Early NFκB Activation Is Inhibited During Focal Cerebral Ischemia in Interleukin-1β–Converting Enzyme Deficient Mice

Feng-Peng Huang,[1,4] Zhi-Qiu Wang,[1,4] Du-Chu Wu,[1] Gerald P. Schielke,[1,3] Yi Sun,[2] and Guo-Yuan Yang[1★]

[1]Department of Surgery (Neurosurgery), University of Michigan, Ann Arbor
[2]Department of Molecular Biology, Pfizer Global Research and Development, Ann Arbor, Michigan
[3]Department of CNS pharmacology, Pfizer Global Research and Development, Ann Arbor, Michigan
[4]Department of Neurosurgery, Institute of Neurology, Hua-Shan Hospital, Shanghai Medical School, Fudan University, Shanghai, China

Our previous study demonstrated that the inhibition of interleukin-1β (IL-1β) reduces ischemic brain injury; however, the molecular mechanism of the action of IL-1 in cerebral ischemia is unclear. We are investigating currently the role of NFκB during focal cerebral ischemia, using mutant mice deficient in the interleukin-1 converting enzyme gene (ICE KO) in a middle cerebral artery occlusion (MCAO) model. Adult male ICE KO and wild-type mice ($n = 120$) underwent up to 24 hr of permanent MCAO. Cytoplasmic phospho-NFκB/p65 expression in ischemic brain was examined using Western blot analysis and immunohistochemistry. NFκB DNA-binding activity was detected using electrophoretic mobility shift assay (EMSA). Furthermore, ICAM-1 expression was examined in both the ICE KO and wild-type mice (WT). Western blot analysis and immunostaining showed that the level of cytosolic phosphorylated NFκB/p65 increased after 2 and 4 hr of MCAO in WT mice; however, NFκB/p65 was significantly reduced after MCAO in the ICE KO mice ($P < 0.05$). EMSA showed that NFκB DNA-binding activity increased after MCAO in WT mice; but this effect was reduced in the ICE KO mice. The number of ICAM-1-positive vessels in the ischemic hemisphere was greatly attenuated in the ICE KO mice ($P < 0.05$), which paralleled the results of immunohistochemistry. Our results demonstrate that NFκB phosphorylation is reduced in ICE KO mice, suggesting that ICE or IL-1 are involved in early NFκB phosphorylation. Because cerebral ischemia induced infarction is significantly reduced in ICE KO mice, we conclude that early NFκB phosphorylation plays a disruptive role in the ischemic process. © 2003 Wiley-Liss, Inc.

Key words: intercellular adhesion molecule-1 (ICAM-1); interleukin-1β (IL-1β); IL-1β-converting enzyme (ICE); middle cerebral artery occlusion; nuclear factor kappa B (NFκB)

Cerebral ischemia activates a cascade of intracellular signal transduction pathways through the production of many different stimuli, including proinflammatory cytokines, reactive oxygen species (ROS), neurotrophic factors, and calcium influx (Mattson, 1997). It is well known that proinflammatory cytokines play an important role in the pathogenesis of cerebral ischemia (Dinarello, 1997b). Interleukin-1β (IL-1β), a proinflammatory cytokine, is increased markedly during global and focal cerebral ischemia and postischemic reperfusion (Rothwell and Hopkins, 1995; Szaflarski et al., 1995; Arvin et al., 1996). Exogenous application of IL-1β exacerbates ischemic brain injury, whereas administration or overexpression of IL-1 receptor antagonist (IL-1ra) greatly reduces ischemic brain injury (Minami et al., 1992; Yamasaki et al., 1992, 1996; Betz et al., 1995; Loddick and Rothwell, 1996; Feuerstein et al., 1997). IL-1β–converting enzyme (ICE) cleaves inactive 31–kD pro-IL-1β to the active 17–kD IL-1β form and processes the pro–interferon γ–inducing factor. ICE is a member of a family of cysteine proteases, the caspases, several of which are associated with the apoptotic cell death pathway. This pathway has been implicated in ischemic brain damage (Linnik et al., 1993). Our previous studies have demonstrated that ischemic brain injury, edema formation, and inflammatory responses were reduced significantly in ICE knockout (ICE KO) mice (Schielke et al., 1998; Yang et al., 1999c). It is uncertain, however, whether ICE-deficient mice are protected from ischemic damage by a reduction of inflammatory response, apoptosis, or both (Hara et al., 1997b; Schielke et al., 1998).

Responses to a variety of stimuli associated with stress and injury such as ischemia are mediated through numerous intracellular signaling molecules(Carroll et al., 2000). An important signaling molecule among them is nuclear transcription factor kappa B (NFκB), which is activated by cytokines, mitogens, physical or oxidative stress, and chemical agents (Siebenlist et al., 1994). Potent inducers of NFκB include cytokines, tumor necrosis factor-alpha (TNFα), IL-1β, lipopolysaccharide, oxygen free radicals, ultraviolet light, and γ-irradiation (Baeuerle and Baichwal, 1997). NFκB is classified as a Rel protein and is distinguished structurally by a Rel homology domain that is involved in DNA binding and dimerization (Verma et al., 1995). NFκB is composed normally of a dimer of 50- and 65-kD subunits that reside in the cytoplasm of most cells and are bound to an inhibitory protein, I kappa B (IκB). Upon exposure to stimuli, IκB protein is phosphorylated, followed by ubiquitination and degradation. As a consequence, NFκB is phosphorylated and translocated into the nucleus, where it initiates gene transcription. It is known that phospho-NFκB participates in the control of transcription of over 150 target genes, including proinflammatory cytokines, chemokines, immunoreceptors, acute phase proteins, stress response genes, and regulators of apoptosis (Gilmore et al., 1996; Gilmore, 1997).

To identify the relationship between decreased levels of ICE and the phosphorylation of NFκB, we designed the present experiments to determine the levels of phospho-NFκB after middle cerebral artery occlusion (MCAO) in mutant mice deficient in the ICE gene. Furthermore, we examined the role of NFκB in regulating the inflammatory response during focal cerebral ischemia.

MATERIALS AND METHODS

Animal Preparation

The institutional animal care and use committee approved the procedures for the use of laboratory animals. The ICE KO mice were developed and characterized by the BASF Corporation, (Worcester, MA) (Li et al., 1995). These mice contain a null mutation in the ICE gene (ICE$-$/$-$) generated by homologous recombination in embryonic stem cells. The wild-type (WT, ICE$+$/$+$) mice were generated from the same chimeric founder, and interbred amongst themselves. Both strains have a mixed background of SV129- and C57BL/6-derived genes.

Experimental Groups

Three separate paradigms were used in this study. The first experiment measured the time course of NFκB expression after MCAO using Western blot analysis. Adult ICE KO ($n = 20$) and WT mice ($n = 20$) weighing 28–35 g underwent 0.5, 2, 4, 8, and 24 hr of permanent MCAO ($n = 4$ at each point). The second experiment localized NFκB and intercellular adhesion molecule-1 (ICAM-1) expression using double-labeled fluorescence staining or stable 3,3′-diaminobenzidine tetrahydrochloride (DAB; Research Genetics, Huntsville, AL) immunostaining. ICE KO ($n = 20$) and WT mice ($n = 20$) underwent 0, 2, 4, 8, and 24 hr of permanent MCAO ($n = 4$ at each time point).

The third experiment examined the NFκB activity using electrophoretic mobility gel-shift assay (EMSA). The time points and animal numbers were the same as Experiment 1 and 2.

Middle Cerebral Artery Occlusion

Adult ICE KO and WT mice were anesthetized with 4% isoflurane in 70% N_2O/30% O_2 and maintained with 1.5% isoflurane through a facemask. Body temperature was maintained with a heated blanket using a feedback control system (73A, Yellow Springs, Ohio). MCAO was carried out as described previously (Yang et al., 1994). Briefly, the left common carotid artery was exposed through a midline incision. The internal carotid artery was isolated and the pterygopalatine branch ligated close to its origin. A 2-cm length of 5-0 rounded tip nylon suture was gently advanced from the external carotid artery through the common carotid artery and into the internal carotid artery for a distance of 11.0 ± 0.5 mm.

Surface Cerebral Blood Flow Measurement

Surface cerebral blood flow (sCBF) was measured using a laser-Doppler flowmetry monitor (Model BPM2 System; Vasamedics, St. Paul, MN) equipped with a small caliber probe of 0.7-mm diameter (P-433; Vasamedics). Surface CBF was measured 5 min before and 15 min after MCAO at three points: 1) the ischemic core: 3.5 mm lateral and 1 mm posterior to the bregma on the ipsilateral hemisphere; 2) the control area: corresponding site on the contralateral hemisphere; and 3) the perifocal area: 1 mm lateral and 1 mm posterior to the bregma on the ipsilateral hemisphere. Blood flow values are expressed as a percentage of baseline values (Yang et al., 1997).

Western Blot Analysis

Mice were sacrificed at 0.5, 2, 4, 8, and 24 hr of MCAO. The brains were removed immediately and divided into contralateral and ipsilateral hemispheres. Tissue samples were homogenized and lysed in a buffer containing 150 mM NaCl, 50 mM Tris-Cl, 1% NP-40, 0.5% DOC, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml polymethylsulfonylfluoride, as well as 2 μg/ml each of pepstatin, leupeptin, and chymostatin. Lysates were cleared by centrifugation at 14,000 $\times$ g for 10 min at 4°C. The protein concentration of each sample was determined with a Bio-Rad assay (Bio-Rad, Hercules, CA). Western blot analysis was carried out using standard techniques (Sambrook et al., 1989). The sample was boiled at 100°C in SDS gel loading buffer (100 mM Tris-Cl, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) for 5 min. The protein sample was loaded onto a 7.5% SDS polyacrylamide gel. After electrophoresis, size-fractionated proteins were electrotransferred to a polyvinylidene difluoride membrane (PVDF; Bio-Rad Laboratories, Hercules, CA) in a transfer buffer. The membrane was placed in 5% powdered milk in 100 mM phosphate-buffered saline (PBS) solution with 0.1% Tween 20 (PBST) for 60 min at room temperature to block nonspecific binding. The transferred PVDF membrane was probed with polyclonal goat anti-activate NFκB/p65 antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer overnight at 4°C. After washing, the membrane was immunoprobed with peroxidase-conjugated anti–goat IgG-HRP (1:2,000 dilution; Santa Cruz Biotechnology). An enhanced ECL kit (Amersham, Bucking-

hamshire, UK) was applied to detect the active NFκB protein according to the manufacturer's instructions. Protein amount was quantified by a gel densitometric scanning program using NIH 1.62 image software.

Immunohistochemistry

ICE KO and WT mice were anesthetized and perfused transcardially with heparinized saline, followed by 2% paraformaldehyde in 100 mM PBS, pH 7.4 (Sigma, St. Louis, MO), after 0.5, 2, 4, 8, and 24 hr of MCAO. Brains were removed, postfixed overnight in the same solution, immersed in 25% sucrose at 4°C until they sunk to the bottom, and then embedded in a 60%/40% sucrose/OCT (Sakura Finetek, Torrance, CA) solution. Coronal sections (20 μm in thickness) were cut on a cryostat (CM1800; Leica, Germany) and mounted on precleaned microscope slides (Fisher Scientific, Pittsburgh, PA). For single NFκB/p65 immunohistochemistry, sections were incubated for 30 min with 10% goat serum to reduce nonspecific binding. Sections were then washed in PBST (PBS containing 0.5% Triton X-100) and incubated overnight at 4°C with a rabbit polyclonal anti-active NFκB/p65 antibody (1:500 dilution; Santa Cruz Biotechnology). Negative controls were carried out using the same concentration of normal rabbit IgG instead of primary antibody. After treatment with 1% H_2O_2 in 30%/70% methanol/PBS solution, the sections were incubated with a biotinylated goat anti–rabbit secondary antibody in 1:200 dilution for 90 min at room temperature, followed by an ABC process (ABC-Elite Kit; Vector, San Francisco, CA). Finally, the sections were treated with stable 3,3′–diaminobenzidine tetrahydrochloride (DAB, Research Genetics, Huntsville, AL) as a peroxidase substrate and counterstained with hematoxylin. ICAM-1 expression was carried out as described above except the first antibody was rabbit polyclonal anti-ICAM-1 antibody (Caltag, San Francisco, CA). The total numbers of deep brown-stained positive vessels were counted in each hemisphere, including the cortex and basal ganglia region. ICAM-1 vessel counting was done manually to exclude the infiltrating cells occasionally expressing ICAM-1.

When double-labeled fluorescent immunohistochemistry was used, sections were incubated for 30 min with 10% donkey serum to reduce nonspecific binding, then incubated overnight at 4°C with the goat anti-active NFκB/p65 antibody (1:200 dilution; Santa Cruz Biotechnology). After washing, fluorescein-conjugated donkey anti-goat IgG (1:100 dilution; Vector) was applied for 60 min at room temperature. Sections were incubated with polyclonal rabbit anti–neuron specific enolase (NSE, 1:200 dilution; Chemicon International, Temecula, CA) antibody for 2 hr at room temperature. NSE immunoreactivity was detected using rhodamine-conjugated donkey anti-rabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA) at room temperature. Double-labeled immunostaining was evaluated using a fluorescence microscope (Nikon Microphoto-SA) with a filter cube (excitation filter, 450−490 nm; suppression filter, 515–560 nm) for fluorescein isothiocyanate labeling and another filter cube (excitation filter, 515−560 nm; suppression filter, 590 nm) for rhodamine. Photomicrographs for double labeling illustrations were obtained by changing the filter cube without altering the position or focus of the section (Gong et al., 1998).

Nuclear Extraction and Electrophoretic Mobility Shift Assay

The nuclear extraction and electrophoretic mobility shift assay (EMSA) was carried out as specified by the manufacturer's instructions (Promega, Minneapolis, MN). After the animals were sacrificed, the brains were removed immediately and homogenized in a microfuge tube with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 1 mM AEBSF, and 10 μg/ml leupeptin). The nuclei were collected by centrifugation at 500 × g for 10 min and washed once with buffer A containing 0.2% NP-40. The nuclear proteins were then extracted with buffer B (20 mM HEPES, pH 7.9, 0.42 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM AEBSF, and 10 μg/ml leupeptin) for 30 min at 4°C, and microfuged for 30 min. The nuclear extracts were microdialyzed against buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM AEBSF) with an ultrafree centrifugal filter (Millipore) and frozen at −80°C. For determination of protein-DNA interactions, a double-stranded oligonucleotide containing NFκB consensus binding sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was used (Promega) and end-labeled with gamma [^{32}P]-ATP according to manufacturer's recommendation. The NFκB binding reactions were carried out in a final volume of 20 μl mixtures containing buffer (10 mM HEPES, pH 7.5, 0.25 μg/μl poly [dI/dC], 0.1 mM NaCl, 0.8 mM EDTA, 1 mM DTT, 0.05% NP-40, 4% glycerol), 10 μg nuclear proteins, and 10^6 cpm of radiolabeled probe. The mixtures were incubated for 20 min at room temperature. The DNA–protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5× Tris-borate/EDTA buffer. The gel was dried and exposed to Kodak X-ray film overnight at −70°C. EMSA was carried out on nuclear extracts from each sample after ischemia. Semiquantitation of the EMSA was carried out with densitometry.

Statistical Analysis

Microcomputer based statistical programs (Statview II; ABACUS, Berkeley, CA and Excel; Microsoft, Redmond, WA) were used. All data are presented as mean ± SD. Parametric data at different points during MCAO between the ICE KO and the WT mice were compared using ANOVA followed by Student's t-test. A probability value of <5% was considered significant.

RESULTS

Physiological Parameters

ICE KO and WT mice developed normally with no visible neurological deficit detected in either group. The blood pressure, blood gases, and body temperature changes were similar and there were no statistical differences between the ICE KO and the WT mice at 0.5, 2, 4, 8, and 24 hr after MCAO (data not shown). Surface CBF dropped sharply to below 20% of baseline in the ischemic core, and to 35–50% of baseline in the ipsilateral perifocal region. CBF was maintained at approximately 100% of baseline in the contralateral hemisphere after MCAO in both ICE KO and WT mice. There were no statistical
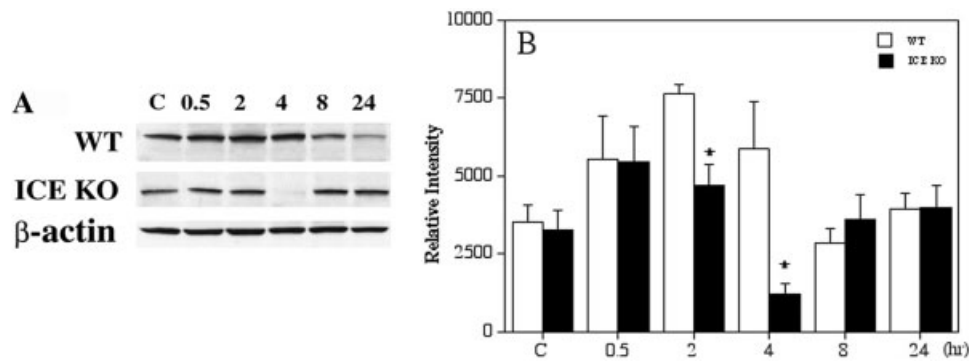
Fig. 1. Increase of p65/NFκB activation in mouse brain after ischemia. **A:** Activated p65/NFκB expression during ischemia in WT and ICE KO mouse brain. Cytoplasmic protein extracts were prepared from the ischemic hemisphere in mouse brain after 0.5, 2, 4, 8, and 24 hr of MCAO; shows a 65-kD active NFκB band detected by Western blot analysis with a polyclonal anti-active p65/NFκB antibody in a representative experiment. C, sham control animals. An equal amount of extract protein was used in each sample (see β-actin). **B:** The bar graph shows densitometric quantity of the p65/NFκB induction during cerebral ischemia in ICE KO and WT mice. C, sham control animals. Values are mean ± SD; $n = 4$ for each time. $\star P < 0.01$, relative density of activated p65/NFκB in ICE KO mice vs. that in WT mice.

differences between the two groups ($P > 0.05$, data not shown).

Phospho–P65/NFκB Is Decreased in ICE KO Mice After Ischemia

The levels of phospho-p65/NFκB were measured using Western blot analysis in mouse brain samples collected from both ICE KO and WT mice that underwent 0.5, 2, 4, 8, and 24 hr of MCAO. Phospho-p65/NFκB was expressed at a very low level in the sham animals or in the contralateral hemisphere of mouse brain after MCAO (data not shown). The phospho-p65/NFκB expression in the ischemic hemisphere, however, shown as a major band at approximately 65 kDa, increased as early as 0.5 hr of MCAO, peaked at 2 and 4 hr, and then decreased gradually in the WT mice (Fig. 1A, as a representative experiment). Interestingly, phospho-p65/NFκB expression was reduced significantly in ICE KO mice after 2 and 4 hr of MCAO compared to WT mice ($P < 0.05$). Densitometric quantitative data ($n = 4$ at each time) is shown in Figure 1B. Phospho-p65/NFκB expression was reduced significantly after 2 and 4 hr of MCAO in ICE KO mice, demonstrating that ICE or IL-1 is critical to the process of phosphorylation of NFκB during cerebral ischemia.

Cellular Localization of Phospho-p65/NFκB

Localization of phospho-p65/NFκB during ischemia was assessed by immunostaining brain sections prepared from both the ICE KO and the WT mice that underwent MCAO for different lengths of time. As shown in Figure 2, few phospho-p65/NFκB-positive cells could be detected in brain sections from sham–operated mice or in the contralateral hemisphere in ischemic mice (Fig. 2A,F). Phospho-p65/NFκB-positive cells could be detected, however, in both the ischemic cortex and the caudate

putamen after 2 hr of MCAO (Fig. 2C,H). The regions of p65/NFκB-positive cells extended to the perifocal region at 4 hr of MCAO (Fig. 2E,J) in the WT mice. These results correlated with the result from Western blot analysis. The increase of phospho-p65/NFκB immunoreactivity was greatly attenuated, however, in both the ischemic cortex and the caudate putamen in ICE KO mice (2 hr of MCAO: Fig. 2A,G; 4 hr of MCAO: Fig.2D,I). No immunoreactivity was detected in the negative control sections (data not shown). Further study showed that the phospho-p65/NFκB was located mainly in cytoplasm after 2 hr of MCAO (Fig. 3C), and translocated into the nucleus after 4 hr of MCAO (Fig. 3D). Double-labeled immunohistochemical studies revealed that early expression of p65/NFκB was colocalized with NSE, a neuron specific marker (Fig. 4). Phospho-p65/NFκB-positive cells appeared in both the ischemic cortex and the caudate putamen after 2 hr of MCAO in WT mice. These results demonstrate that p65/NFκB was phosphorylated during the early acute ischemic period and early phospho-p65/NFκB expression was detected mainly in the neurons.

NFκB DNA Binding Activity

After release from IκB, NFκB translocates into the nucleus when it binds to its consensus sequence and transactivates target genes. We next examined NFκB DNA-binding activity by EMSA using samples from the ischemic brain tissue in both the ICE KO and the WT mice. Relative intensities of protein-DNA complex increased after 4 hr of MCAO and remained elevated during 24 hr of MCAO in the WT mice (Fig. 5). This correlates with the immunostaining data showing p65/ NFκB translocation into the nucleus; however, there is no significant induction of NFκB DNA binding after 4 hr of MCAO in ICE KO mice ($P < 0.05$, Fig. 5). This data indicates that
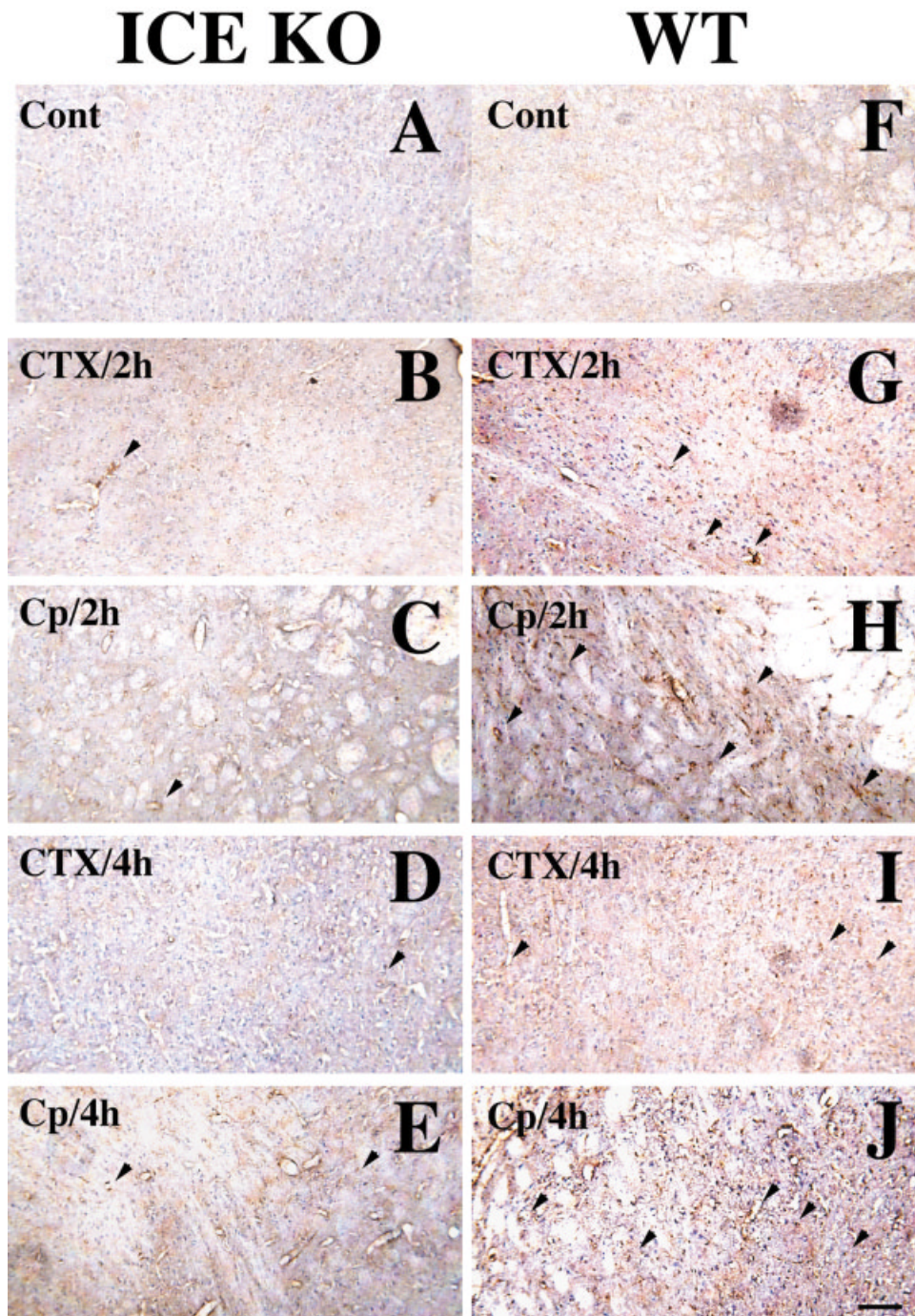
Fig. 2. Localization of p65/NFκB activation in mouse brain after ischemia. Photomicrographs showed that active p65/NFκB–positive staining in the ICK KO (left column) and the WT mice (right column) after 2 (**B,C,G,H**) and 4 hr (**D,E,I J**) of MCAO in the ischemic cortex (B,D,G,I) and caudate putamen (C,E,H,J), respectively. Cont, negative control section (blocking peptide): showed no positive staining in either the ischemic cortex or caudate putamen. CTX, cortex; Cp, caudate putamen; 2h, 2 hr of MCAO; 4h, 4 hr of MCAO. Arrowheads indicate these dark brown cells are phospho-p65/NFκB-positive cells. Many phospho-p65/NFκB-positive cells in both cortex and caudate putamen regions were detected in WT mice after 2 and 4 hr of MCAO; however, ischemia-induced p65/NFκB phosphorylation was greatly attenuated in ICE KO mice at the same points. Scale bar = 100 μm.
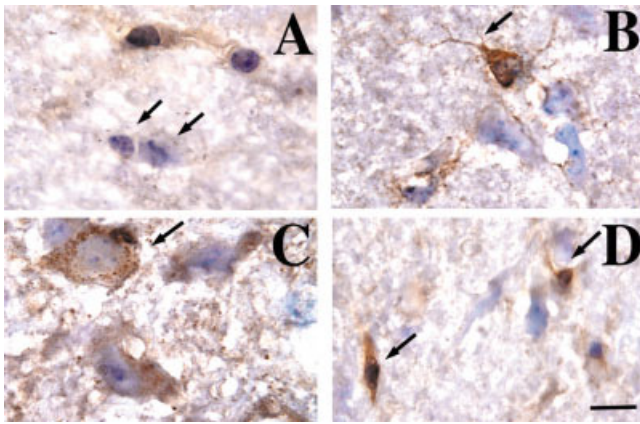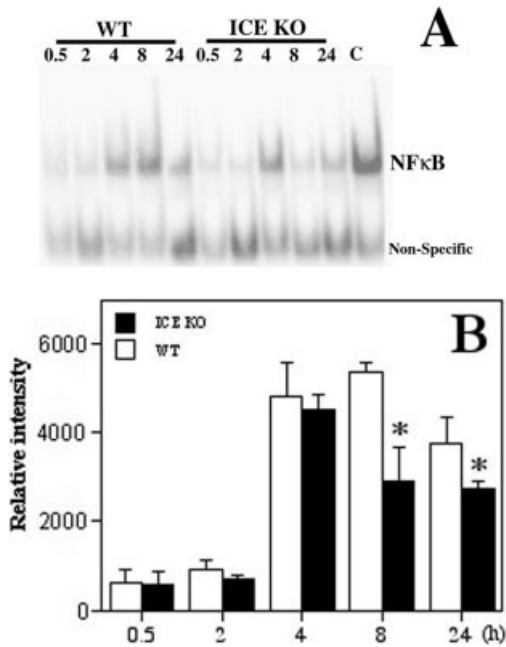
Figure 3.

Figure 4.

Figure 5.

Figure 6.

NFκB was phosphorylated after cerebral ischemia in WT mice, but this ischemia-induced NFκB phosphorylation was inhibited significantly in the ICE-deficient mice.

ICAM-1 Immunostaining

One known NFκB target gene involved in inflammatory regulation is ICAM-1. Therefore, we examined the expression of ICAM-1 in WT and ICE KO mice. Results showed that ICAM-1-positive vessels in the ipsilateral hemisphere of ICE KO mice were fewer than those in the WT mice after 24 hr of MCAO (148 ± 29 vs. 247 ± 25; $P < 0.05$). As shown in Figure 6, ICAM-1 expression in the ischemic hemisphere after 24 hr of MCAO was increased in the WT mice; however, the increase of 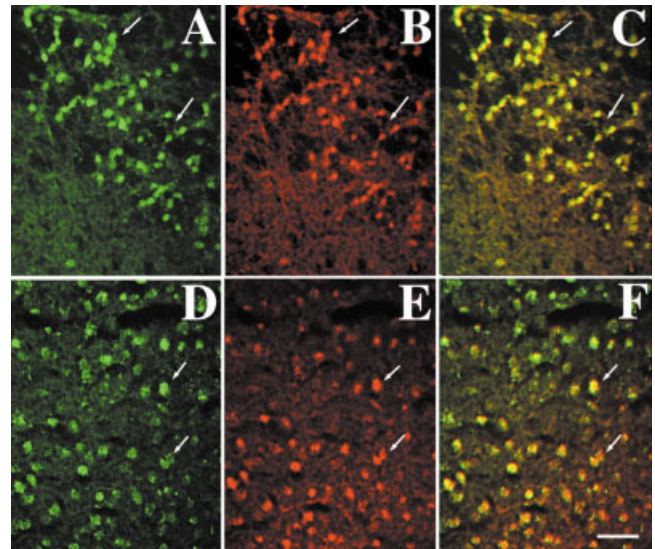ICAM-1-positive vessels in the ICE KO m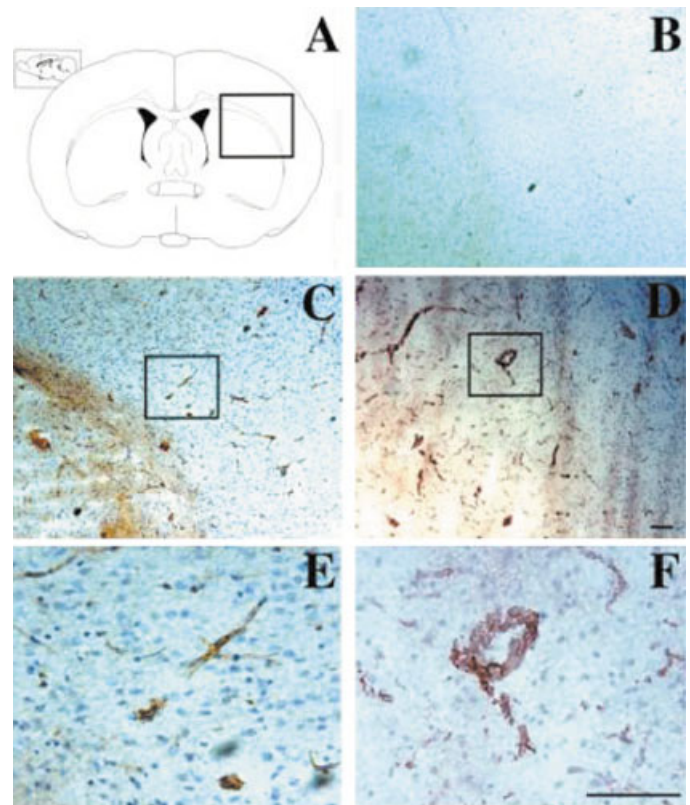ice was significantly decreased. The negative control section showed no ICAM-1-positive staining (data not shown). Few ICAM-1-positive vessels were detected in the ischemic hemisphere after 24 hr of MCAO in the sham animals.

DISCUSSION

This study demonstrates that the NFκB is phosphorylated in WT mice at the early stage of focal cerebral ischemia. This early NFκB phosphorylation is localized in neurons in the ischemic core and perifocal regions; however, NFκB phosphorylation is greatly reduced in ICE KO mice. Reduced ICAM-1 production, a NFκB target gene protein in the ICE KO mice, may be due to the reduction of NFκB phosphorylation. These findings suggest that the mature IL-1β and ICE genes may be important mediators of NFκB phosphorylation during early ischemia. Considerable evidence points to ICAM-1 as crucial mediator of ischemic brain damage. Thus, reduction of early NFκB phosphorylation may be one of the mechanisms through which ICE KO mice are protected from damaging inflammatory responses during focal cerebral ischemia.

Gene knockout mice provide useful tools for defining signaling pathways leading to ischemia-induced neuronal death (Huang et al., 1994; Yang et al., 1994). In a previous study, we demonstrated that after permanent focal ischemia, ICE KO mice had less brain edema and smaller infarction compared to WT controls (Schielke et al., 1998). A possible mechanism underlying this observation is reduced mature IL-1β production by the ischemic brain. ICE is a member of the caspase family of cysteine proteases, which are associated with both proinflammatory and apoptotic actions (Dinarello, 1992, 1996; Hara et al., 1997a; Yang et al., 1999a). ICE is critical for processing pro-IL-1 to the biologically active form of the cytokine, IL-1β. Previous studies have demonstrated that macrophages isolated from the ICE KO mice are not capable of producing IL-1β. Similarly, in a model of endotoxic shock, no IL-1β is detected in the plasma of the ICE KO

mice, and survival is greatly enhanced. Interestingly, IL-1α production is reduced also in these mice, although not to the extent of IL-1β(Dinarello, 1997a). The possibility of differences, however, in the genetic background of the knockout mice and their wild-type controls is a limitation in the use of gene knockout models. The ICE KO and WT mice used in this study were derived from the same progenitors, a mixed SV/129 and C57BL/6 background. Previous observations found that these ICE KO and WT mice developed normally (Schielke et al., 1998). Additionally, our data demonstrated that the changes in physiological parameters, cortical cerebral blood flow and body temperature, in response to MCAO, were similar in the two groups. Thus, the resistance to ischemia induced brain damage and activation of the NFκB pathways in the ICE KO mice is likely due to deletion of the ICE gene and not the differences in the genetic background of the KO and WT mice.

NFκB phosphorylation has been associated with immune and inflammatory responses. It is activated by a variety of stimuli known to occur in focal ischemia, such as cytokines, ROS, and glutamate (Baeuerle and Henkel, 1994). Expression of NFκB has been described in a variety of brain cell types, such as murine primary astrocytes, microglia, Schwann cells, neurons, and human endothelial cells (O'Neill and Kaltschmidt, 1997; Howard et al., 1998). NFκB activates the transcription of many genes thought to be involved in the pathogenesis of cerebral ischemia, including IL-1β, TNFα, iNOS, VCAM, ICAM-1, and IL-6. Thus, as a signaling molecule, NFκB may play a central role in the regulation of inflammatory responses to brain injury and infection. Existing evidence supports this concept. For example, Salminen et al. (1995) reported that NFκB was increased significantly in the

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Fig. 3. Activation of p65/NFκB in both cytoplasm and nucleus. Microphotographs showing DAB-positive staining for phospho-p65/NFκB in the mouse brain after 2 (**C**) and 4 hr (**D**) of MCAO. Arrows indicated no active p65/NFκB-positive cells were observed in the sham animal (**A**). Active p65/NFκB-positive staining could be observed after 2 hr of MCAO and these cells displayed neuronal morphology (**B**; arrow indicates neuronal cell body and axons). Active p65/NFκB-positive staining located in cytoplasm after 2 hr of MCAO in the WT mice (C), and active p65/NFκB-positive staining translocated into the nucleus after 4 hr of MCAO in the WT mice (D). This result demonstrates that early p65 phosphorylation and translocation occurs in the ischemic cells. Scale bar = 20 μm.

Fig. 4. Activation of p65/NFκB in neurons. Double-labeling fluorescence photomicrographs from the ipsilateral cortex showing double-labeled staining for p65/NFκB (green; **A,D**) and NSE (red; **B,E**); p65/NFκB was well colocalized with NSE staining, demonstrating that neurons express p65/NFκB after MCAO (**C,F**). Neurons that are p65/NFκB-positive are detected in both ischemic cortex (A,B) and caudate putamen (D,E). Injured neurons in the ischemic hemisphere after 2 hr of MCAO displayed strong active p65/NFκB immunoreactivity, indicating that the early NFκB activation occurs in the neuronal cells after ischemic insult. Arrows point to injured cells, showing shrunken cell bodies. Scale bar = 10 μm.

Fig. 5. DNA binding of NFκB after MCAO. Nuclear extraction and electrophoretic mobility shift assay (EMSA) of nuclear protein extracts from ischemic brain tissue, probed with ^{32}P, labeled the double-stranded oligonucleotide containing the NFκB-specific binding sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). NFκB binding reactions were carried out in a final volume of 20 μl mixture containing buffer, 10 μg nuclear proteins, and 10^6 cpm of radiolabeled probe. NFκB indicates the bands of specific DNA-protein complexes. The binding corresponding to active NFκB is detected in both the ICE KO and the WT mice after 0.5, 2, 4, 8, and 24 hr of MCAO. This binding is largely reduced after 4, 8, and 24 hr of MCAO in ICE KO mice compared to WT mice. C, positive control.

Fig. 6. Attenuation of ICAM-1 expression in ICE KO mice. Photographs showing ICAM-1-positive vessel staining in the ischemic perifocal areas in ICE KO (**C,E**) and the WT (**D, F**) mice after 24 hr of MCAO. (**A**) shows the area examined, and (**B**) shows there are no ICAM-1-positive vessels in the negative control section (blocking peptide) after MCAO. The brown staining demonstrated that the expression of ICAM-1-positive vessels increased greatly after 24 hr of MCAO in WT mice (D) compared to ICE KO mice (C). Magnification of squared areas in C and D are shown (E,F). Scale bar = 30 μm.

ischemic cortex and hippocampus after 30 min of four-vessel occlusion and 72 hr of reperfusion in rats. Yang et al. (1995) confirmed that NFκB expression was increased in the ipsilateral cortex in the rat brain trauma model, which is related to the production of IL-1β and TNFα; however, Schneider et al. (1999) reported contradictory results: they found that infarct volume after 2 hr of ischemia and 20 hr of reperfusion was reduced significantly in the NFκB/p50 knockout mice (42 vs. 32 mm³). There are several studies that demonstrate that NFκB phosphorylation provided neuroprotection in vitro (Blatteis, 1988). All these results suggest that NFκB phosphorylation plays different roles and transfers different signaling during focal cerebral ischemia. Depending upon the severity and duration of ischemia, NFκB could transfer neuroprotective or neurotoxic genes. These genes will then produce downstream proteins that will repair or further damage tissue.

Our data demonstrates that NFκB is phosphorylated after 2 hr of MCAO in the WT mice, and this phospho-NFκB form is located mainly in neurons in the ischemic core and the perifocal regions. Reduction of the NFκB signal in the ischemic ICE KO mice suggests that early processing of pro-IL-1 to its active form may be a critical trigger for this response. We observed that cytosolic NFκB could translocate into the nucleus after 4 hr of MCAO, and this is followed by an increase of nuclear NFκB DNA-binding activity. Because NFκB phosphorylation occurs earlier than the overexpression of cytokines (Bruce-Keller and Mattson, 1998), NFκB phosphorylation and translocation during MCAO may be the pathway by which an extracellular signal rapidly evokes a transcriptional response. Thus, as a signal molecule, NFκB may play a central role in the regulation of many inflammatory responses during brain injury.

ICAM-1, one of the most studied adhesion molecules, is located on the endothelial surface. Its leukocyte counterpart, integrin CD11/CD18, and many others are currently under investigation (Zhang et al., 1995; Yang et al., 1998). A number of experimental studies have documented that E-selectin (ELAM) and P-selectin are upregulated after focal cerebral ischemia in rodents (Okada et al., 1994; Wang et al., 1994, 1995; Jander et al., 1995; Zhang et al., 1995, 1996). There is considerable evidence supporting the idea that leukocyte infiltration into the brain contributes to the development of ischemic lesions. Blocking ICAM-1 with an antibody reduces both neutrophil accumulation and the size of the lesion (Zhang et al., 1994). The possible relevance of adhesion molecules in the pathogenesis of ischemic brain damage has been demonstrated recently, and mice deficient in the ICAM-1 gene show a marked reduction in cerebral infarction lesions after tMCAO (Connolly et al., 1996; Soriano et al., 1996). ICAM-1 expression is greatly increased in hypoxic endothelial cells in the presence of circulating endotoxin; however, pretreatment with salicylate, a novel NFκB inhibitor, completely abolishes the enhanced ICAM-1 expression (Zund et al., 1998). This study supports that

salicylate administered before cardiopulmonary bypass may protect the heart against ischemia/reperfusion injuries and reduce the load of the overall inflammatory response (Zund et al., 1998). Proinflammatory cytokines IL-1β and TNFα are known to be induced earlier than the adhesion molecules (Wang and Feuerstein, 1995). IL-1β and TNFα can upregulate the expression of ICAM-1, ELAM-1, and P-selectin in vitro (Feuerstein et al., 1997; Yang et al., 1999a,b). The development of the inflammatory response in focal ischemia, therefore, may appear in the following sequence: expression of IL-1β and TNFα, upregulation of adhesion molecules, and then migration of leukocytes. In our study, however, the expression of ICAM-1 protein is significantly lower in ICE KO mice compared to that in WT mice after MCAO. This result suggests that mature IL-1β is a critical mediator in the upregulation of ICAM-1 expression during cerebral ischemia.

In summary, we have demonstrated that NFκB is phosphorylated and translocated to the nucleus after permanent MCAO in WT mice but significantly reduced in ICE KO mice, suggesting that NFκB phosphorylation and translocation during focal cerebral ischemia may depend partially on the existence of mature IL-1β or ICE. Compared to WT mice, in the ICE KO mice, ICAM-1 protein expression during permanent MCAO was reduced significantly, suggesting that NFκB may play an important role in control and regulation of downstream genes including the adhesion molecule ICAM-1. Because ICE is a member of a cysteine protease family that has been implicated in apoptotic cell death (Yuan et al., 1993), a yet to be answered question is whether these deleterious effects are mediated by IL-1β production or by pro–apoptotic mechanisms.

ACKNOWLEDGMENT

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