Induction of Bis, a Bcl-2-binding protein, in reactive astrocytes of the rat hippocampus following kainic acid-induced seizure

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Abbreviations: KA, kainic acid; GFAP, glial fibrillary acidic protein

Abstract

The expression of Bis (also called Bag-3), a Bcl-2-binding protein, was investigated in the rat kainic acid (KA) model of temporal lobe epilepsy. Western blot analysis showed a significant increase in the expression levels of Bis protein in the hippocampus following the systemic administration of KA. Bis immunoreactivity increased preferentially in the CA1 and CA3 regions, as well as in the hilar region of the dentate gyrus. Experiments with double immunofluorescence revealed that, in KA-administered rats, the cells expressing Bis were GFAP-expressing reactive astrocytes. The increase in Bis immunoreactivity was accompanied by increased Bcl-2 in reactive astrocytes in the striatum radiatum, whereas Bcl-2 immunoreactivity in pyramidal neurons was not affected. These results of the co-expression of Bis and Bcl-2 in reactive astrocytes in this seizure model suggest that Bis might modulate the glial reaction under excitotoxic brain injury, probably by interacting with Bcl-2.

Keywords: kainic acid, excitotoxicity, apoptosis, Bis, Bcl-2, reactive astrocytes.

Introduction

The systemic administration of kainic acid (KA) induces limbic seizures in rats, which represents an established animal model for human temporal lobe epilepsy (Ben-Ari, 1985). The neuropathological sequelae include massive neuronal degeneration of pyramidal neurons in the CA1 and CA3 areas of the hippocampus (Altar and Baudry, 1990; Mitchell *et al*., 1993; Zhang *et al*., 1999). Although the molecular regulation of this process remains poorly understood, such seizure-induced cell death has the hallmarks of apoptosis in its biochemical and morphological aspects (Filipkowski *et al*., 1994; Pollard *et al*., 1994; Zhang *et al*., 1998). The expression of a number of cell-death regulatory genes has been investigated in this model of seizures to develop a therapeutic strategy for delaying or preventing neuronal degeneration after seizures in humans (Gillardon *et al*., 1995; López *et al*., 1999; Henshall *et al*., 2000; Chuang *et al*., 2001; Henshall *et al*., 2001).

The *bcl-2* family of genes, that includes *bcl-2* and *bax*, plays an important role in the maintenance of the balance between the death and survival of cells under different physiological or pathological conditions (Adams and Cory, 1998; Tsujimoto, 1998). In a number of systems, naturally occurring or experimentally induced neuronal death can be prevented by the overexpression of Bcl-2 (Dubois-Dauphin *et al*., 1994; Farlie *et al*., 1995; Chen *et al*., 1996). Several studies using Western blot analysis, RT-PCR, or immunohistochemistry have indicated that expression of Bcl-2 is increased in the rat brain following KA-induced seizures or ischemia-reperfusion (Graham *et al*., 1996; Henshall *et al*., 2000; Chung *et al*., 2001). However, neither the exact mechanisms by which Bcl-2 levels are increased nor the subsequent influences on neuronal survival under experimental and pathological conditions are fully understood, despite increasing evidence of the neuroprotective potential of Bcl-2 when its expression is increased by genetic manipulation.

Recently, we have cloned the *bis* (bcl-2-interacting death suppressor) gene, which encodes a novel Bcl-2-binding protein by a protein interaction technique (Lee *et al*., 1999). Bis has also been reported as Bag-3, which interacts with Hsp70 (Takayama *et al*., 1999). DNA transfection experiments have shown that Bis acts synergistically with Bcl-2 in preventing apoptosis, indicating that Bis is a novel modulator of a cellular anti-apoptotic pathway (Lee *et al*., 1999). In support of this finding, Bis is expressed at moderate or high levels in pancreatic cancers, where Bcl-2 is frequently overexpressed, but at low levels in normal pancreatic tissues (Liao *et al*., 2001). These results suggest that Bis may function through its interaction with Bcl-2 to increase cell survival under pathological conditions, including ischemia and seizures.

In the present study, we investigated the cellular and temporal pattern of Bis expression in the rat hippocampus after the administration of KA, to determine whether changes in the expression of Bis are associated with changes in Bcl-2 expression in relation to neuronal apoptosis.

Materials and Methods

Experimental animals and tissue processing

Male Sprague-Dawley rats (250-300 g) received a single intraperitoneal injection of KA (10 mg/kg) dissolved in sterile saline. Their behavior was then observed con-stantly for the next 6 h. Only animals with stage-5 seizures (the rearing and falling stage, as described by Zhang *et al*. [1997]) were used for this study. Control animals were injected with sterile saline. Animals were maintained for 12 h, 1, 2, 3, 5, and 10 days after KA injection. Seven animals were killed at each time-point: three rats were used for Western blot analysis, and four rats for immunohistochemistry. Control animals were treated on the same schedule as the KA-injected rats. The animals were deeply anesthetized with 16.9% urethane (10 ml/kg) and killed either by transcardial perfusion with a fixative containing 4% parafor-maldehyde in 0.1 M phosphate buffer, pH 7.4, or by decapitation. In the latter case, the hippocampus was then dissected out and frozen rapidly in liquid nitrogen. All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea, and were consistent with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23).

Immunoblot analysis

Hippocampi were dissected at the indicated times after KA injection and homogenized in ice-cold RIPA buffer (50 mM Tris buffer, pH 8.0, 150 mM NaCl, 1% nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). Equal amounts of total protein from control and ischemic hippocampi were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto PVDF membrane. Blots were immunostained with rabbit polyclonal anti-Bis antiserum (Lee *et al*., 1999) and anti-β-actin antibody (Sigma, St. Louis, MO, USA) followed by peroxidase-conjugated secondary antibodies (Promega, Madison, CA, USA). The immunoreactive bands were then detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK).

Immunohistochemisty and double-immunofluorescence histochemistry

Coronal sections (25 μm) from the hippocampus were incubated overnight at 4°C with a Bis-directed rabbit polyclonal antibody (1:500), or monoclonal antibodies against anti-glial fibrillary acidic protein (GFAP) (1:50; Roche, Mannheim, Germany), or Bcl-2 (1:40; Dako, Glostrup, Denmark). Primary antibody binding was visualized using peroxidase-labeled goat anti-rabbit antibody (1:200; Jackson, West groove, PA, USA) or peroxidase-labeled goat anti-mouse antibody (1:200; Jackson), with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 as the detection system. The specificity of Bis, GFAP, and Bcl-2 immunoreactivity was confirmed by the absence of immunohistochemical staining in sections from which the primary antibody was omitted, or in which it was substituted with non-specific rabbit IgG. For double-immunofluorescence histochemistry, sections were incubated overnight at 4°C in a combination of Bis-directed rabbit polyclonal antibody and either a mouse monoclonal antibody against GFAP or a mouse monoclonal antibody against Bcl-2. The sections were incubated with a mixture of FITC-conjugated goat anti-mouse IgG (1:100; Jackson) and Cy3-conjugated goat anti-rabbit IgG (1:100; Jackson) for 2 h at room temperature. Control sections were prepared as described above. Slides were viewed using a confocal microscope (MRC-1024, Bio-Rad, Hercules, CA, USA). Images were converted to TIFF format, and the contrast levels of images were adjusted using Adobe Photoshop program.

Results and Discussion

Western blot analysis demonstrated that Bis protein levels were significantly increased in the hippocampus following KA injection (Figure 1). The induction of Bis was detectable as early as 12 h, peaked at 1 day, and the increased levels were sustained for at least 5 days after KA injection. Immunohistochemistry confirmed the increased expression of Bis in this model of seizure. In the hippocampus of control animals, Bis expression was

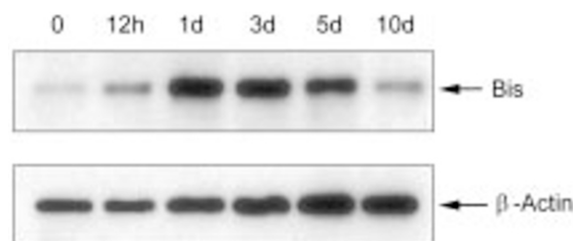

Figure 1. Western-blotting detection of Bis following KA administration. Expression of Bis within the hippocampus was investigated by immunoblotting with anti-Bis antiserum in control and KA-treated rat brains at 12 h, 1, 3, 5, and 10 days (d). β-actin was used as the control to verify identical protein loading.

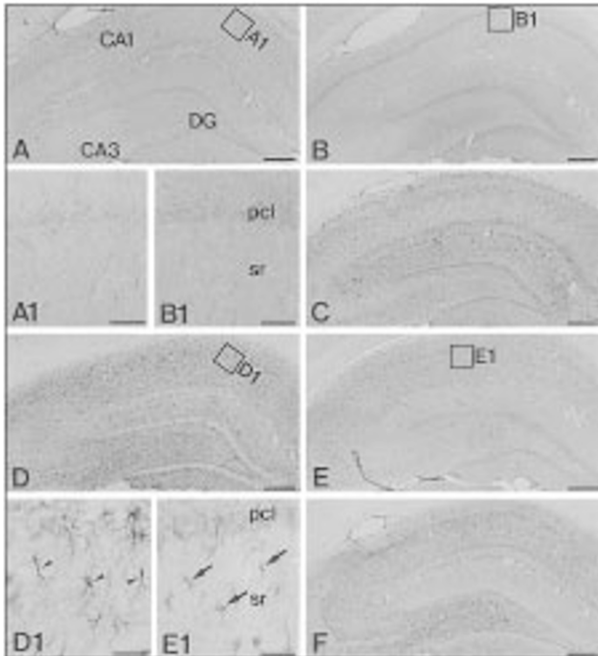

Figure 2. Changes in Bis immunoreactivity in the hippocampus after KA injection (C, D, F) and comparison with Bcl-2 immunoreactivity (B, E). In the hippocampi of control animals, no significant immunolabeling for Bis was detectable (A), but weak Bcl-2 immunoreactivity was observed in neurons of the granule cell and pyramidal cell layers (B). DG, dentate gyrus. (A1, B1) Higher magnification of the CA1 sector from A and B, respectively. pcl, pyramidal cell layer; sr, stratum radiatum. (C) At 1 day after KA injection, Bis immunoreactivity was observed uniformly over the hippocampus. Three days after KA injection, Bis immunoreactivity was more pronounced in the CA1 and CA3 regions and also in the hilar region of the dentate gyrus (D), and the immunoreactivity profile was coincident with that of Bcl-2 in the adjacent section (E). (D1, E1) Higher magnification of the CA1 sector from D and E, respectively. Note the similarity between Bis (arrowheads in D1) and Bcl-2 immunoreactive cells (arrows in E1) with respect to morphology and distribution. (F) At 5 days after KA injection, Bis immunoreactivity remained intense in the CA1 and CA3 regions and in the dentate hilar region. Scale bars: 300 μm for A, B, C, D, E, and F; 50 μm for A1, B1, D1, and E1.

too low for detection by immunolabeling (Figure 2A, A1), whereas weak Bcl-2 immunoreactivity was localized to neurons of the granule cell and pyramidal cell layers of the hippocampus proper (Figure 2B, B1). After KA injection, significant changes in both the staining intensity and the distribution of Bis and Bcl-2 immunoreactivity were observed. Bis immunoreactivity was visible within 12 h, and the signal intensity increased further for 5 days after KA injection. At 12 h, Bis immunoreactivity was distributed rather homogeneously over the hippocampus, and this pattern remained with elevation of staining intensity at 24 h (Figure 2C). At 3 days after KA injection, Bis immunoreactivity had increased preferentially in the CA1 and CA3 regions, and in the hilar region of the dentate gyrus (Figure 2D). Inspection of immunolabeled sections under high magnification revealed that Bis immunoreactivity was localized in cells with the morphology of reactive astrocytes (Figure 2D1). Double-immunofluorescence histochemical analysis of Bis and GFAP identified these cells as GFAP-positive astrocytes (Figure 3A-C). At each time point after KA injection, Bcl-2 immunoreactivity was investigated relative to the patterns of Bis immunoreactivity. Bcl-2 immunoreactivity increased in glia-like cells around the CA1 and CA3 areas and the dentate hilus, which were morphologically similar to Bis-immunoreactive cells, whereas Bcl-2 immunoreactivity in the neurons of the pyramidal cell and granule cell layers appeared unchanged (Figure 2E, E1). Double-immunofluorescence histochemistry and confocal imaging confirmed the colocalization of Bis and Bcl-2 immunofluorescence in reactive astrocytes in the stratum radiatum, whereas cells in the pyramidal cell layer were immunopositive for Bcl-2 but not for Bis (Figure 3D-F). Bis immunoreactivity remained intense in the CA1 and CA3 regions and in the dentate hilar region for 5 days after KA injection (Figure 2F). Similar changes were found in Bcl-2-immunostained sections at the same time point. Bcl-2 immunoreactivity was also observed predominantly in the CA1 and CA3 regions, as well as in the dentate hilar region (data not shown).

The present results demonstrate that the expression of Bis, a Bcl-2-binding protein, is increased in reactive astrocytes in the hippocampus of the rat KA model of human temporal lobe epilepsy. Even though the precise role of Bis expressed in the brain following seizures is unknown, the spatial profile of Bis expression in astrocytes in the vulnerable region, including the CA1 and CA3 areas, indicates that Bis may be involved in the astroglial reaction to excitotoxic neuronal damage. Furthermore, our results show that Bis is co-expressed with Bcl-2 in reactive astrocytes. The induction of Bcl-2 in reactive astrocytes has been previously reported in traumatic injury or in the diseased brain (Burne *et al*., 1996; Krajewski *et al*., 1997). Recently, Xu *et al*. (1999) observed that overexpression of Bcl-2 in astrocytes reduced the neuronal injury induced by combined oxygenglucose deprivation. Taken together, the co-expression of Bis and Bcl-2 in reactive astrocytes following seizure might provide a protective mechanism against excitotoxic neuronal damage. This proposition is supported by our earlier observation (Lee *et al*., in press) that Bis is increased together with Bcl-2 in reactive astrocytes after transient forebrain ischemia.

In response to various type of brain injury, astrocytes exhibit the morphological and functional changes, called astrocytic reaction, including the increased expression of GFAP, nuclear hypertrophy, and cell hyperplasia (Landis, 1994). Reactive astrocytes participate in creating an environment to protect neurons against many kinds of injury, by the re-uptake and inactivation of excitatory neurotransmitters, the maintenance of water and ionic homeostasis, and the reduction of oxygen free radicals (Nicholls and Attwell, 1990; Desagher *et al*.,

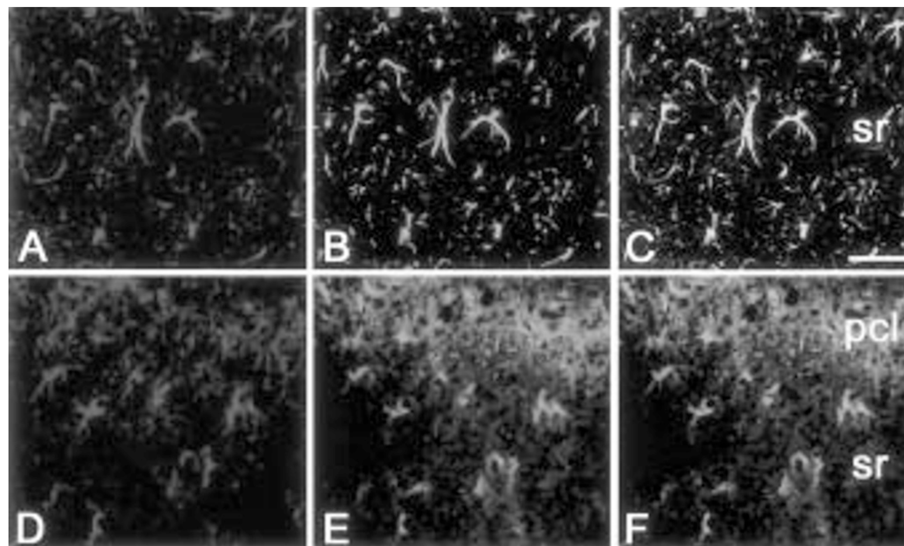

Figure 3. Confocal imaging of immunofluorescence for Bis (A, D) and either GFAP (B) or Bcl-2 (E) in the hippocampus 3 days after KA injection. (C, F) Superimposed images of Cy3 (A, D) and FITC (B, E) labeling. Note that Bis immunoreactivity is colocalized both in GFAP-immunoreactive astrocytes and in Bcl-2-immunoreactive cells in the stratum radiatum (sr) in the CA1 sector, but not in Bcl-2-immunoreactive cells in the pyramidal cell layer (pcl). Scale bars: 50 μm for A-F.

1996; Tsacopoulos and Magistretti, 1996). Therefore, our results showing the co-expression of Bcl-2 and Bis in astrocytes after seizures or forebrain ischemia raise the possibility that the interaction of Bcl-2 and Bis may be involved in enhancing astrocyte function, to modulate and/or repair neuronal damage in various pathological conditions. Although Bis increases the anti-apoptotic activity of Bcl-2 in DNA transfer experiments (Lee *et al*., 1999), the molecular mechanisms by which Bis modulates Bcl-2 functions in reactive astrocytes must be determined to define the precise role of Bis in the brain under pathophysiological conditions.

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