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Neuronal Expression of the DNA Repair Protein Ku 70 After Ischemic Preconditioning Corresponds to Tolerance to Global Cerebral Ischemia

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Background and Purpose—Oxidative stress after ischemia/reperfusion has been shown to induce DNA damage and subsequent DNA repair activity. Ku 70/86, multifunctional DNA repair proteins, bind to broken DNA ends and trigger a DNA repair pathway. We investigated the involvement of these proteins in the development of neuronal tolerance to global cerebral ischemia after ischemic preconditioning.

Methods—Adult male Sprague-Dawley rats were subjected to either 5 minutes of lethal global ischemia with or without 3 minutes of sublethal ischemic preconditioning or 3 minutes of ischemia only. Neuronal injury was histologically assessed, and DNA damage was visualized by in situ labeling of DNA fragmentation and DNA gel electrophoresis. Ku expression was also examined by immunohistochemistry and Western blot analysis.

Results—Hippocampal CA1 neurons underwent DNA-fragmented cell death 3 days after 5 minutes of ischemia. However, these neurons showed a strong tolerance to 5 minutes of ischemia 1 to 3 days after ischemic preconditioning. Immunohistochemistry showed virtually no constitutive expression of Ku proteins in CA1 neurons; however, ischemic preconditioning induced neuronal Ku 70 expression 1 to 3 days later. Western blot confirmed an increase in Ku 70 in this region at the same time.

Conclusions—The temporal and spatial expression of Ku 70 corresponded to tolerance of the hippocampal CA1 neurons to subsequent ischemia, suggesting the involvement of Ku proteins in the development of neuronal tolerance after ischemic preconditioning. **(*Stroke*. 2001;32:2388-2393.)**

Key Words: apoptosis ■ cerebral ischemia, global ■ DNA fragmentation ■ DNA repair ■ rats

Ischemic preconditioning (IPC) has been shown to ameliorate damage from subsequent global ischemia in the hippocampal CA1 subregion.[1,2] A number of reports have shown upregulation of certain genes and proteins after IPC, such as heat shock proteins (HSPs)[3–5] and Jun-related proteins,[6] and have speculated on their involvement in the development of ischemic tolerance. Neuronal death in the CA1 subregion after lethal global ischemia has been shown to occur in a delayed manner,[7] and recent studies have demonstrated that these neuronal deaths are in part caused by apoptosis.[8,9] DNA fragmentation and cell death are thought to occur downstream of cytochrome *c* release from mitochondria[10] and after activation of caspases.[11,12] On the other hand, ischemia/reperfusion injury directly causes oxidative DNA damage, which precedes DNA fragmentation in the brain,[13,14] suggesting the possibility that oxidative DNA damage can lead cells to apoptotic cell death. A recent study showed that IPC reduces the oxidative DNA damage of lethal global ischemia[15]; however, the involvement of DNA repair mechanisms after IPC has not been well studied.

The DNA repair protein Ku is one of the DNA end-binding proteins in mammalian cells. Ku 70 (70 kDa) and Ku 86 (86 kDa) proteins are DNA binding regulatory subunits of the DNA-dependent protein kinase (DNA-PK), which is composed of the 470-kDa catalytic subunit and Ku proteins.[16,17] Ku 70 and Ku 86 proteins contribute to the repair of DNA double-strand breaks as a part of the DNA-PK.[16] However, both Ku 70 and Ku 86 heterodimers have single-strand DNA-dependent ATPase activity and bind to single-strand DNA, single-strand nicks, gaps in DNA, and single- to double-strand transitions in DNA.[16] It has been reported that a short duration of ischemic insult to rabbit spinal cord induced reversible neurological deficits and increased the DNA-binding activity of Ku, whereas a long duration of ischemia caused permanent deficits and decreased the DNA-binding activity of Ku.[18] We recently reported that early reduction of Ku proteins preceded DNA fragmentation after focal cerebral ischemia.[19] These results suggest that Ku may play a role in DNA repair mechanisms after ischemia/reperfusion.

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To investigate the possible involvement of Ku proteins in DNA damage/repair pathway after lethal ischemia and IPC, we examined Ku expression by immunohistochemistry and Western blot.

Materials and Methods

Experimental Paradigms

A "lethal" 5 minutes of ischemia was used as a test insult, and a "sublethal" 3 minutes of ischemia was used for IPC. First, ischemic cell damage was characterized by cresyl violet staining, in situ labeling of DNA fragmentation (terminal deoxynucleotidyl transferase–mediated uridine 5′-triphosphate-biotin nick end labeling [TUNEL]), and DNA gel electrophoresis 1 and 3 days after the test ischemia and 1, 3, and 5 days after IPC. Ku immunohistochemistry was also performed at the same time points. Second, double staining was also performed to clarify Ku-immunopositive cell populations. Third, expression of Ku proteins was confirmed by Western blot at the same time points as immunohistochemistry after the test ischemia and IPC. Finally, Ku-positive neuronal cells were counted 1, 3, and 5 days after IPC, and preconditioning effects on neuronal survival were also evaluated. A test insult was induced 1, 3, and 5 days after IPC, and morphologically viable cells and TUNEL-positive cells in the hippocampal CA1 pyramidal cell layer were counted 5 days after the test insult.

Surgery

Transient global ischemia was induced in male Sprague-Dawley rats (weight, 275 to 350 g) by bilateral common carotid artery occlusion and bleeding to lower the mean arterial blood pressure to 30 to 35 mm Hg by the method originally described by Smith et al[20] with some modifications.[21,22] The rectal temperature was controlled at 37.0±0.5°C during surgery with a feedback-regulated heating pad. All animals were treated in accordance with Stanford University guidelines, and the animal protocol was approved by Stanford University's Administrative Panel on Laboratory Animal Care.

Histological Assessment

Anesthetized animals were perfused with 10 U/mL heparin and subsequently with 4% formaldehyde in PBS (pH 7.4). Brains were removed, postfixed for 24 hours in 4% formaldehyde, and sectioned at 50 μm on a vibratome. For histological assessment of damage in the hippocampus, the brain sections were stained with cresyl violet.

In Situ Labeling of DNA Fragmentation

The brains were rapidly frozen and sectioned on a cryostat into a thickness of 20 μm. Frozen brain sections at the level of the hippocampus were stained with the use of an in situ technique (TUNEL reaction) to detect the DNA free 3′-OH ends as previously described.[19] Staining was visualized with the use of 0.025% diaminobenzidine (DAB) and 0.075% H_2O_2 in PBS with 0.4 mg/mL nickel sulfate. The sections were then counterstained with methyl green.

DNA Gel Electrophoresis

Approximately 50 mg wet weight of tissue was taken from the hippocampal CA1 subregion after the brain was cut coronally. DNA in the samples was labeled with biotin, subjected to electrophoresis, and transferred to a nylon membrane. The membrane was first blocked by 5% powdered milk (BioRad) in 0.1 mol/L PBS for 30 minutes and then incubated with streptavidin-horseradish peroxidase conjugate (Trevigen) for 30 minutes. Finally, labeled DNA was visualized by the chemiluminescence method with the use of PeroxyGlow (Trevigen). The nylon membrane was exposed to x-ray film.

Immunohistochemistry

Free-floating coronal vibratome sections, 50 μm thick, were exposed to goat anti–Ku 70/86 antibody (1:200; Santa Cruz Biotechnology) in PBS for 24 hours at 4°C, followed by procedures previously described.[19] The sections were finally exposed to 0.025% DAB and 0.075% H_2O_2 in PBS for 1 minute. We also performed the preabsorption method using Ku blocking peptides (Santa Cruz Biotechnology) to confirm the specificity of the antibodies. Additional sections were also processed for double staining to clarify the subpopulations of Ku-immunopositive cells. To confirm the nuclear distribution of Ku, Ku 70/86 was labeled with fluorescein-conjugated anti-goat IgG (1:100; Jackson Immunoresearch Laboratories), and sections were mounted with a DNA dye, 4′, 6 diamidino-2-phenylindole (DAPI), containing mounting medium (Vectashield, Vector Laboratories). For Ku and glial fibrillary acidic protein (GFAP) double staining, Ku 70/86 was immunohistochemically visualized with DAB and nickel sulfate as described above, and the sections were incubated in anti-GFAP (1:100; Santa Cruz Biotechnology) for 1 hour and then in horseradish peroxidase–conjugated anti-goat antibody (1:100; Vector Laboratories). GFAP was visualized with DAB as described above.

Western Blot Analysis

Approximately 50 mg of samples from the CA1 subregion of the hippocampus was processed as described.[19] The primary antibody was a 1:1000 dilution of goat polyclonal antibody against Ku 70/86 (Santa Cruz Biotechnology). As a secondary antibody, horseradish peroxidase–conjugated anti-goat IgG was used, and signals were detected with a chemiluminescence kit (Amersham International). The signals were exposed on x-ray film (Hyperfilm; Amersham International). Subsequently, the membrane was processed in the stripping solution (100 mmol 2-mercaptoethanol, 2% SDS, 62.5 mmol Tris-HCl, pH 6.7) for 30 minutes at 60°C and further stained for β-actin to confirm the consistent protein loading per each lane. After the film was scanned with a GS-700 imaging densitometer (Bio-Rad), a quantitative analysis was performed with the use of Multi-Analyst software (Bio-Rad).

Cell Counting and Statistical Analyses

To evaluate the neuronal expression of Ku in the pyramidal cell layer at the center of the CA1 subregion (250 μm in length), the Ku-positive large nuclei (>5 μm) were counted by a blinded examiner. The number of positive nuclei was expressed as percentage of positive nuclei of that in the normal CA1 subregion. Viable cells and DNA-fragmented cells were also counted by the same procedure on cresyl violet–stained and TUNEL-processed sections, respectively.

Cell counting was performed in 2 coronal brain slices (4 hippocampi, approximately at 1.0 and 2.0 mm posterior from the bregma) for each animal, and the results were averaged. The averaged results from 5 animals at each time point were then presented as the mean±SD. For densitometric analyses of Western blot, the optical density of each band was measured on the same membrane at each time point (n=4 each), and the results were also presented as the mean±SD. The statistical significance between controls and each group was established with the F test followed by the unpaired Student's *t* test. The significance was accepted as $P<0.05$.

Physiological Variables Before and After 5 Minutes of Global Ischemia With or Without IPC

	MABP	pH	Po_2, mm Hg	Pco_2, mm Hg
5 Minutes of ischemia without ICP				
Before	89.3±6.5	7.369±0.034	117.8±5.8	44.87±2.95
After	84.5±12.1	7.363±0.017	119.4±11.9	47.12±4.81
5 Minutes of ischemia with ICP				
Before	85.3±10.1	7.371±0.054	110.2±9.1	43.12±5.14
After	87.5±8.7	7.360±0.044	121.4±13.6	46.97±4.40

MABP indicates mean arterial blood pressure. Values are mean±SD (n=5).

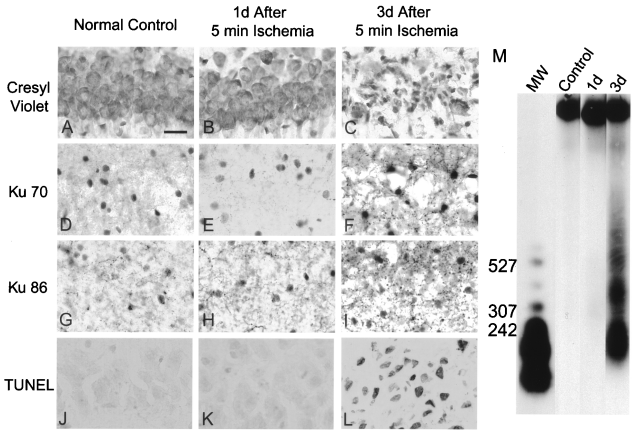

Figure 1. Cresyl violet staining (A to C), Ku 70/86 immunohistochemistry (D to I), TUNEL (J to L), and DNA gel electrophoresis (M) of the normal and ischemic hippocampal CA1 pyramidal cell layer after test ischemia (5 minutes). Most of the neurons in the pyramidal cell layer were morphologically damaged 3 days after ischemia (A to C). These neurons became virtually all TUNEL-positive (J to L), and DNA gel electrophoresis showed DNA laddering at 3 days. Ku 70/86 was abundant in small nuclei (diameter <5 μm) in the normal brain as well as in the ischemic brain (D to I). Bar=20 μm. MW indicates molecular weight.

Results

Physiological Data

Physiological parameters showed no significant differences in mean arterial blood pressure and arterial blood gas analysis between the 5-minute ischemia without IPC group and the 5-minute ischemia with IPC group before and 5 minutes after ischemia (Table).

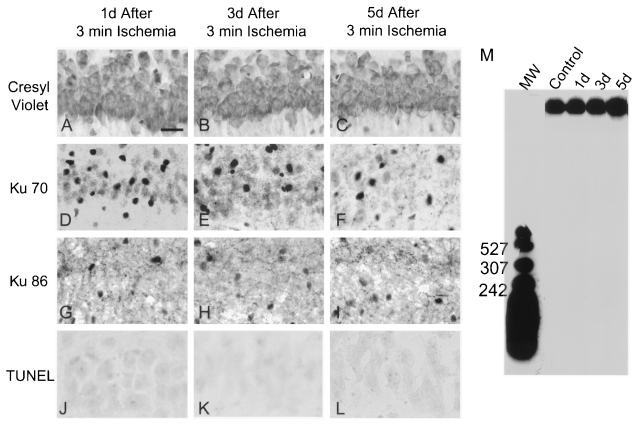

Figure 2. Cresyl violet staining (A to C), Ku 70/86 immunohistochemistry (D to I), TUNEL (J to L), and DNA gel electrophoresis (M) of the hippocampal CA1 pyramidal cell layer after IPC. Neither morphological cell damage nor DNA fragmentation was observed 1 to 5 days after ischemia by cresyl violet staining, TUNEL, and DNA gel electrophoresis (A to C, J to M). Large nuclei (diameter >5 μm) in the hippocampal CA1 pyramidal cell layer became Ku 70–positive 1 and 3 days after IPC (D, E), but the expression was attenuated at 5 days (F). Ku 86–positive large nuclei were not observed 1 to 5 days (G to I) after IPC. Small nuclei in this region (diameter <5 μm) were strongly Ku 70– and Ku 86–positive on days 1 through 5. Bar=20 μm. MW indicates molecular weight.

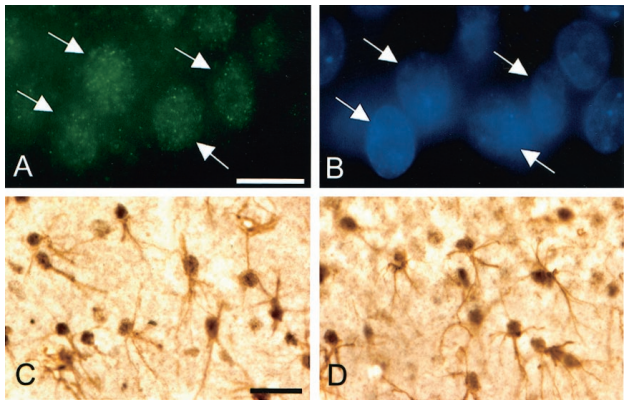

Figure 3. Nuclear and Ku 70 double staining (A, B) and GFAP and Ku 70/86 double immunostaining (C, D). DAPI nuclear staining (A) and Ku 70 fluorescent immunostaining (B) of the same field 3 days after IPC showed that Ku 70–positive large nuclei of the hippocampal CA1 pyramidal cell layer were pyramidal neurons (arrows). GFAP (brown) and Ku 70 (black) double staining (C) showed that the cells with Ku 70–positive small nuclei also had GFAP-positive processes, indicating that they were virtually all astrocytes. GFAP (brown) and Ku 86 (black) double staining (D) also confirmed that the cells with Ku 86–positive small nuclei were astrocytes. Bar=20 μm.

Nonneuronal Ku Expression and DNA Fragmentation After 5 Minutes of Ischemia

Cresyl violet staining showed normal features of hippocampal CA1 neuronal nuclei 1 day after 5 minutes of ischemia; however, at 3 days a majority of the neurons showed shrunken, small nuclei (Figure 1A to 1C). Ku 70– and Ku 86–positive cells were seen mainly outside of the pyramidal cell layer, and the size of the positive cell nuclei was obviously smaller (<5 μm) than in the CA1 pyramidal neurons (Figure 1D to 1I). Most of the pyramidal cell neurons were not positive for Ku 70/86 before or after ischemia (Figure 1D to 1I). Most of the CA1 pyramidal neurons became TUNEL-positive 3 days after ischemia, and DNA gel electrophoresis revealed a laddering pattern 3 days but not 1 day after ischemia (Figure 1J to 1M). With the preabsorption method, Ku 70/86 blocking peptides totally eliminated immunoreactivity of the sections (not shown in Figure 1), confirming the specificity of the antibodies.

Increased Expression of Ku 70 in Large Nuclei After IPC

There were no morphologically damaged cells in the hippocampal CA1 subregion after IPC (Figure 2A to 2C). DNA fragmentation was not observed by TUNEL or DNA gel electrophoresis (Figure 2J to 2M). Many large nuclei (>5 μm) in the CA1 pyramidal cell layer became faintly Ku 70–positive 1 and 3 days after 3 minutes of ischemia (Figure 2D and 2E); however, only a few positive cells were seen at 5 days (Figure 2F). In contrast, there were no Ku 86–positive large nuclei after 3 minutes of ischemia (Figure 2G to 2I). There were numerous strongly Ku 70/86–positive small nuclei (<5 μm) seen at all time points (Figure 2D to 2I).

Double Staining Confirmed Neuronal and Astroglial Expression of Ku

Ku 70–positive large nuclei 3 days after ischemia were confirmed to be neurons by immunofluorescent labeling.

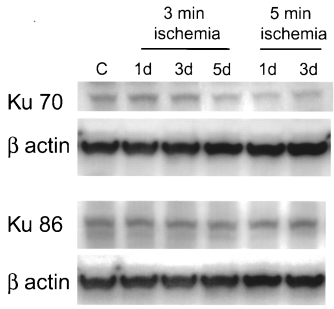

Figure 4. Western blot analysis of Ku 70 and Ku 86 in the hippocampal CA1 subregion. Ku 70/86 immunoreactivity was evident as bands at 70/86 kDa in the whole cell fraction. After 3 minutes of ischemia (IPC), Ku 70 increased at 1 to 3 days and returned to the control level at 5 days; however, no change was seen after 5 minutes of ischemia. The increase in Ku 70 was statistically confirmed by a densitometric study ($P<0.05$). Ku 86 immunoreactivity was always at the same level after 3 or 5 minutes of ischemia. A consistent amount of β-actin immunoreactivity is also shown. C indicates normal control.

Large nuclei in the hippocampal CA1 pyramidal cell layer visualized by DAPI were also Ku 70–positive 3 days after 3 minutes of ischemia, confirming that Ku 70 was expressed in the nuclei of pyramidal neurons (Figure 3A and 3B). Ku 70/86–positive small nuclei in this region were virtually all astrocytes, since they were double stained with GFAP-positive processes (Figure 3C and 3D).

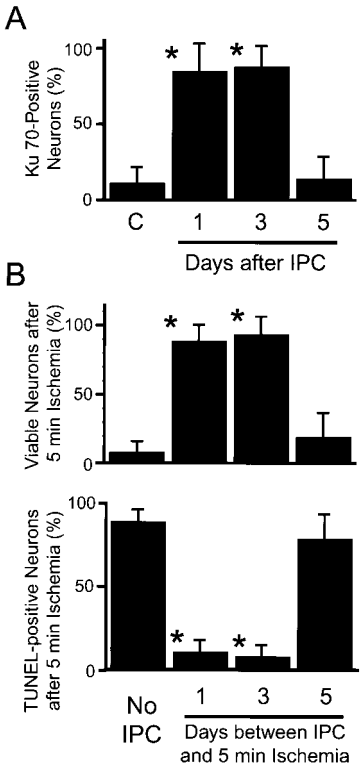

Figure 5. Ku 70–positive cell counting after 3 minutes of IPC and neuronal survival after 5 minutes of ischemia in the hippocampal CA1 subregion. The number of Ku 70–positive cells was increased 1 to 3 days after IPC ($P<0.001$) but returned to the control level at 5 days (A). Without IPC, <10% of the neurons in the pyramidal cell layer survived after 5 minutes of ischemia. However, 80% to 90% of them survived when 5 minutes of ischemia was induced 1 to 3 days after IPC (B, top). TUNEL-positive cells were decreased at the same time (B, bottom). *$P<0.001$ vs normal control or without IPC level. C indicates normal control.

Western Blot Analysis of Ku 70/86 Expression

After 3 minutes of ischemia, Western blot showed increased expression of Ku 70 3 days after ischemia, but it returned to the normal control level at 5 days (Figure 4, top panel, lanes 1 to 4). However, Ku 70 expression was not modified after 5 minutes of ischemia (Figure 4, top panel, lanes 5 and 6). Statistical analysis (n=4 each) confirmed the significant increase ($P<0.05$) of Ku 70 3 days after 3 minutes of ischemia (optical density at 1 and 3 days=0.299±0.061 and 0.313±0.040, respectively) compared with that in normal control brain (optical density=0.210±0.058). Ku 86 expression was not changed at any time after 3 or 5 minutes of ischemia. A consistent amount of β-actin immunoreactivity is also shown in Figure 4.

Ku 70–Positive Cell Counting After IPC and Preconditioning Effects

The number of Ku 70–positive cells was increased 1 to 3 days after IPC ($P<0.001$) but returned to the normal control level at 5 days (Figure 5A). Without IPC, <10% of the neurons in the pyramidal cell layer survived after 5 minutes of ischemia; however, 80% to 90% of them survived when 5 minutes of ischemia was induced 1 to 3 days after IPC (Figure 5B, top panel). TUNEL-positive neurons were significantly decreased at the same time (Figure 5B, bottom panel).

Discussion

Neuronal Ku 70 Expression Corresponds to Development of Tolerance

The present study demonstrated that IPC protected hippocampal CA1 neurons from delayed death when the test insult was induced 1 to 3 days after IPC and, at the same time, there was a corresponding increase in neuronal Ku 70 expression. Ku immunohistochemistry showed almost no expression of Ku 70/86 in normal hippocampal CA1 neurons; however, IPC induced neuronal expression of Ku 70 1 to 3 days after IPC. Western blot also showed an increase in Ku 70 in the hippocampal CA1 subregion at this time. Since immunohistochemistry did not show substantial changes in staining density of the Ku 70 protein in astrocytes, we assume that the increase is primarily from increased expression in neurons. The relatively high level of Ku 70/86 protein expression in normal brain was probably due to astroglial expression, and it may explain the reason for the modest overall Ku 70 changes after IPC. Furthermore, an increase in the number of Ku 70–positive neurons corresponded to the number of cells protected by IPC.

IPC and Subsequent Upregulation of Proteins/Genes

IPC has been reported to show protective effects on hippocampal CA1 neurons from 30 minutes to 7 days after reperfusion.[1,2,23,24] A number of proteins and their genes have been reported to be upregulated after IPC. HSPs and their mRNA[3,25–27] and neurotrophic factors and their mRNA[28]

were upregulated, and Jun-related proteins increased[6] after IPC, suggesting an involvement of these proteins in tolerance induction. Recently, ischemic tolerance has also been reported to be associated with a modulation at the level of gene transcription. The binding activity of transcription factor activator protein-1, which is a homodimeric/heterodimeric complex consisting of c-Fos and c-Jun protein families,[29] was drastically modulated after IPC,[30,31] and tumor suppressor gene p53 and its response genes were also activated.[32]

Apoptotic Death of Hippocampal CA1 Neurons After Transient Global Ischemia

In Figure 1, TUNEL and DNA gel electrophoresis show that lethal transient global ischemia induced DNA fragmentation in the hippocampal CA1 subregion. These results, especially the DNA laddering pattern, suggest that delayed cell death in this region was caused by an apoptotic pathway. Recent studies support this idea[8–12]; however, this hypothesis has been challenged by an electron microscopic study.[33] Nevertheless, protective effects of caspase inhibitors on delayed neuronal death[11] and expression of caspase-3 and caspase-9 in the hippocampal CA1 subregion[11,12] before DNA fragmentation may argue for the biochemical evidence of apoptosis.

Role of Ku and Other DNA Repair Proteins After Ischemia/Reperfusion Injury

Ku plays a critical role by triggering the DNA repair process as a regulatory component of DNA-PK. Ku itself is thought to stabilize broken DNA ends, bring them together, and prepare them for ligation.[16] Recent studies showed that Ku-deficient cells were extremely sensitive to apoptotic stimuli because of the deficiency of DNA double-strand break repair.[34] These reports suggest that the reduction in Ku might contribute to DNA-fragmented cell death. After transient global ischemia, free radicals cause oxidative damage and mutagenic lesions in nuclear DNA,[13] and oxidative DNA damage precedes DNA fragmentation after focal cerebral ischemia.[14] There is DNA repair activity after global ischemia,[13] and IPC was reported to reduce oxidative DNA damage in the hippocampus.[15] Taken together, IPC may reduce oxidative DNA damage by certain DNA repair activity after transient global ischemia and therefore reduce subsequent DNA-fragmented apoptotic cell death in the hippocampal CA1 subregion. Our previous studies demonstrated that the early reduction of DNA repair proteins such as apurinic/apyrimidinic endonuclease[35,36] or x-ray repair cross-complementing group 1[37] after focal or global cerebral ischemia might be responsible for DNA fragmentation and subsequent cell death. Furthermore, overexpression of DNA recombination repair protein 1 reduced the somatic mutation and recombination frequency induced by oxidative DNA damage,[38] strongly suggesting the involvement of DNA repair proteins in the repair process of the oxidative DNA damages. However, Ku protein may only be one of a number of upregulated DNA repair enzymes after IPC, and its role is unclear. Further investigation using genetically modified animals overexpressing certain DNA repair protein is needed to clarify their roles.

We recently reported that Ku 70 and Ku 86 were constitutively expressed in the cortex and caudate putamen; however, the expression was dramatically reduced 4 hours after focal cerebral ischemia and preceded DNA fragmentation in mice.[19] In the present study we did not observe the constitutive expression of Ku 70 and Ku 86 in hippocampal CA1 neurons, but upregulation of Ku 70 was observed 1 to 3 days after IPC. This unique neuronal expression of Ku 70 after brief ischemia may suggest the involvement of Ku proteins in the possibly upregulated DNA repair activity after IPC. Furthermore, a constitutive high expression of Ku in astrocytes may partly explain their strong tolerance to global ischemia. However, it is unclear whether upregulation of Ku 70 solely increases the neuronal DNA repair function, since Ku 70 and Ku 86 form a tightly associated heterodimer as a part of DNA-PK.[16,17] Further studies are necessary to address this issue.

Acknowledgments

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