular-weight standards (Bio-Rad, Mini-protean System) was transferred to polyvinylidene difluoride membranes (Bio-Rad) and then incubated in 5% skimmed dry milk for 1 hour. The membranes were incubated in a 1:1000 dilution of mouse anti-Bcl-2 (Transduction Laboratories, catalog No. B46620) and mouse anti-Bcl-xl and anti-Bax antibody (both Santa Cruz Biotechnology Inc, catalog No. sc-8392 and sc-7480, respectively). After they were washed, the secondary antibody (American Qualex, catalog No. A106PU) was applied. Membranes were then developed with enhanced chemiluminescence (ECL, Amer sham) plus reagents and exposed to RX film. The density of each band was quantified as described above.

Immunocytochemistry

At 48 hours after reperfusion, mice (n=6 per group) were anesthetized and then transcardially perfused with 0.1 mol/L PBS containing heparin (4 U/mL), followed by 0.01 mol/L periodate/0.075 mol/L lysine/2% paraformaldehyde in 0.0375 mol/L PBS (pH 6.3). Brains were removed and post-fixed. After they were washed in gradually increasing concentrations of sucrose, brains were rapidly frozen at −80°C. Consecutive coronal sections (10 μm) were prepared and frozen until they were used.

To block nonspecific reactions, sections were incubated in 0.1 mol/L PBS containing 1.5% bovine serum albumin, 0.2% gelatin, and 0.1% Tween-20 for 1 hour. Sections were then incubated in the same Bcl-2, Bcl-xl, or Bax antibody that was used for Western blot at a dilution of 1:200. After several washes, a 1:200 dilution of goat anti-mouse FITC immunonjugate (Santa Cruz Biotechnology Inc, catalog No. sc-2010) was added. Sections were then mounted and observed under light microscopy with excitation/emission wavelengths of 494/520 (green).

To identify whether striatal neuronal decay occurred after 6 minutes of ischemia, immunofluorescent staining for neuron-specific nuclear protein NeuN (mouse anti-NeuN, Chemicon International, Inc, catalog No. MAB377) was performed on day 7 after reperfusion. The procedures were essentially the same as described above. The number of NeuN-positive cells was counted in 5 assigned subregions (250×125 μm²) in the striatum, as described previously.

Double-Label Immunocytochemistry

Terminal deoxynucleotidyl transferase–mediated dUTP-nick end-labeling (TUNEL) was performed with an ApopTag Red apoptosis in situ detection kit (Intergen Company) to covisualize DNA strand breaks and Bcl-2 family proteins on the same slide. Sections obtained from mice (n=6 per group) at 4 or 48 hours after 18-minute ischemia were processed for TUNEL staining according to the protocol of Intergen Company, and then immunofluorescent staining as described above was performed for Bcl-2 family proteins.

To indicate whether Bcl-2 family proteins colocalized to neurons or other cell populations after ischemia, double-label immunofluorescent staining was performed for Bcl-2 family proteins with either the NeuN or glial marker glial fibrillary acidic protein (GFAP) (Dako, catalog No. Z0334).

Statistical Analysis

Quantitative data were expressed as mean±SD. Differences between means were determined by 1-way ANOVA followed by Dunn’s post hoc test for multiple comparisons. A probability value of <0.05 was considered statistically different.

Results

RT-PCR Analysis

RT-PCR was performed to detect the changes of Bcl-2, Bcl-xl, and Bax mRNA transcription at 6, 12, 24, 48, and 72 hours after sublethal ischemia. As shown in Figure 1, 6 minutes of sublethal ischemia induced marked transcriptions of Bcl-2 and Bcl-xl genes. There were modest increases in the Bcl-2 and Bcl-xl mRNA levels at 6 hours that become significant 12 hours after 6 minutes of BCCAO. Bcl-2 mRNA levels increased 2.2-fold at 12 hours and 2.1-fold at 24 hours. Bcl-xl mRNA levels increased 1.8- and 2.3-fold at 12 and 24 hours, respectively. Both Bcl-2 and Bcl-xl mRNA returned to basal level at 72 hours. No significant change in Bax mRNA levels was found between sham control and animals in the 6-minute ischemia group over the designed time course.

Western Blot Analysis

Western blot analysis showed that expression of Bcl-2, Bcl-xl, and Bax proteins was present in all samples examined. Notably, expression of Bcl-2 and Bcl-xl proteins was increased markedly in ischemic striatum from 48 hours after reperfusion compared with those of sham controls. Figure 2A shows the bands at approximately 26 kDa, which was the predicted size for Bcl-2 protein. Compared with sham controls, the ischemic striatum showed increased Bcl-2 immuno-
reactivity at 48- and 72-hour time points. Figure 2B shows that the expression of approximately 28 kDa Bcl-xl protein was weakly detected in sham controls and at 4 hours after ischemia. It increased at 48 and 72 hours after 6 minutes of ischemia. Both Bcl-2 and Bcl-xl proteins returned to the basal level on day 7. However, Bax protein (approximately 21 kDa) did not show any remarkable change after sublethal forebrain ischemia compared with sham controls (Figure 2C). Densitometry was performed, and the ratio of ischemic animals to sham controls was obtained. Changes in the density of Bcl-2 family proteins at each time point are shown in Figure 2D. Bcl-2 protein was increased 1.7-fold at 48 hours (P<0.05 versus control) and increased 1.6-fold at 72 hours (P<0.05 versus control) in ischemic samples. Bcl-xl was increased 2.3- to 2.5-fold from 48 to 72 hours, respectively, after reperfusion. No significant difference of Bax levels was found between sham controls and the experimental groups.

**Immunohistochemistry**

NeuN staining showed that 6 minutes of BCCAO did not induce any detectable loss of neurons compared with sham controls. The numbers of neurons in control striatum and those at 7 days after 6 minutes of BCCAO were 98.8±10.2/250×125 μm² and 95.6±8.1/250×125 μm², respectively. Basal Bcl-2 (Figure 3A), Bcl-xl (Figure 3E), and Bax (Figure 3G) immunoreactivity was detectable in control striatum. At 48 hours after 3 minutes of BCCAO, Bcl-2 (Figure 3B), Bcl-xl, and Bax (data not shown) immunoreactivity showed no increase compared with controls. Bcl-2 (Figure 3C) and Bcl-xl (Figure 3F) immunoreactivity increased in striatal neurons at 48 hours after 6 minutes of BCCAO, while Bax immunoreactivity showed no increase (Figure 3H). Although Bcl-2 immunoreactivity increased at 48 hours after 8-minute BCCAO (Figure 3D), TUNEL-positive cells could be also observed (Figure 3D [red]). Bcl-xl exhibited approximately the same change as Bcl-2 after 8 minutes of BCCAO (data not shown). Bcl-2/NeuN double staining and Bcl-2/GFAP double staining at 48 hours after 6-minute BCCAO showed that Bcl-2 colocalized to neurons (Figure 3I) but not astrocytes (Figure 3J).

**Double-Label Immunocytochemistry**

In non-preconditioned mice, double-label staining of TUNEL and Bcl-2 revealed basal Bcl-2 immunoreactivity at 4 hours after 18 minutes of lethal ischemia; no TUNEL-positive cells were present (Figure 4A). The most TUNEL-positive cells (29.3±4.8/250×125 μm²) were found at 48 hours after 18 minutes of BCCAO, and few intact neurons (7.2±2.4/
250×125 μm²) were found, showing increased Bcl-2 expression with weaker or no TUNEL reactivity (Figure 4B). In preconditioned mice, the intensity of Bcl-2 immunoreactivity increased at 4 hours after 18 minutes of BCCAO (Figure 4C), while again no TUNEL-positive cells were found. Consistent with the results in non-preconditioned mice, some intact neurons showed increased Bcl-2 immunoreactivity, and some TUNEL-positive cells were found. However, the number of intact neurons (20.7±6.3/250×125 μm²) was greater and the number of TUNEL-positive cells (9.2±3.2/250×125 μm²) was smaller in preconditioned animals (Figure 4D). Bcl-xl was also detected, and the findings were quite similar to those of Bcl-2 (Figure 4E and 4F). Figure 4E and 4F show images of TUNEL-positive cells (red) and Bcl-xl (green) at 48 hours after 18 minutes of BCCAO with or without preconditioning.

Bax immunoreactivity appeared to show no change between preconditioned and non-preconditioned animals (data not shown).

Discussion

The major finding of this study is that upregulation of Bcl-2 and Bcl-xl, but not Bax protein, was identified in C57BL/6 murine striatum after 6 minutes of BCCAO, which indicates that Bcl-2 and Bcl-xl proteins may participate in protective ischemic preconditioning induced by sublethal forebrain ischemia.

Figure 2. Representative Western blots showed immunoreactivity of Bcl-2 family proteins in controls and in striatum at 4, 24, 48, and 72 hours and 7 days after 6 minutes of BCCAO. A, Bcl-2 protein was detected in all samples at the predicted molecular size of 26 kDa. Compared with sham controls, the ischemic striatum showed increased Bcl-2 immunoreactivity at 48- and 72-hour time points. B, At approximately 28 kDa, Bcl-xl protein was weakly detected in sham control and at 4 hours after ischemia. It was increased at 48 and 72 hours after 6 minutes of ischemia. C, Bax protein was detected in all samples at 21 kDa. Bax immunoreactivity did not significantly change in ischemic striatum at the various time points. D, Relative changes in Bcl-2 family protein expression. Data are expressed as mean±SD of ratio of ischemic animals to sham controls (n=6 per group). Levels of Bcl-2 and Bcl-xl proteins significantly increased at 48 and 72 hours, but no significant change of Bax levels was found. *P<0.05 vs control, Dunnett’s post hoc test.

Figure 3. Immunofluorescent images of Bcl-2 (A, B, C, D), Bcl-xl (E, F), and Bax (G, H) in striatal sections. Bcl-2, Bcl-xl, and Bax were weakly expressed in sham control striatum (A, E, G). Bcl-2 expression was not increased at 48 hours after 3 minutes of ischemia (B). Upregulation of Bcl-2 and Bcl-xl expression was detected at 48 hours after 6 minutes of ischemia (C, F). Double staining of TUNEL and Bcl-2 showed TUNEL-positive cells (red) at 48 hours after 8 minutes of ischemia (D). Note that some TUNEL-positive cells displayed nuclear condensation without Bcl-2 expression in cytoplasm (arrows), and some intact neurons with cytoplasm Bcl-2 immunoreactivity showed weaker nuclear TUNEL reaction (arrowheads). No increased Bax immunoreactivity was detected in 6-minute ischemic striatum (H). Double staining of Bcl-2 and NeuN proved that Bcl-2 was expressed in neuronal cytoplasm (I), and double staining of Bcl-2 and GFAP showed no Bcl-2 expression in astrocytes (J). Bar=25 μm.
Among these proteins, Bcl-2 and Bcl-xl suppress apoptosis, whereas Bax promotes it. Therefore, attention has been paid to the fact that the counterregulated expression of Bcl-2 family proteins determines whether neurons undergo survival or apoptosis. Since striatal neuronal injury is more severe, symmetrical, and reproducible in comparison to the hippocampus in C57BL/6 mice, we focused on the striatum to examine Bcl-2 family protein expression rather than the hippocampus in the present study. Our results showed that Bcl-2, Bcl-xl, and Bax proteins were all expressed under normal conditions. These findings are consistent with previous studies with hippocampus of rats and gerbils. Immunoactivities of Bcl-2 and Bcl-xl proteins increased significantly from 48 hours after 6 minutes of ischemia and returned to basal levels within 7 days. The expression patterns of Bcl-2 and Bcl-xl proteins in this study are consistent with a previous study that indicated that intact neurons show an increase in Bcl-2 and Bcl-xl proteins in rat lethal ischemic cortex. We also examined the hippocampus in our preliminary experiments and found that changes of Bcl-2 family protein expression in the dorsal CA1, CA2, and CA3 regions of hippocampus were similar to that in striatum. However, changes in CA1 region were asymmetrical in 4 of 6 animals. Another finding is that 6-minute ischemia did not cause neuronal injury (data not shown).

We found that 3 minutes of BCCAO did not increase Bcl-2 and Bcl-xl expression, while 8 minutes of BCCAO did. However, neuronal injury was also found after 8 minutes of BCCAO. Indeed, double labeling of TUNEL and Bcl-2 family proteins revealed that lethal ischemia–induced DNA fragmentation was attenuated by the conditioning ischemia–induced Bcl-2 and Bcl-xl overexpression. Although TUNEL staining alone is not specific for apoptotic cell death, cell death with or without DNA fragmentation may implicate different underlying mechanisms. Among TUNEL-positive cells without Bcl-2 or Bcl-xl protein expression, a few showed the chromatin condensation or aggregation that are generally accepted as hallmarks of apoptotic cell death. In addition, few intact neurons with weaker or without TUNEL reactivity showed increased Bcl-2 or Bcl-xl expression. The attenuation of lethal ischemia–induced DNA fragmentation by a conditioning ischemia–induced Bcl-2 and Bcl-xl overexpression indicates that Bcl-2 family proteins may take part in protective ischemic preconditioning induced by sublethal forebrain ischemia.

In many experimental paradigms, level changes in the Bcl-2 family genes have been reported as early as 6 to 8 hours in hippocampus after lethal ischemia, but the increased Bcl-2, Bcl-xl, and Bax mRNA levels were not consistent with protein levels, probably because of the decreased protein synthesis due to lethal ischemia. In our experiment the Bcl-2 family mRNA level changes are observed to be consistent with those of the proteins. Here we consider that this discrepancy is due to the different severity of ischemia. We have proved that 6 minutes of BCCAO did not cause any neuronal loss, and therefore the protein synthesis inhibition might also be too slight to be detected in our experiment if such inhibition occurs at all.

Many studies have demonstrated that Bcl-2 family proteins are related to the formation of channels in mitochondrial membranes and regulate cytochrome c release. The released cytochrome c from mitochondria may activate the intrinsic apoptotic pathway via apoptosome formation and caspase-9 activation and thus drive cells to apoptosis. A recent study indicates that isolated mitochondria incubated with Bcl-2 show only very low cytochrome c release, and incubation of mitochondria with calpain-truncated Bcl-2 induces substantial or almost complete release of cytochrome c compared with total mitochondrial cytochrome c content. In addition, sublethal ischemia can prevent cytochrome c release from mitochondria. We speculate that Bcl-2 and Bcl-xl proteins are increased in cytoplasm after 6 minutes of sublethal forebrain ischemia; thereafter, when the subsequent lethal ischemia is performed, increased Bcl-2 and Bcl-xl proteins

\[ \text{Figure 4. Double staining of Bcl-2 family proteins (green) and TUNEL (red) in striatal sections obtained from non-preconditioned (A, B, E) and preconditioned (C, D, F) mice. A, Basal Bcl-2 immunoreactivity was found at 4 hours after 18 minutes of BCCAO, and few intact neurons with weaker or no TUNEL reactivity (arrowheads) showed increased Bcl-2 expression. C, In preconditioned mice, Bcl-2 immunoreactivity increased at 48 hours after 18-minute BCCAO. D, At 48 hours after 18-minute BCCAO in preconditioned mice, intact neurons showed increased Bcl-2 immunoreactivity, and few TUNEL-positive cells were found. However, the number of intact neurons (arrowheads) increased, and the number of TUNEL-positive cells (arrows) decreased, which contrasted with non-preconditioned animals. E, TUNEL-positive cells (arrows) and Bcl-xl immunoreactivity at 48 hours after 18 minutes of BCCAO, without preconditioning. F, Compared with non-preconditioned animals, the number of TUNEL-positive cells was smaller and Bcl-xl immunoreactivity was greater at 48 hours after 18 minutes of BCCAO in preconditioned animals. Note that TUNEL-positive cells demonstrated condensed, pyknotic nuclei and a clumped nuclear pattern suggestive of apoptosis (arrows). Bar=25 mm.} \]
are translocated to neuronal mitochondria membranes, and cytochrome c release is inhibited.

Additionally, Shimizu et al. demonstrated that the protective effect was diminished after the administration of Bcl-2 antisense oligodeoxynucleotides into cerebral ventricle, which further confirmed the involvement of Bcl-2 in ischemia preconditioning. Meanwhile, this study has provided a therapeutic strategy for the treatment of brain ischemia in the near future.

In conclusion, upregulation of Bcl-2 and Bcl-xl was detected in murine striatum after a sublethal forebrain ischemia, demonstrating that different expression patterns of Bcl-2, Bcl-xl, and Bax proteins may be associated with the protective effect of ischemic preconditioning. The present study may be useful for the further development of clinical therapies of neuronal injury induced by cerebral ischemia.

References


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