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Bruce/apollon promotes hippocampal neuron survival and is downregulated by kainic acid

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Abstract

Prolonged or excess stimulation of excitatory amino acid receptors leads to seizures and the induction of excitotoxic nerve cell injury. Kainic acid acting on glutamate receptors produces degeneration of vulnerable neurons in parts of the hippocampus and amygdala, but the exact mechanisms are not fully understood. We have here investigated whether the anti-apoptotic protein Bruce is involved in kainic acid-induced neurodegeneration. In the rat hippocampus and cortex, Bruce was exclusively expressed by neurons. The levels of Bruce were rapidly downregulated by kainic acid in hippocampal neurons as shown both in vivo and in cell culture. Caspase-3 was activated in neurons exhibiting low levels of Bruce causing cell death. Likewise, downregulation of Bruce using antisense oligonucleotides decreased viability and enhanced the effect of kainic acid in the hippocampal neurons. The results show that Bruce is involved in neurodegeneration caused by kainic acid and the downregulation of the protein promotes neuronal death.

Keywords: Bruce/apollon; Hippocampus; Kainic acid; Excitotoxicity; Neuronal death; Caspase-3; Cytochrome *c*

Overactivation of glutamate receptor that occurs in epilepsy and in trauma produces seizures leading to cell death in the nervous system and in various neurological disorders [1,2]. Kainic acid has been used as a model for seizure-induced excitotoxic injury, and hippocampus and amygdala are particularly vulnerable to kainic acid in vivo [3–7]. Cell demise following kainic acid insults is partly apoptotic and accompanied by caspase-3 activation and nuclear condensation [5,8–10]. Increases in calcium and alterations in neuronal gene expression are thought to underlie excitotoxic damage but the exact mechanisms involved remain unclear.

Kainic acid upregulates a number of genes and decreases others but the functional relevance of these changes to the ensuing cell death is not fully understood. Previous studies have shown that kainic acid alters the expression of anti-apoptotic proteins, such as Bcl-2 and Bax that contribute to cell death of vulnerable neurons [11,12]. Apart from the Bcl-2 proteins, the inhibitor of apoptosis proteins (IAPs) are endogenous proteins with an anti-apoptotic function and are expressed by most cells [13]. The inhibition of cell death is due to inactivation of caspases, such as caspase-9 and-3 by the baculovirus IAP repeat (BIR) domains in the IAPs [14–16]. X-chromosome linked IAP (XIAP) contain three such domains and is also present in the brain [10].

Bruce/apollon belongs to the IAPs having one BIR-domain in the aminoterminal and a ubiquitin conjugation motif in the carboxyterminal region [17]. Recent studies on gene deleted mice for Bruce suggest a role for this protein in control of cell death during early development [18]. However, little is known about the expression and function of Bruce in the nervous system.

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We have previously shown that XIAP and the rat homologue for cIAP-1, Riap-2, are regulated by kainic acid in the rat hippocampus [10,19]. The expression of these proteins was downregulated in the CA3 region of hippocampus accompanying nerve cell death in this region. In this study, we have investigated the expression of Bruce in rat brain after kainic acid and in cultured hippocampal neurons. The results show that Bruce is rapidly downregulated in hippocampal neurons by kainic acid. Neurons lacking Bruce showed caspase-3 activation and increased cell death. Downregulation of Bruce by antisense oligonucleotides also reduced neuronal viability. The results show that Bruce is neuroprotective and that this protein is linked to the process of excitotoxicity induced by kainic acid.

Materials and methods

Animals. All animal experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Local Ethical Committee.

Kainic acid injections. Adult male Wistar rats (200–300 g; B&K Sollentuna, Sweden) were injected into the lateral ventricle with kainic acid (0.35 μg/ml/0.5 μl, Sigma, Sweden) or an equal volume of saline [10]. Rats were anaesthetised by urethane and killed by decapitation at 12, 24, and 48 h post-injection. Brains were frozen and stored at −70 °C until analysis.

Neuronal cultures and cell viability. Hippocampus was dissected from E17 old Wistar rats (B&K, Sollentuna, Sweden), and neurons were prepared and plated at a density of 2×10^6 cells per well in a poly-ornithine coated six-well plates as described before [10]. The incubation was carried out for 5–7 days using the Neurobasal medium (Invitrogen) supplemented with B27 ingredients (Invitrogen). Different concentrations of kainic acid (Sigma, Sweden) were then added for 24 h. The viability of cell was determined by the MTT assay or by counting cells with condensated or fragmented nuclei after staining cells with DAPI (Sigma, Sweden).

Downregulation of Bruce. To downregulate Bruce in hippocampal neurons, we employed stable anti-sense oligonucleotides (Sigma-Genosys) and transfections using the Amaxa Rat Neuron Nucleofection kit (AMAXA GmbH, Germany). Briefly, 6×10^6 cells were suspended into 100 μl nucleofection solution and transfected with 4 μM of a mixture of antisense oligonucleotides according to the manufacturer's instructions. The sequences of the oligonucleotides against Bruce corresponded to nucleotides 52–70 and 149–167 in the rat sequence. Following transfections, equal number of cells were plated onto poly-ornithine coated plates and incubated for 4–5 days followed by stimulation with kainic acid. The viability of cells was estimated as above. The degree of downregulation was determined by Western blotting, showing a clear reduction in Bruce using this approach.

Western blotting. Tissue and cultured hippocampal neurons were homogenized in ice-cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, and 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor cocktail (Roche, Sweden). Equal amounts of protein determined by DC Protein Assay (Bio-Rad, Sweden) were boiled, separated by SDS–PAGE and blotted onto a nitrocellulose filter (Amersham, Sweden). Filters were blocked for 1 h in blocking solution (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 5% skimmed milk), followed by incubation overnight at 4 °C with primary anti-Bruce antibody (diluted 1:500, BD Biosciences, Sweden). After washing, the filter was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2500, Pierce/Boule Nordic, Sweden) followed by detection with enhanced chemiluminescent method (Pierce). Filters were stripped for 30 min at 60 °C (62.5 mM Tris–HCl, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS) and reprobed with an anti-spectrin or β-actin antibody (1:1000; Chemicon, Sweden).

Immunocytochemistry. Hippocampal neurons were plated on poly-ornithine (Sigma) coated coverslips and treated as indicated in the text followed by fixation with 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized with 0.1% Triton X-100, blocked for 1 h in 5% bovine serum albumin–5% goat serum, and incubated with primary anti-Bruce (1:200; BD Biosciences), anti-cytochrome *c* (1:200, BD Biosciences), and active caspase-3 (1:300; Cell Signaling). After incubation overnight, cells were washed with PBS followed by a 2-h incubation with secondary Cy2- and Cy3-conjugated antibodies (1:500, Jackson Laboratories, CA, USA). Cells were counterstained for 5 min with Hoechst 33342 (4 μg/ml; Sigma) and mounted using gel mounting medium (Sigma). Control staining without primary antibodies showed no detectable signal.

Immunohistochemistry. Freshly frozen 13 μm sections from adult rat brains were cut on a cryostat (Leitz Digital, Germany), mounted onto Superfrost slides (Metzel-Gläser, Germany), and fixed using acetone–methanol (1:1) for 10 min at −20 °C. The slides were incubated with 5% bovine serum albumin (BSA; Sigma) for 2 h at room temperature (RT), followed by an overnight incubation at +4 °C with anti-Bruce antibody (1:300; BD Biosciences, Sweden). After washing with TBS-0.05% Tween 20, secondary Cy2-conjugated anti-mouse antibody (diluted 1:200, Jackson Laboratories, USA) was added for 2 h at RT. Slides were dehydrated in a series with graded alcohol and mounted using gel-mounting medium (Sigma, Sweden). Control slides without primary antibody showed no staining. A similar protocol as above was used to stain cultured hippocampal neurons. For double staining, the Bruce antibody was combined with the Tau polyclonal antibody (1:200, Roche, USA) or with the antibody against glial fibrillary acidic protein (GFAP; 1:200, Sigma, Sweden). The incubation was carried out overnight at +4 °C. Secondary Cy3-conjugated anti-rabbit antisera (Jackson laboratories, Sweden) or Cy2-conjugated anti-mouse antisera (Jackson Laboratories, Sweden) were added for 2 h at RT, and the cells were analysed with a Zeiss Axiovision fluorescence microscope or Zeiss LSM confocal Microscope.

Image quantification and statistics. Western blot images were analysed using ImageQuant program (Bio-Rad, Sweden) to obtain mean values for the different bands. Four animals were used for each time point, and values are shown as means ± SD and analysed by ANOVA and Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Bruce is expressed by neurons in the rat nervous system

To study the expression of Bruce in the rat brain, immunohistochemistry was made using an antibody specific for Bruce in combination with neuronal and glial markers. The results showed that the immunoreactivity for Bruce was co-localized with neuronal marker Tau, but not with astrocyte marker GFAP. This suggests that Bruce is expressed predominantly by neurons in the mature rat brain (Fig. 1). This was further confirmed by staining of cultured hippocampal neurons as shown below.

Kainic acid decreases expression of Bruce in rat hippocampal neurons undergoing cell death

Administration of kainic acid induces seizures and degeneration of neurons in parts of the hippocampus within 24 h after treatment (Fig. 2A). Data showed that the expression of Bruce was downregulated by kainic acid at 12 h and remained low thereafter (Fig. 2B). Similarly, immunocytochemistry revealed decreased levels in the CA3 region of rat hippocampus (Fig. 2C). Cells with reduced levels of Bruce were shown to undergo cell degeneration, whereas viable cells expressed the protein (Fig. 2C).