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Nuclear translocation and calpain-dependent reduction of Bcl-2 after neonatal cerebral hypoxia–ischemia

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ABSTRACT

Apoptosis-related mechanisms are important in the pathophysiology of hypoxic–ischemic injury in the neonatal brain. Caspases are the major executioners of apoptosis, but there are a number of upstream players that influence the cell death pathways. The Bcl-2 family proteins are important modulators of mitochondrial permeability, working either to promote or prevent apoptosis. In this study we focused on the anti-apoptotic Bcl-2 protein after neonatal cerebral hypoxia–ischemia (HI) in 8-day-old rats. Bcl-2 translocated to nuclei and accumulated there over the first 24 h of reperfusion after HI, as judged by immunohistochemistry and immuno-electron microscopy. We also found that the total level of Bcl-2 decreased after HI *in vivo* and after ionophore challenge in cultured human neuroblastoma (IMR-32) cells *in vitro*. Furthermore, the Bcl-2 reduction was calpain-dependent, because it could be prevented by the calpain inhibitor CX295 both *in vivo* and *in vitro*, suggesting cross-talk between excitotoxic and apoptotic mechanisms.

1. Introduction

Hypoxic–ischemic (HI) brain injury resulting from perinatal asphyxia remains a clinical problem, in spite of increased knowledge of the pathophysiology (Blomgren et al., 2003; Johnston et al., 2002). Hypothermia has been the only effective means for neuroprotection (Shankaran et al., 2005), but recently erythropoietin treatment has also emerged as a promising strategy (Zhu et al., 2009). However, additional strategies and treatments are needed. It is today acknowledged that apoptosis-related mechanisms contribute to the tissue damage that occurs after HI injury, especially when it affects the developing brain (Hu et al., 2000; Zhu et al., 2005), but the molecular mechanisms need to be investigated further. Apoptotic cell death includes caspase-dependent and -independent pathways (Blomgren et al., 2007, 2001; Cheng et al., 1998; Zhu et al., 2003). Several intracellular apoptosis-related pathways have been identified in which mitochondria are pivotal regulators in the process of cell death and survival, both through their roles in energy metabolism and calcium homeostasis, but also because of their capacity to release pro-apoptotic proteins (Blomgren et al., 2003; Zhu et al., 2003). The different pathways for induction of apoptosis converge upon the activation of caspase-3 or the translocation of apoptosis-inducing factor (Blomgren et al., 2007; Zhu et al., 2007).

The Bcl-2 family proteins are involved in the control of apoptosis both upstream of and at the level of mitochondria, representing a critical proximal intracellular checkpoint. The Bcl-2 family consists of at least 20 pro- or anti-apoptotic members. Induction of cell death signaling in neurons is prevented by death suppressors (e.g. Bcl-2), and promoted by for example Bax or Bid (Galluzzi et al., 2009). Over-expression of the anti-apoptotic Bcl-2, deletion of

Abbreviations: Apaf-1, apoptosis protease-activating factor 1; Bax, Bcl-2-associated protein X; Bcl-2, B-cell lymphoma 2; COX IV, cytochrome c oxidase subunit IV; DAB, diaminobenzidine; GOD, glucose oxidase DAB method; ER, endoplasmic reticulum; HI, hypoxia–ischemia; MAP-2, microtubule associated protein 2.

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the pro-apoptotic Bax or inhibition of the Bax-induced mitochondrial pore formation increases the resistance to ischemic insults, indicating that mitochondrial pathways are involved in neuronal cell death (Gibson et al., 2001; Hetz et al., 2005; Martinou et al., 1994). HI induces Bax translocation from cytosol to mitochondria leading to cytochrome c release from the mitochondrial intermembrane space, which in turn activates caspase-9 and subsequently caspase-3 (Northington et al., 2001). The anti-apoptotic protein Bcl-2 can interfere with the Bax translocation to mitochondria and thereby block the release of apoptotic proteins (Ferrer and Planas, 2003), and Bcl-2 can probably prevent opening of the mitochondrial membrane permeability transition pore (Zoratti and Szabo, 1995). The balance of the pro- *vs* anti-apoptotic Bcl-2 family proteins seems critical for the development of neonatal HI brain injury (Gibson et al., 2001; Hallin et al., 2006).

Calpains are calcium-dependent cysteine proteases usually implicated in necrosis, but they have also been shown to be involved in cell death after HI, either in concert with caspases or independently (Blomgren et al., 2001; Leist and Jäättelä, 2001; Nakagawa and Yuan, 2000). Lately, calpains have also been implicated in caspase-independent apoptosis, in the release of AIF from mitochondria (Norberg et al., 2008), although this concept has been challenged (Joshi et al., 2009; Wang et al., 2009). Calpains can function upstream of caspases and procaspase-3 has been demonstrated to be a calpain substrate *in vitro* (McGinnis et al., 1999) and *in vivo* (Blomgren et al., 2001). Some of the Bcl-2 family proteins are known to be cleaved by calpains (Wood et al., 1998) or caspases (Cheng et al., 1997; Choi et al., 2001) influencing their function. Understanding the interaction between calpains and caspases will help unravel the molecular mechanisms that activate the death executioners and will provide new insights for the design of strategies to ameliorate the effects of HI brain injury. The aim of this study was to investigate the cellular distribution of Bcl-2 after HI in the neonatal rat brain and explore the possible interaction between calpains and Bcl-2.

2. Materials and methods

2.1. Induction of unilateral hypoxic–ischemic brain injury

Wistar rats of both genders were operated according to the Rice-Vannucci model (Rice et al., 1981; Zhu et al., 2003) on postnatal day 8 (P8). In brief, the left common carotid artery was cut between double ligatures of prolene sutures (6–0) under enflurane anesthesia, 3–4% for induction followed by 1–2% for maintenance. The wound was sutured and infiltrated with lidocaine for analgesia. The pups were left with their dam to recover for about 1 h and then placed in a humidified chamber, pre-warmed to 36 °C. The chamber was perfused with air for 10 min followed by a period of 55 min in 7.8% oxygen in nitrogen followed by another 10 min with air. The air and the gas mixture were humidified and preheated (36 °C) before entering the chamber. Control animals were subjected neither to ligation nor hypoxia. All animal experimentation was approved by the Ethical Committee of Gothenburg (182-99 and 288-02).

2.2. Calpain Inhibition in vivo

Pups were treated with the calpain inhibitor CX295 (Z-Leu-aminobutyric acid-CONH(CH2) 3-morpholine; Cortex Pharmaceuticals, Irvine, CA) or vehicle ($n = 8$/group). The first dose, 200 μl of 5 mM CX295 in 100 mM NaCl (equivalent to 80 μmol/kg or 40 mg/kg body weight), was administered subcutaneously immediately after HI. Subsequently, animals were injected with 100 μl of the CX295 solution (equivalent to 40 μmol/kg or 20 mg/kg body weight) every 3 h for 24 h. Control animals were injected with 100 mM NaCl.

2.3. Immunoblotting

Animals were sacrificed by decapitation 2, 6 or 24 h after HI. Control animals were sacrificed on postnatal day 8. The brains were rapidly dissected out on a bed of ice. The parietal cortex and diencephalon were dissected out from each hemisphere and ice-cold isolation buffer was added [15 mM Tris–HCl, pH 7.6, 320 mM sucrose, 1 mM dithiothreitol (DTT), 3 mM EDTA-K, 0.5% protease inhibitor cocktail (Sigma) and 2.5 μM cyclosporine A (Sigma)]. Homogenization was performed gently by hand (to preserve mitochondrial integrity) in a 2-mL glass/glass homogenizer (Merck Eurolab, Göteborg, Sweden) using, sequentially, two different pestles with a total clearance of 0.12 and 0.05 mm, respectively. The homogenates were centrifuged at 800*g* at 4 °C for 10 min. The supernatants were further centrifuged at 9200*g* for 15 min at 4 °C, producing mitochondrial and synaptosomal fractions in the pellets (P2) and crude cytosolic fractions in the supernatants (S2). The P2 (mitochondrial) fractions were washed and recentrifuged. The S2 fraction was centrifuged for 40 min at 100,000*g* at +4 °C yielding a cytosolic fraction (S3) in the supernatant. A set of control animals (aged 0, 3, 7, 14, 21, 42 and 60 days) were sacrificed and their brains collected as described above. All fractions were stored at −80 °C. The protein concentrations were determined according to Whitaker and Granum (Whitaker and Granum, 1980), adapted for microplates, using a Spectramax Plus plate reader (*Molecular Devices, Sunnyvale, CA, USA*). When brain samples were pooled, equal amounts of total protein from 6 animals per group were mixed.

In the case of IMR-32 cells all experiments were repeated in triplicate. For sample preparation, please see below. SDS–PAGE was performed using 4–12% Tris–glycine gels (*Novex, San Diego, CA*) and proteins were transferred to enhanced nitrocellulose membranes (*Schleicher & Schuell, Dassel, Germany*). For detection of Bcl-2 and Bax, polyclonal rabbit antibodies (sc-492 and sc-493, respectively, *Santa Cruz Biotechnology*) were used, diluted 1:1000. Active m-calpain was detected by immunoblotting using a rabbit polyclonal antibody diluted 1:1000 (a kind gift from Dr. Mitsushi Inomata, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) (Manya et al., 2002). Anti-caspase-12 (Oncogene, PC558) was diluted 1:2000. Anti-α-fodrin (Biomol, Plymouth Meeting, PA, USA) was diluted 1:500. Equal loading of protein was confirmed using rabbit anti-actin (1:200, A2066, Sigma, Stockholm, Sweden) or mouse anti-Cox IV (1:500, A21344, Molecular Probes, Eugene, USA). Peroxidase-labeled secondary goat-anti-rabbit IgG (1:2000), horse anti-goat (1:2000) horse anti-mouse (1:4000) antibodies were from Vector Laboratories (*Burlingame, CA, USA*). Immunoreactive bands were visualized using Super Signal West Dura (*Pierce, Rockford, IL, USA*) chemiluminescent substrate and a LAS-3000 cooled CCD-camera (*Fujifilm, Tokyo, Japan*).

2.4. Immunohistochemistry

At 2, 6, 14 or 24 h after HI or on postnatal day 8 for controls, the animals were deeply anesthetized with an intraperitoneal injection of phenobarbital and sacrificed by perfusion fixation with 4% neutral buffered formaldehyde (*Histofix, Histolab Products AB Sweden*). Brains were dissected out and immersed in the same fixative overnight, dehydrated by passing through a graded series of ethanol, cleared in xylene, embedded in paraffin and cut frontally to 5 μm sections. Sections were deparaffinized, rehydrated and stained for Bcl-2 (polyclonal rabbit-anti-Bcl-2; *Santa Cruz Biotechnology: sc-783*). Antigen retrieval was performed by heating the sections in 10 mM citrate buffer, pH 6.0 for 10 min. Non-specific protein bind-

ing was blocked using 4% normal goat serum in PBS. Biotinylated goat anti-rabbit secondary antibodies were from Vector (*Burlingame, CA, USA*). Endogenous peroxidase activity was blocked by 0.6% hydrogen peroxide in methanol and the signal was enhanced using biotinylated avidin–biotin complex (Vectastain ABC Elite, *Vector*) with 0.5 mg/mL 3,3′-diaminobenzidine (DAB) enhanced with 15 mg/mL ammonium nickel sulfate, 2 mg/mL β-D-glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL beta-glucose oxidase (Sigma). Negative controls were performed by excluding the primary antibody as well as by preabsorption of the primary antibodies with the antigen used to raise the antibody. The number of Bcl-2 immunopositive cells was counted in a blinded manner in the cortex and dentate gyrus of ipsilateral hemispheres. In the cortex, cells were counted in four adjacent visual fields, each with an area of 180×10^3 μm^2, in the injured, MAP-2-negative (or corresponding) area. The whole dentate gyrus was similarly investigated.

2.5. Fluorescence microscopy

After dehydration and antigen retrieval as above, non-specific protein binding was blocked using 4% normal donkey serum in PBS. Incubation with anti-Bcl-2 (diluted 1:150) was performed over night at 4 °C. A secondary antibody (Alexa Fluor 555, donkey-anti-rabbit IgG; diluted 1:500) (*Jackson ImmunoResearch, PA, USA*) was applied for 60 min at 22 °C. A second blocking step with 0.1 M Tris–HCl, pH 7.5 containing 3% BSA for 30 min was followed by washing with PBS and incubation with TUNEL reaction mixture for 60 min at 37 °C. After washing, the sections were mounted using Vectashield mounting medium with DAPI (*DAKO Corporation, Carpinteria, CA, USA).*

2.6. Electron microscopy

Pups were deeply anesthetized with an intraperitoneal injection of phenobarbital and sacrificed by perfusion fixation with a freshly made solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 2 or 24 h after HI. The brains were dissected out, post-fixed in the same solution for 2 days at +4 °C and stored in 0.1 M phosphate buffer at +4 °C up to 24 h until sectioning. Control animals were sacrificed on postnatal day 8. The hemispheres were separated, the left hemisphere was mounted and cut frontally to 50 μm sections on an *Oxford* vibratome. All subsequent incubations were performed with free floating 50 μm thick sections in a 12-well cell culture plate (*Costar*) on a rocking plate at room temperature. After rinsing in PBS, sections were incubated with 0.05% glycine in PBS, rinsed again with PBS, blocked and permeabilized over night at +4 °C in a solution of 1% PVP 40 and 0.5% Tween 20 in PBS (PBS-PT). Sections were washed with PBS-T (0.01% Tween 20) and incubated with polyclonal rabbit-anti-Bcl-2 (1:100 in 1% PVP 40, PBS-T, *Santa Cruz Biotechnology: sc-783*) at +4 °C over night. Negative control sections were incubated in PBS-PT only. After rinsing in PBS-T, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:500 in PBS-PT) for 4 h at 22 °C. They were washed in PBS-T and incubated with avidin–biotin complex (*Vectastain ABC Elite kit, DAKO*) for 60 min. A final rinse in PBS was performed before the sections were washed in 0.1 M sodium acetate buffer, pH 6.0 and the immunoreactivity developed as above. Finally, the sections were washed with PBS and stored in PBS at +4 °C over night. Sections were rinsed in 0.1 M phosphate buffer (pH 7.4) for 20 min, post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 30 min and rinsed again in phosphate buffer for 15 min. Dehydration was performed using a graded series of acetone: 15 min each in 50%, 70%, 80% and 90% acetone, then three times 15 min in 100% acetone. Sections were infiltrated with 30% *Vestopal W* plastic (*Promochem, Boras, Sweden*) (diluted in 100% acetone) for 60 min followed by over night (16 h) infiltration with 60% *Vestopal W* at +4 °C, 7 h of infiltration with 100% *Vestopal W* at 22 °C after which they were put in +4 °C over night. The sections were flat-embedded in *Vestopal W*, and the plastic was allowed to polymerize, at 45 °C for 2 h, then 60 °C for 2 days. Samples were selected for ultramicrotomy from the immunostained and embedded vibratome sections. After cutting from the vibratome section, specimens were mounted on premoulded *Vestopal W* holders using "Loctite Super Attak" glue (*Loctite Corporation, Loctite Sweden AB*). Thin sections (1000 or 1500 Å) were cut using an LKB III ultrotome and placed on formvar-coated one-hole copper grids. The 1000 Å sections were contrasted with uranyl acetate and lead citrate for evaluation of cellular morphology, while the 1500 Å sections were used for evaluation of the immunohistochemical staining. The samples were evaluated using a Philips 400 transmission electron microscope.

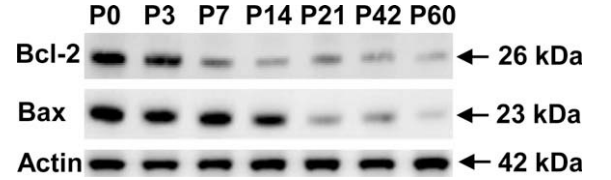

Fig. 1. Postnatal expression of Bcl-2 and Bax in rat brain. Immunoblots of pooled homogenate samples from postnatal day (P) 0, 3, 7, 14, 21, 42 and 60 rats demonstrating downregulation of both Bcl-2 and Bax during normal brain development. Actin immunoblots demonstrate equal loading.

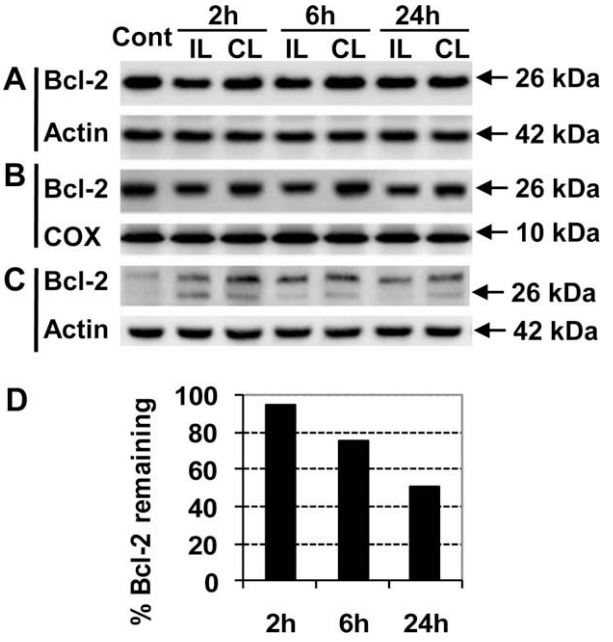

Fig. 2. Bcl-2 immunoblots of pooled cortical homogenates, mitochondrial fractions and cytosolic fractions after HI. (A) Bcl-2 expression in pooled brain homogenates (n = 6/group) from 8-day-old normal control rats (Cont) or 2, 6 and 24 h after HI, demonstrating degradation in the ipsilateral (IL) hemisphere at early recovery. During late recovery, 24 h post-HI, there was no longer any obvious reduction compared with the contralateral (CL) hemisphere. (B) Bcl-2 was reduced in the mitochondrial (P2) fractions from ipsilateral hemispheres as early as 2 h post-HI, and was even more reduced after 24 h. (C) Bcl-2 increased dramatically in the soluble, cytosolic (S3) fraction of both hemispheres, most pronounced at 2 h post-HI. The nature of the heavier (approx. 28 kDa) band is not clear. The actin and COX IV immunoblots demonstrate equal loading. (D) The bar graph shows the relative amount of Bcl-2 remaining in the cytosolic fraction of the ipsilateral compared to the contralateral hemisphere after densitometric quantification of Bcl-2 bands from the respective hemispheres.

2.7. Cell culture

Human neuroblastoma cells (IMR-32) were cultured and amplified in RPMI-1640 medium (BE12-167, Bio-Whittaker, Verviers, Belgium). On day 5, when cells had reached 80–90% confluence, they were treated with 0, 50 or 100 μM calpain inhibitor, cx-295 (Cortex Pharmaceuticals, Irvine, CA) for 60 min, then stimulated with 0, 25 or 50 μM thapsigargin for 4 h. Adherent cells were resuspended by mechanical scraping and then suspended in fresh medium. The suspension was centrifuged at 1500 rpm for 10 min, the supernatant was discarded and the pellet mixed with an equal volume of lysis buffer (20 mM Tris, 5 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 10 μM APMSF, 150 mM NaCl, 8 mM CHAPS, 0.025% protease inhibitor cocktail and 0.25 M sucrose). Samples were mixed with equal volumes of concentrated (3×) buffer, heated at 96 °C for 5 min and then stored at −20 °C. SDS–PAGE electrophoresis and immunoblotting were performed as above. Three independent sets of experiments were performed to validate the findings. Alternatively, subcellular fractionation was performed by sequential centrifugation steps: the lysate was centrifuged for 10 min at 800*g* at +4 °C, yielding P1 and S1 fractions. The S1 fraction was centrifuged for 15 min at 9200*g* at +4 °C resulting in P2 and S2 fractions. Finally, the S2 fraction was centrifuged for 2 h at 54,000*g* at +4 °C yielding P3 and S3 fractions. Pellets were resuspended in lysis buffer and protein concentrations determined as above. Samples were mixed with equal volumes of concentrated (3×) buffer, heated at 96 °C for 5 min and then stored at −20 °C.

2.8. Statistics

All data are expressed as mean ± s.e.m. Cell counting was analyzed using ANOVA and Fisher's post hoc test. The percentage of Bcl-2 reduction after HI in vehicle- or CX295-treated rats was analyzed using Mann–Whitney *U* test. Statistical significance was assumed at $p < 0.05$.

3. Results

3.1. Developmental changes

Both Bcl-2 and Bax were highly expressed in the rat cortex during postnatal brain development and the expression decreased as brain growth leveled out (Fig. 1). Bcl-2 dropped 70% between the day of birth and postnatal day 7, whereas Bax expression decreased more slowly, displaying a 70% reduction between the day of birth and postnatal day 21 (Fig. 1).

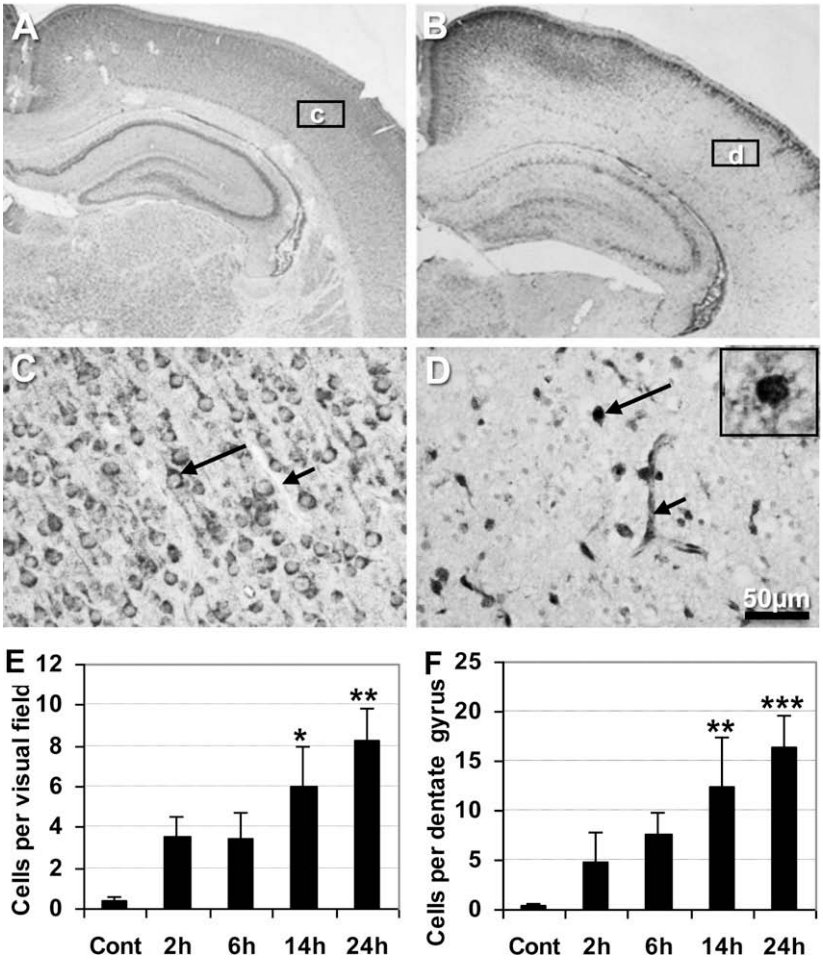

Fig. 3. Bcl-2 immunohistochemistry staining after HI. Representative pictures of Bcl-2 immunostaining in a normal control brain (A) and 24 h after HI (B). Higher magnification demonstrates cytoplasmic staining in neurons (long arrow) and absence of immunoreactivity in endothelium (short arrow) in the normal control cortex (C). In areas of injury, nuclear Bcl-2 staining in neurons (long arrow) and staining in the endothelium (short arrow) were observed 24 h after HI (D). The areas shown in (C) and (D) are indicated in (A) and (B), respectively, at lower magnification by rectangles designated "c" and "d". The number of cells with nuclear Bcl-2 immunoreactivity, as in (D) (long arrow), was counted in the cortex (E) and in the dentate gyrus (F). The numbers increased several-fold during reperfusion. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control.

3.2. Bcl-2 downregulation and nuclear translocation after HI

The 26 kDa Bcl-2 band decreased 20% in homogenates from the ipsilateral hemisphere 6 h after HI, but there was no obvious change 24 h after HI in the ipsilateral compared to the contralateral hemispheres (Fig. 2A). In mitochondrial (P2) fractions, Bcl-2 decreased after HI in ipsilateral hemispheres, more pronounced at 6 and 24 h of recovery than 2 h after HI. This indicates a progressive and sustained loss of Bcl-2 in mitochondria even after the overall levels have normalized (Fig. 2B). In cytosolic (S3) fractions, double bands of Bcl-2 were detected, the heavier band being the more prominent, both in controls and after HI (Fig. 2C). Increased levels of both the heavier band and the lower band were found in cytosolic fractions from both hemispheres 2 and 6 h after HI, most pronounced at 2 h in the contralateral hemisphere, indicating release of Bcl-2 into the soluble fraction (Fig. 2C). The lower band was most pronounced after 2 h reperfusion (Fig. 2C). Densitometry analysis of the lower band showed a decrease in the ipsilateral hemisphere with prolonged reperfusion (Fig. 2D). In tissue sections, Bcl-2 was found to be widely distributed in all brain regions of normal neonatal rats, displaying the strongest staining in neurons, whereas endothelial cells were left clearly unstained (Fig. 3A and C). Following HI, the overall Bcl-2 staining decreased in the damaged areas (Fig. 3B and D), as confirmed by immunoblotting (Fig. 2A). The few, strongly immunopositive cells remaining in the injured areas displayed a predominantly nuclear staining, particularly 24 h post-HI, including both neurons and endothelial cells (Fig. 3B and D). The number of nuclei exhibiting Bcl-2 immunoreactivity increased significantly in all investigated brain areas after the HI insult (Fig. 3E and F). Immunofluorescent triple labeling demonstrated that Bcl-2 immunoreactivity could be detected in the nucleus mainly in cells displaying DNA damage, as judged by TUNEL staining (Fig. 4). TUNEL-labeled nuclei in the penumbral region displayed a high degree of colocalization with Bcl-2 immunoreactivity both at 2 h (Fig. 4A) and 24 h (Fig. 4B) after HI. The number of double-labeled nuclei increased during reperfusion, as shown also for the number of nuclei with Bcl-2 staining (Fig. 3E and F). Immuno-electron microscopy confirmed the results obtained by immunoblotting and immunohistochemistry. Bcl-2 immunoreactivity in the control tissue was distributed in a relatively weak and diffuse pattern throughout the cytoplasm, without any distinctly labeled organelles (Fig. 5A and B). Immunoreactivity was neither found in association with neuronal nuclei (Fig. 5A), nor in endothelial cells (Fig. 5B) in the non-ischemic brain. After 2 h of reperfusion, distinct immunoreactivity also appeared in association with nuclei and membrane-bound organelles (Fig. 5C), some of which appeared to be mitochondria. Furthermore, labeling now appeared in endothelial cells (Fig. 5D). After 24 h of reperfusion, even stronger immunoreactivity was present closely associated with the nuclear membrane, and some intranuclear immunoreactivity was also observed (Fig. 5E). A number of more strongly labeled, membrane-bound organelles appeared 24 h post-HI (Fig. 5E), occasionally at structures appearing to be nuclear pores (Fig. 5E and G). Increased cytoplasmic staining was found in endothelial cells 24 h post-HI (Fig. 5F). No immunoreactivity was found in the negative control sections (Fig. 5H).

3.3. Calpain-dependent reduction of Bcl-2 in vivo and in vitro

The amount of soluble Bcl-2, the lower band, in the cytosolic fraction was reduced in the ipsilateral hemisphere 24 h after HI, as indicated by immunoblotting (Fig. 6). Treatment with the calpain inhibitor CX295 prevented this reduction of Bcl-2 in the ipsilateral hemisphere. To confirm this calpain-dependent downregulation of Bcl-2 *in vivo*, we challenged IMR-32 cells with the ionophore thapsigargin and analyzed the levels of Bcl-2 protein in the lysates. The active form of m-calpain increased in a dose-

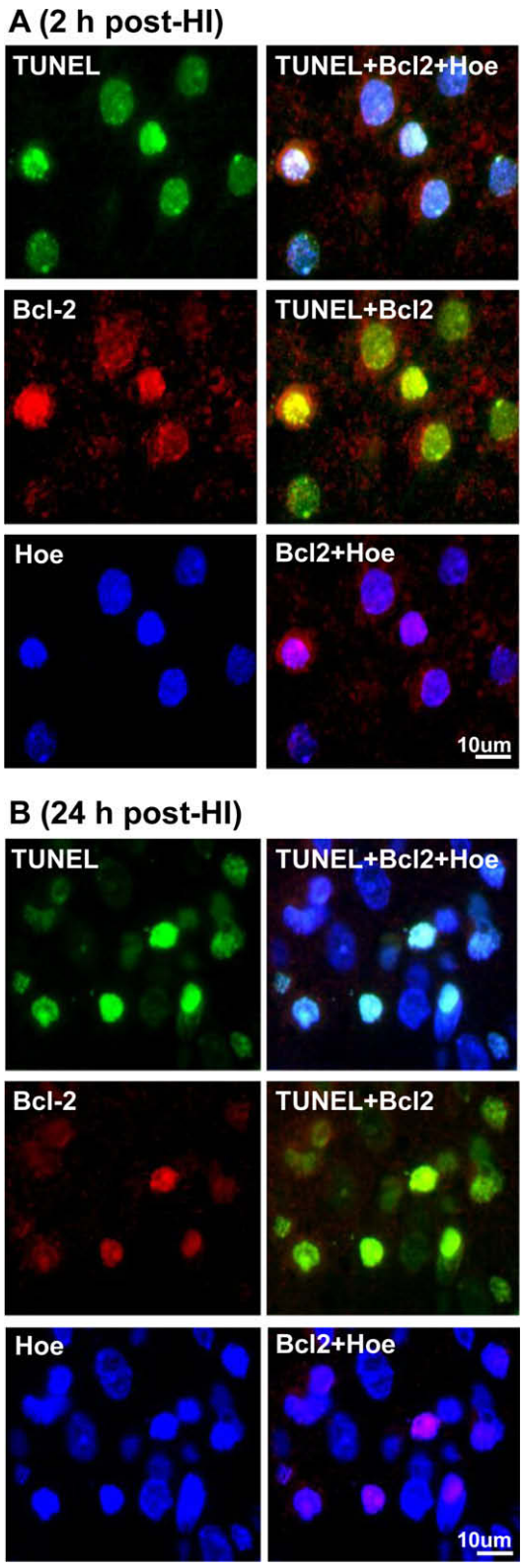

Fig. 4. Double labeling of Bcl-2 and TUNEL in injured cortex at 2 h and 24 h after HI. Representative immunoreactivity for TUNEL (green), Bcl-2 (red), Hoechst 33342 (blue) and overlays in the ipsilateral cortex 2 h (A) and 24 h (B) after HI. Nuclear Bcl-2 staining was largely colocalized with the DNA damage marker TUNEL in cells displaying a pyknotic nuclear morphology at early recovery after HI (A) and, even more pronounced at later recovery, in penumbral areas 24 h after HI (B). Scale bar = 10 μm.

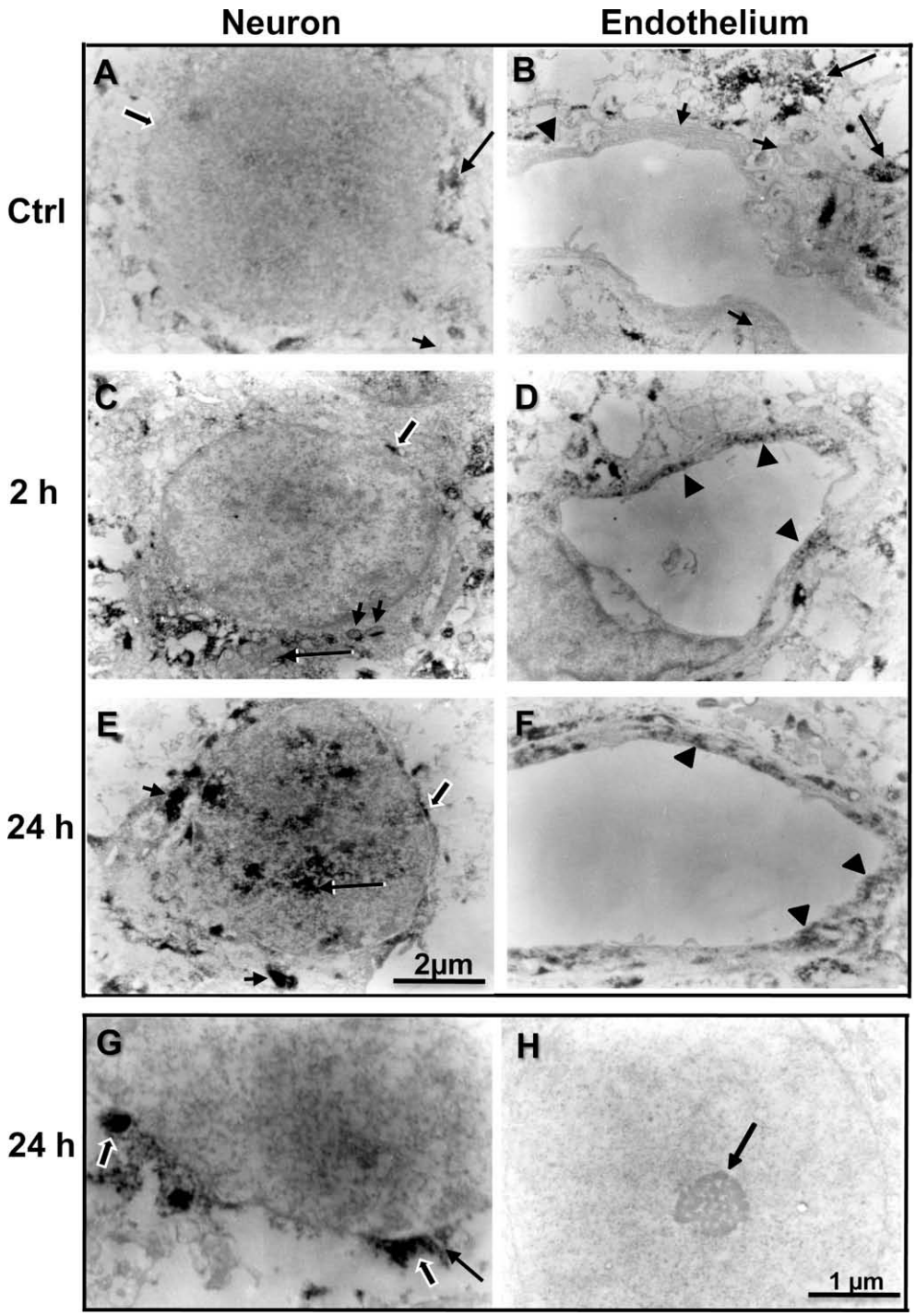

Fig. 5. Bcl-2 immuno-electron microscopy in the cortex. (A) Diffuse cytoplasmic staining is present in normal control neurons, not clearly associated with any organelles. The thin arrow points to a non-labeled membranous organelle. No immunoreactivity is seen at the nuclear membrane (bold arrow). (B) The arrowhead shows endothelium without labeling. Short arrows show unlabeled organelles. Long arrows show diffuse cytoplasmic labeling in non-endothelial cells. (C) A neuronal mitochondrion (long arrow) and other membrane-bound organelles (short arrows) exhibit membrane-associated labeling 2 h after HI. The bold arrow shows a site of immunoreactivity close to the nucleus. (D) Staining appearing in endothelial cytoplasm (arrowheads) 2 h after HI. (E) Intranuclear neuronal staining (long arrow), labeling of nuclear membrane (bold arrow) and strong immunoreactivity in some organelles, possibly mitochondria (short arrows) 24 h after HI. (F) Stronger staining in the endothelial cytoplasm (arrowheads) 24 h after HI. (G) Close-up view of immunoreactivity associated with the nuclear membrane (bold arrows), possibly at the site of a nuclear pore (thin arrow). (H) No immunoreactivity was detected in negative control sections, without primary antibody, 24 h after HI. The arrow indicates a nucleolus.

dependent manner when thapsigargin was added, as expected, and this activation was largely prevented by CX295 (Fig. 7). We confirmed that the Bcl-2 levels decreased upon thapsigargin treatment and that this could be prevented by pretreatment with the calpain inhibitor. Also, release of caspase-12 from cellular membranes into the cytosol was demonstrated in a calpain-dependent manner (Fig. 7). There was no change in the levels of Bax in this paradigm, neither by thapsigargin treatment, nor calpain inhibition (Fig. 7).

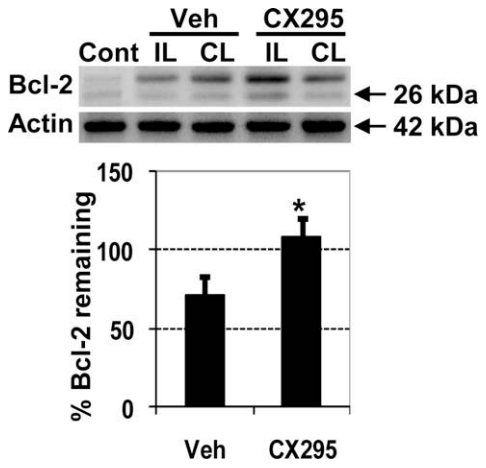

Fig. 6. Calpain inhibition prevented Bcl-2 reduction after HI. Bcl-2 in the cytosolic fraction from cortical tissue, both from ipsilateral and contralateral hemispheres at 24 h after HI, was prevented from downregulation by calpain inhibition (CX295) in the ipsilateral hemisphere after HI. The graph shows the relative amount of Bcl-2 remaining in the ipsilateral compared with the contralateral hemisphere. Actin was used as a loading control ($n = 8$ for each group).

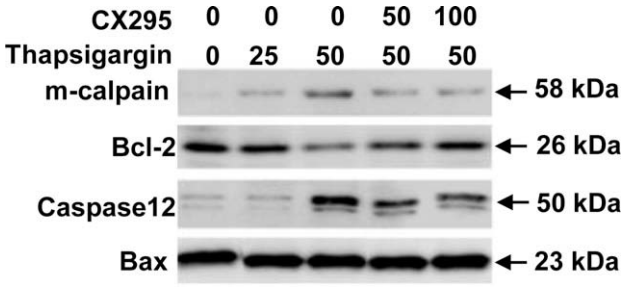

Fig. 7. Calpain inhibition prevented loss of Bcl-2 and decreased caspase-12 activation in IMR-32 cells after ionophore (thapsigargin) treatment. Expression of the active 58 kDa m-calpain increased with ionophore stimulation and decreased with concurrent calpain inhibition. The total level of Bcl-2 decreased with increasing calpain activation and returned towards normal levels with increasing calpain inhibition. The expression of caspase-12 in the cytosolic fraction increased with calpain activation and decreased with calpain inhibition. The total levels of Bax did not change after calpain activation.

4. Discussion

Previous studies have demonstrated that the major anti-apoptotic Bcl-2 is expressed in the brain and that its expression levels can regulate cell death following ischemic injury (Martinou et al., 1994). It has been shown that Bcl-2 is localized on the outer mitochondrial membrane, is highly expressed in the brain during development and subsequently downregulated (Soane et al., 2008). Bcl-2 acts by inhibiting pro-apoptotic Bcl-2 family member, Bax, through heterodimerization (Sedlak et al., 1995). Bax is widely expressed in the brain and Bax deficiency attenuated neonatal HI brain injury (Gibson et al., 2001). Together, these and other studies indicate that Bcl-2 family proteins play a crucial role in the regulation of apoptotic signal transduction.

The expression of Bcl-2 and Bax after neonatal cerebral HI was investigated by Ferrer and coworkers, using immunohistochemistry and immunoblotting of whole brain homogenates, but they found only slight changes in Bcl-2 at 12 and 24 h after the injury (Ferrer et al., 1997). This led them to conclude that these proteins did not play a major role in determining the fate of the cells. In an adult model of cerebral focal ischemia (Chen et al., 1995), Bcl-2 was found to be upregulated and located in the cytoplasmic (non-nuclear) compartment 24 h after the insult, in cells sublethally injured in non-infarcted areas or just outside the border of the infarct. Within the infarct, they found only endothelial cells showing Bcl-2 immunoreactivity. Control animals did not exhibit any expression of Bcl-2. In this neonatal model, we detected positive cells also within the injured area after 24 h, in contrast to results from the adult ischemia model (Chen et al., 1995). However, the intracellular location had changed from diffusely cytoplasmic to nuclear. The increased expression of Bcl-2 in the injured area may reflect an adaptive response of surviving neurons to an ischemic insult. In accordance with the results from the adult model, clearly positive endothelial cells could also be found exclusively within the injured area.

Our findings, demonstrating a translocation of Bcl-2 to neuronal nuclei in damaged areas after HI, support the notion that Bcl-2 is involved in mechanisms other than those based on direct interaction with Bax. The translocation was particularly apparent at 24 h after HI, a time point at which the caspase-3 activity has reached its maximum in this paradigm (Blomgren et al., 2001; Wang et al., 2001). At this time point the bulk of Bcl-2 in injured areas appeared to be concentrated to nuclei. The physiological impact of the intracellular redistribution of Bcl-2 after neonatal cerebral HI is unclear. One hypothesis is that it is involved in regulating nuclear transport (Krajewski et al., 1993). An injured cell might respond by sending a number of "SOS" signals to the nucleus. If it is possible to rescue the cell, transcription factors (for example Hypoxia-Inducible Factor, HIF) would need to reach their target genes to activate genes coding for "first aid"-proteins and for this to occur, functional nuclear pores must exist. Possibly, Bcl-2 could, through its ability to interact with other proteins, act as a bait to attract and escort them to the nuclear gate. Alternatively, Bcl-2 gating the nuclear pores might *prevent* traffic from cytosol to nucleus, an observation previously made in an over-expression study, where Bcl-2 prevented the nuclear translocation of the apoptosis-inducing transcription factor p53 (Beham et al., 1997). We used subcellular fractionation to elucidate the expression and possible translocation of Bcl-2. We found that the levels of Bcl-2 in homogenates were decreased in the ipsilateral compared with contralateral hemispheres at early recovery post-HI, but not at 24 h post-HI. In the mitochondrial fraction, the decrease was more pronounced, but delayed, such that the difference between the ipsi- and contralateral hemispheres was greatest at 24 h post-HI. The soluble, cytosolic fraction contained a smaller amount of Bcl-2 than the particular fractions, which facilitated quantification after immunoblotting. The nature of the heavier band, migrating at an apparent molecular mass of 28 kDa, detected primarily in the soluble fraction, is not clear. It might represent post-translational modification of Bcl-2, such as phosphorylation (Hallin et al., 2006). Apparently, early after HI, Bcl-2 is translocated from mitochondria to the cytosol and to nuclei. Later during reperfusion, nuclear translocation of Bcl-2 continues, whereas cytosolic Bcl-2 is reduced. Nuclear translocation was demonstrated using immunohistochemistry and immunoelectron microscopy. We tried to demonstrate nuclear translocation also in nuclear fractions by using immunoblotting, but we could not obtain pure nuclear fractions, free from mitochondrial contamination (not shown). We did succeed in producing nuclear fractions from adult brains without mitochondrial contamination, but not from immature brains. We speculate that there is a pool of mitochondria closely associated with the nuclei in the immature brain, but this remains to be shown. For the same reasons, we have been unable in other studies to produce immunoblots demonstrating nuclear translocation of AIF and therefore had to settle for immunoblots showing decreased levels of AIF in mitochondrial fractions, plus immunohistochemical evidence of nuclear translocation (Zhu et al., 2003, 2007, 2005).

Calpains are cysteine proteases and have been demonstrated to be involved in neuronal cell death together with caspase-3

(Blomgren et al., 2001). One possible mechanism is calpain-mediated, ischemia-induced cleavage of procaspase-12 or the loop of Bcl-xL, demonstrating cross-talk between the calpain and caspase families during neuronal cell death (Nakagawa and Yuan, 2000). Calpain-induced cleavage of Bax has been shown earlier (Wood and Newcomb, 2000; Wood et al., 1998), but there are very few reports demonstrating calpain-dependent cleavage of Bcl-2 (Gil-Parrado et al., 2002). Our *in vitro* experiments revealed calpain-dependent reduction of Bcl-2. The results may be interpreted such that a massive influx of calcium, similar to the conditions in the core of an ischemic infarct, promoting necrotic cell death, will "short-circuit" apoptotic mechanisms. This line of reasoning was suggested earlier by Lankiewicz et al. reporting calpain-dependent cleavage and inactivation of Apaf-1 (Lankiewicz et al., 2000). Concurrently, calpain-dependent release of caspase-12 (Nakagawa and Yuan, 2000) or partial cleavage and enhanced activation of caspase-3 (Blomgren et al., 2001) may also contribute to the cross-talk between excitotoxic and apoptotic mechanisms. Downregulation, for example through proteolytic degradation, of the anti-apoptotic Bcl-2 may be interpreted as an injurious mechanism, promoting apoptotic cell death, provided that the cleavage products are inactive or even pro-apoptotic. The physiological implication of this finding was confirmed by *in vivo* administration of the calpain inhibitor, CX295, which prevented downregulation of Bcl-2 after HI. Earlier we have shown that CX295 treatment could, at least partially, prevent activation of caspase-3 after HI (Blomgren et al., 2001).

In summary, we demonstrate calpain-dependent reduction of Bcl-2 as well as nuclear translocation of this protein after HI in the immature brain. These changes might be of importance for the life/death decision in the cell.

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