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Research report

Co-involvement of mitochondria and endoplasmic reticulum in regulation of apoptosis: changes in cytochrome *c*, Bcl-2 and Bax in the hippocampus of aluminum-treated rabbits

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Abstract

Neurodegenerative diseases, including Alzheimer's disease, are characterized by a progressive and selective loss of neurons. Apoptosis under mitochondrial control has been implicated in this neuronal death process, involving the release of cytochrome *c* into the cytoplasm and initiation of the apoptosis cascade. However, a growing body of evidence suggests an active role for the endoplasmic reticulum in regulating apoptosis, either independent of mitochondrial, or in concert with mitochondrial-initiated pathways. Members of the Bcl-2 family of proteins have been shown to either inhibit apoptosis, as is the case with Bcl-2, or to promote it, in the case of Bax. Investigations in our laboratory have focused on neuronal injury resulting from the intracisternal administration of aluminum maltolate to New Zealand white rabbits, an animal system relevant to a study of human disease in that it reflects many of the histological and biochemical changes associated with Alzheimer's disease. Here we report that treatment of young adult rabbits with aluminum maltolate induces both cytochrome *c* translocation into brain cytosol, and caspase-3 activation. Furthermore, as assessed by Western blot analysis, these effects are accompanied by a decrease in Bcl-2 and an increase in Bax reactivity in the endoplasmic reticulum.

Theme: Disorder of the nervous system

Topic: Neurotoxicity

Keywords: Bcl-2; Bax; Mitochondria; Endoplasmic reticulum; Alzheimer's disease; Aluminum

1. Introduction

Apoptosis, which plays a critical role in the normal development and maintenance of tissue homeostasis, plays an important role in neurodegenerative diseases and aging [26]. Alzheimer's disease, a common neurodegenerative disorder, is characterized typically by intraneuronal neurofibrillary tangles, neuritic plaques and selective neuronal death, with evidence of apoptosis being observed as an early event preceding the formation of these classical neuropathological features [32]. Mitochondrial changes following cytotoxic stimuli represent a primary event in apoptotic cell death, since the apoptogenic factor, cytochrome *c*, is released into the cytoplasm [2,15,20,21]. Once this translocation occurs, cytochrome *c* binds to another cytoplasmic factor, Apaf-1, and the formed complex activates the initiator caspase-9 that in turn activates the effector caspases, of which caspase-3 is a prominent member [17,31]. Release of cytochrome *c* from the mitochondria has been shown to involve two distinct pathways. One implicates the opening of the mitochondria permeability transition pore (MTP), and the second, trig-

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gered by the proapoptogenic Bax, is independent of the MTP opening [6]. While Bax has been shown to trigger cell death [7,35], the antiapoptotic Bcl-2 can block cytochrome *c* release and caspase activation [1,25]. Bcl-2 resides in the mitochondria and prevents activation of the effector caspases by mechanisms such as blockade of the MTP opening [19,30], or by functioning as a docking protein [24].

Although Bcl-2 may have a direct action on the mitochondria, it also resides in the endoplasmic reticulum [4,36]. A growing body of evidence suggests an active role for the endoplasmic reticulum in the regulation of apoptosis. Indeed, stress in the endoplasmic reticulum has been shown to induce apoptosis [34] and, furthermore, the endoplasmic reticulum induces activation of caspase-12, an effect not triggered by mitochondrial stress [22]. Recently, it has been reported that the drug brefeldin induces endoplasmic reticulum dilatation and leads to cytochrome *c* release and caspase-3 activation [8]. This effect was blocked by the wild-type Bcl-2 and, surprisingly, a Bcl-2 variant that is exclusively targeted to the endoplasmic reticulum, was also able to accomplish the same task [8]. The authors suggested the existence of 'cross-talk' under Bcl-2 control between the endoplasmic reticulum and mitochondria, and that Bcl-2 may exert its protective effect by controlling calcium homeostasis in the different cell compartments.

Studies in our laboratory have focused on neuronal injury resulting from the intracisternal administration of aluminum (Al) maltolate to New Zealand white rabbits. This animal system is relevant to a study of human disease in that it reflects many of the histological and biochemical changes associated with Alzheimer's disease [10]. In the present study we have investigated the effect of Al maltolate-induced neurotoxic injury on cytochrome *c* release and caspase-3 activation in hippocampus of young adult rabbits. Changes in Bcl-2 and Bax responses in mitochondria and endoplasmic reticulum were further assessed to determine whether cross-talk between these intracellular organelles is also involved in the control of programmed cell death in this animal system.

2. Material and methods

2.1. Animals and treatment

All animal procedures were in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the University of Virginia Animal Care and Use Committee. Young adult (8–12 months old) female New Zealand white rabbits received either intracisternal injections of 100 μl saline (controls, $n=6$) or 100 μl of 50 mM Al maltolate in saline (treated, $n=6$). Al maltolate is soluble at neutral pH, thus avoiding precipitation of insoluble Al hydroxide as encountered with most of other Al compounds used for neurotoxicological studies [29]. The injection was carried out under ketamine anesthesia according to the method described previously [27]. Al-treated animals and matched controls were sacrificed on days 2 or 3, depending on the time required for the development of severe neurological symptoms in the Al-treated group. Rabbits were euthanized and then perfused with Dulbecco's phosphate-buffered saline (PBS, Gibco, Grand Island, NY) as described previously [10,11,28]. Brains were immediately removed after sacrifice, and a coronal section cut and bisected to yield two symmetrical hippocampal segments, one for immunohistochemistry and the other for immunoblot analysis. The respective sides chosen for these studies were alternated between successive animals. Each brain hemisphere intended for histology was immediately frozen on a liquid nitrogen-cooled surface, placed into a zipper-closure plastic bag, and buried in dry ice pellets until transferring to −80°C before sectioning. For immunoblot analysis, tissue from the hippocampus was rapidly dissected, homogenized and subjected to ultracentrifugation as described below.

2.2. Western blot analysis

Proteins from the mitochondrial, cytosolic and endoplasmic fractions were extracted as described previously [18]. Approximately 100 mg of brain tissue from hippocampus was gently homogenized, using a teflon homogenizer (Thomas, Philadelphia PA), in seven volumes of cold suspension buffer (20 mM Hepes–KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin and 12.5 μg/ml of *N*-acetyl-Leu-Leu-Norleu-Al). The homogenates were first centrifuged at 750×*g* at 4°C for 5 min, and then at 8000×*g* for 20 min at 4°C. The 8000×*g* pellets were resuspended in cold buffer without sucrose and used as the mitochondrial fraction. The supernatant was further centrifuged at 100 000×*g* for 60 min at 4°C to separate the cytosolic from the endoplasmic reticulum fractions. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, IL). Proteins (7.5 μg) from the mitochondrial, cytosolic and endoplasmic fractions were separated by SDS–PAGE (15% gel) under reducing conditions followed by transfer to a polyvinylidene difluoride membrane (Millipore) at 30 mA for 210 min in transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). Following transfer, membranes were incubated with mouse monoclonal antibody (mAb) to human cytochrome *c* (Pharmingen, San Diego, CA) at a 1:250 dilution, or to a 1:100 dilution of mAbs recognizing either Bcl-2 or Bax (Santa Cruz Biotechnology, CA). Cytochrome oxidase subunit IV (COX) mAb obtained

from a commercial source (Molecular Probes, Eugene, OR) was used as a marker of mitochondrial contamination at 1:1000 dilution. A calnexin mAb (Transduction Laboratories, Lexington, MD) was used at 1:500 dilution as an endoplasmic reticulum marker, and a mAb to anti-β actin (Sigma, St. Louis, MO) was used at a 1:250 dilution as a gel loading control. Following washes with Tris-buffered saline (TBS) containing 0.1% Triton X-100, the blots were developed using enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA). The bands of cytochrome *c*, Bax and Bcl-2 which had been developed on radiographic film, were scanned and densitometrically analyzed using Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA), and these quantitative analyses were expressed as mean±S.E.M. values. Unpaired Student's *t*-test was used to compare levels of each protein between controls and Al-treated groups in the same subcellular fraction.

2.3. Fluorometric assay of caspase-3 activities

Lysates were prepared by homogenizing hippocampal tissue in 20 mM Hepes–KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF. Lysates were centrifuged for 30 min at 160 000×*g* and proteins in the supernatant were quantified using the Bradford method. Lysates (50 μg protein) were incubated for 1 h at 37°C in 1 ml of 1× Hepes buffer containing 10 μl of the fluorogenic substrate Ac-DEVD-AMC (Caspase-3 assay kit, Pharmingen, San Diego, CA). Cleavage of the substrate was monitored at an excitation wavelength of 380 nm and emission wavelength of 440 nm using a Model 450 fluorometer (BioMolecular). Each sample was incubated with or without 10 μl of the caspase-3 inhibitor Ac-DEVD-CHO (Pharmingen, Saint Diego, CA). Caspase-3 activity for each sample was calculated as the difference between the rate of cleavage in the absence and presence of the inhibitor.

2.4. Immunohistochemistry

Serial 14-μm thick coronal frozen sections from control and Al-treated animals were cut at the level of the hippocampus and stored at −80°C prior to immunostaining. The sections were air-dried at room temperature, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in PBS and incubated with a blocking solution of 1.5% normal serum, also in PBS. Subsequently, sections were reacted overnight at 4°C with a mouse mAb against caspase-3 (CPP32, Transduction Laboratories, Lexington, KY) at a 1:500 dilution. After washing with 50 mM TBS, and incubating with the biotinylated secondary antibody, sections were processed with a Vectastain Elite avidin–biotin complex technique kit (Vector Laboratories, Burlingame, CA) and visualized by 3,3′-diaminobenzidine/hydrogen peroxide, with light hematoxylin counterstaining. All procedures were performed at room temperature unless otherwise noted.

3. Results

3.1. Western blot analysis

To confirm the purity of the subcellular fractions, we used antibodies against organelle-specific marker proteins; cytochrome *c* oxidase for mitochondria and calnexin for endoplasmic reticulum. As shown in Fig. 1, the fractions were pure, and the β-actin staining, used as a gel loading control, shows similar protein loading in all the wells.

The immunoreactivity of cytochrome *c* is evident as a single band with a molecular weight of 15 kDa. In controls, cytochrome *c* immunoreactivity is not detectable in the cytosolic fraction, but is strongly positive in the mitochondrial and endoplasmic fractions (Fig. 2A). In the Al-treated group, cytochrome *c* is distributed in the cytosolic, mitochondrial, and endoplasmic reticulum fractions (Fig. 2B).

The antibody to Bcl-2 identifies a protein band with an apparent molecular weight of 27 kDa. Bcl-2 is not detected in the cytoplasmic fractions, but relatively intense bands are found in the mitochondrial and endoplasmic fractions in controls (Fig. 2C). In the Al-treated group, Bcl-2 is restricted to the mitochondria and is barely detected in either the cytosolic or endoplasmic reticulum fractions (Fig. 2D). Bax, with an apparent molecular weight of 21 kDa, is distributed in all the fractions, but with a higher intensity in the cytosolic than in the mitochondrial or endoplasmic reticulum fractions (Fig. 2E). In the Al-

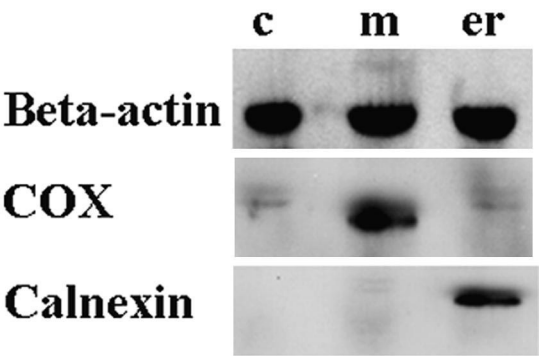

Fig. 1. Western blot analysis for β-actin, cytochrome *c* oxidase subunit IV (COX) and calnexin in cytoplasmic (c), mitochondrial (m) and endoplasmic reticulum (er) fractions from control hippocampal lysates. β-Actin, used as a control loading gel, shows similar protein loading in the mitochondrial, cytoplasmic and endoplasmic reticulum fractions. COX, used as a marker for mitochondrial contamination, is only present in the mitochondrial fractions. Calnexin, used as a marker for the endoplasmic reticulum, stains only the endoplasmic reticulum fractions.

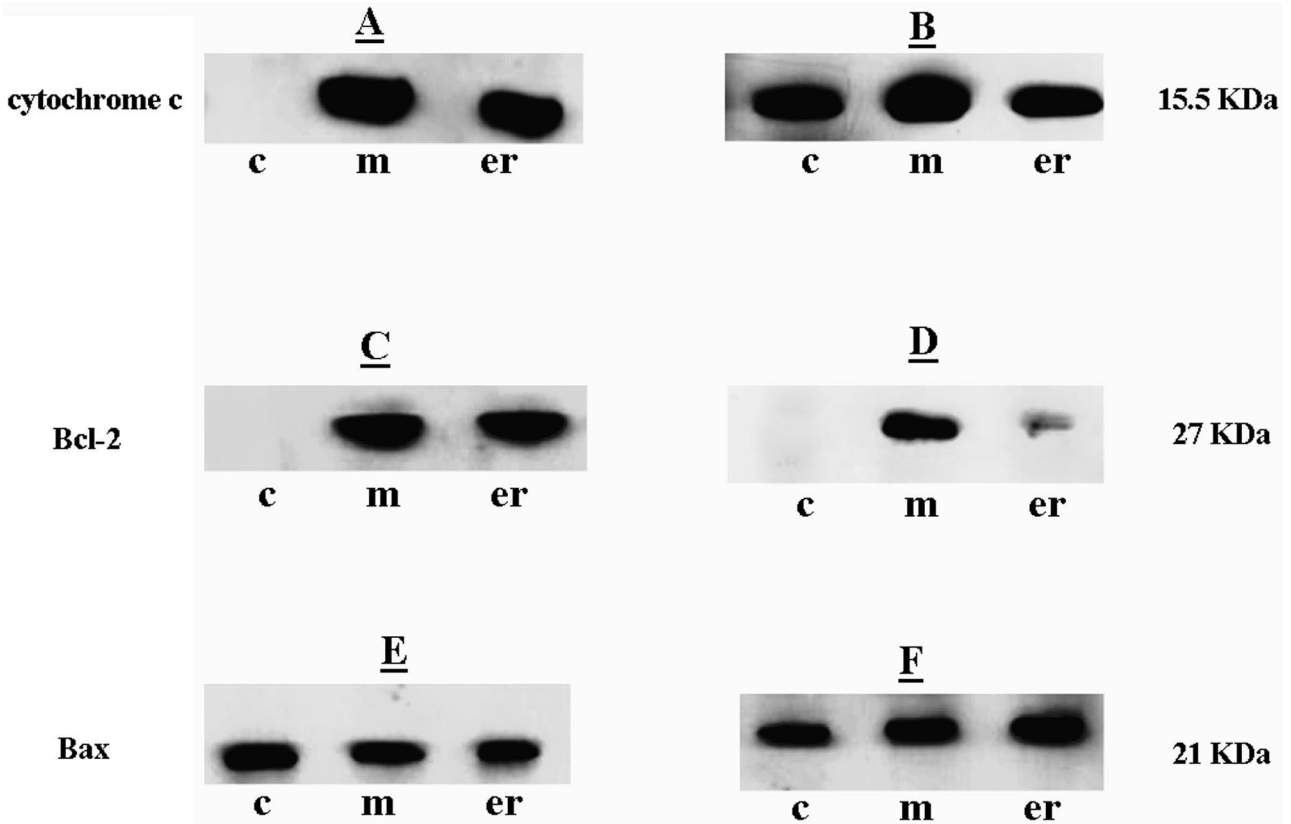

Fig. 2. Representative immunoblots for cytochrome *c*, Bcl-2 and Bax in controls (A,C,E) and in Al-treated groups (B,D,F) in cytoplasmic (c), mitochondrial (m) and endoplasmic reticulum (er) fractions from hippocampus lysates. (A) Cytochrome *c* immunoreactivity is not detected in the cytoplasm but is highly positive in the mitochondria and endoplasmic reticulum in controls. (B) Al treatment induces translocation of cytochrome *c* into the cytoplasm, and positive immunoreactivity for cytochrome *c* is also detected in the mitochondria and endoplasmic reticulum. (C) Bcl-2 staining, which is not detected in the cytoplasm, is highly positive in the mitochondrial and endoplasmic reticulum fractions. (D) Following Al treatment, Bcl-2 is decreased in the mitochondria and is barely detectable in the endoplasmic reticulum. (E) Bax is present in the cytoplasm as well as the mitochondria and endoplasmic reticulum in controls. (F) In the Al-treated group, Bax immunoreactivity is slightly decreased in the cytoplasm and increased in the mitochondria and endoplasmic reticulum.

treated animals, Bax immunoreactivity is detectable also in all the fractions; relative amounts are: endoplasmic reticulum>mitochondria>cytosol (Fig. 2F). The results of the densitometric analysis of cytosolic, mitochondrial and endoplasmic reticulum cytochrome *c*, Bcl-2 and Bax in controls and Al-treated rabbits, are shown in Table 1. Cytochrome *c* is absent in the cytoplasm of controls and its intensities in the other fractions are mitochondria> endoplasmic reticulum. Following Al treatment, cytochrome *c* is distributed as following, cytoplasm= mitochondria>endoplasmic reticulum. In controls, Bcl-2 is not present in the cytoplasmic fraction and levels in the other fractions are mitochondria>endoplasmic reticulum. In the Al-treated group, Bcl-2 is slightly decreased in the mitochondria and barely present in the endoplasmic reticulum. In controls, Bax is present in the three subcellular fractions as follows: cytoplasm>endoplasmic reticulum> mitochondria. Following the administration of Al maltolate the distribution of Bax is endoplasmic reticulm> mitochondria>cytoplasm.

3.2. Caspase-3 activity

The activity of caspase 3-like proteases as assessed by measuring the cleavage of the fluorogenic substrate Ac-DEVD-AMC, demonstrates significant elevations in Al-treated animals approaching 5-fold greater levels than in the controls (Fig. 3).

3.3. Immunohistochemistry

The immunohistochemical localization of caspase-3 in the pyramidal cell layer of the hippocampus of all animals was examined (Fig. 4A). No reaction for caspase-3 is observed in sections processed without incubation with primary antibody (data not shown), or in sections from untreated animals (Fig. 4B). In brains from Al-treated rabbits, there is marked positive immunostaining for caspase-3 in the pyramidal cell layers of the hippocampus (Fig. 4C, arrows).

Table 1
Densitometric scanning analysis of cytochrome *c*, Bcl-2 and Bax in the cytoplasm, mitochondria and endoplasmic reticulum of controls ($n=6$) and of Al-treated rabbits ($n=6$)

	Cytoplasm	Mitochondria	Endoplasmic reticulum
Cytochrome *c*			
Controls	–	2.0±0.15	1.60±0.18
Al-treated	1.75±0.16	1.75±0.22	0.85±0.11
Bcl-2			
Controls	–	1.50±0.10	1.29±0.16**
Al-treated	–	1.10±0.08	0.11±0.06**
Bax			
Controls	1.61±0.22*	0.72±0.13	1.02±0.08*
Al-treated	0.96±0.18*	1.00±0.10	1.42±0.23*

While cytochrome *c* is not detectable in the cytoplasm of controls, it is released into the cytosol following Al treatment. Bcl-2 resides in the mitochondria and endoplasmic reticulum in controls; Al treatment decreases Bcl-2 levels in the endoplasmic reticulum. Bax is distributed in the cytoplasm>endoplasmic reticulum>mitochondria in controls. Following Al administration, Bax is redistributed in the cytoplasm< mitochondria<endoplasmic reticulum.
–, undetectable levels. Data are expressed as mean±S.D. *$P<0.05$; **$P<0.01$ (Student's *t*-test).

4. Discussion

The current study provides evidence that neurotoxic injury, induced in rabbits by the intracisternal administration of Al-maltolate, results in cytoplasmic cytochrome *c* translocation, endoplasmic reticulum Bcl-2 down-regulation and Bax up-regulation, as well as caspase-3 activation. In a previous report from our laboratory [29] we presented immunohistochemical evidence for a similar decrease in the Bcl-2:Bax ratio, together with evidence of apoptosis, in aged rabbits treated with Al maltolate, but this effect was not observed in young adult animals. In the present study, by using a higher concentration of Al maltolate, we now report a dramatic effect following Al administration in young rabbits. There is considerable support for mitochondria playing a key role in the process of cell death, with much attention being focused on cytochrome *c*. When released into the cytoplasm, cytochrome *c* forms a complex with the cytosolic molecule Apaf-1 and activates caspase-9 [33,39]. Subsequently, this complex triggers the activation of effector caspases, in particular caspase-3 [17].

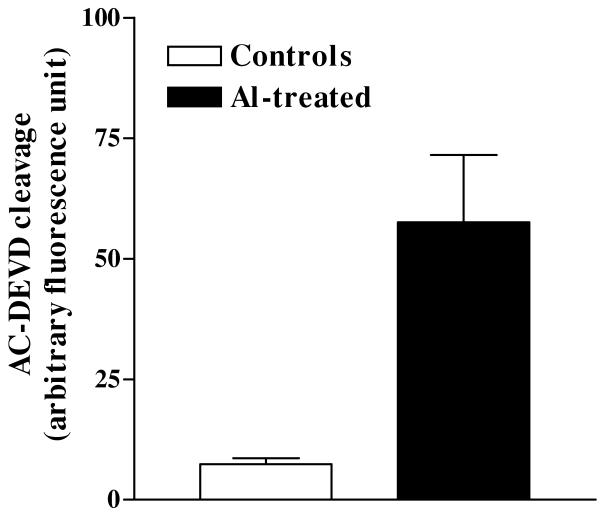

Fig. 3. Caspase-3 activity is shown in hippocampal lysates of controls (white bar) and Al-treated animals (black bar). In controls, low caspase-3 activity is detected. Al treatment induces a 5.5-fold increase in the caspase-3 activity. Data are presented as mean±S.E.M.

Release of cytochrome *c* from mitochondria into the cytosol has been reported following brain injury in mice [21] and addition of the the parkinsonian neurotoxin MPP+ to isolated brain mitochondria [2]. The mechanism by which cytochrome *c* is released from mitochondria to the cytosol is unclear. It has been suggested that following cytotoxic stimuli, the MTP opens and rapidly causes depolarization, uncoupling of oxidative phosphorylation, and subsequent pronounced mitochondrial swelling. Subsequent events are the diffusion of cytochrome *c* into the cytosol through the MTP from its location between the inner and outer mitochondrial membranes [19,38]. On the other hand, cytochrome *c* may also translocate from mitochondria into the cytosol but by a mechanism distinct from the opening of the MTP. Indeed, it has been shown that the pro-apoptotic Bax is able to trigger the release of cytochrome *c* from isolated mitochondria, and that this cytochrome *c* release is not blocked by inhibitors of the MTP opening [6]. Furthermore, it has been reported, in staurosporine-induced apoptosis of HeLa cells, that Bid, a BH3 domain-containing protein, translocates from the cytosol to mitochondria and binds to Bax. This direct binding of Bid to Bax is a prerequisite for Bax structural changes and subsequent cytochrome c release [5]. Our results show that following Al-maltolate treatment, Bax is redistributed to be present at higher levels in endoplasmic reticulum and mitochondria than in the cytosol. Changes in Bax distribution may then initiate cytochrome *c* release and caspase-3 activation. However, whether the cytochrome *c* release we report here results from opening of the MTP, or from an alternate pathway, remains to be determined.

Another interesting finding is the observation of decreased positivity of the anti-apoptotic protein, Bcl-2, in the endoplasmic reticulum following Al treatment. This protein is considered to be a key factor regulating apoptosis. It has been shown that Bcl-2-deficient mice underwent fulminant apoptosis of lymphoid tissue in the thymus and spleen, while mice overexpressing Bcl-2 demonstrated extended cell survival [13]. In other studies of cells treated with staurosporine, overexpression of Bcl-2 has been demonstrated to prevent the efflux of cytochrome *c* from the mitochondria and subsequent initiation of apoptosis [37]. In a cell-free apoptosis system, Bcl-2 over-expression has also been reported to prevent both the release of cytochrome c from mitochondria and activation of cas-

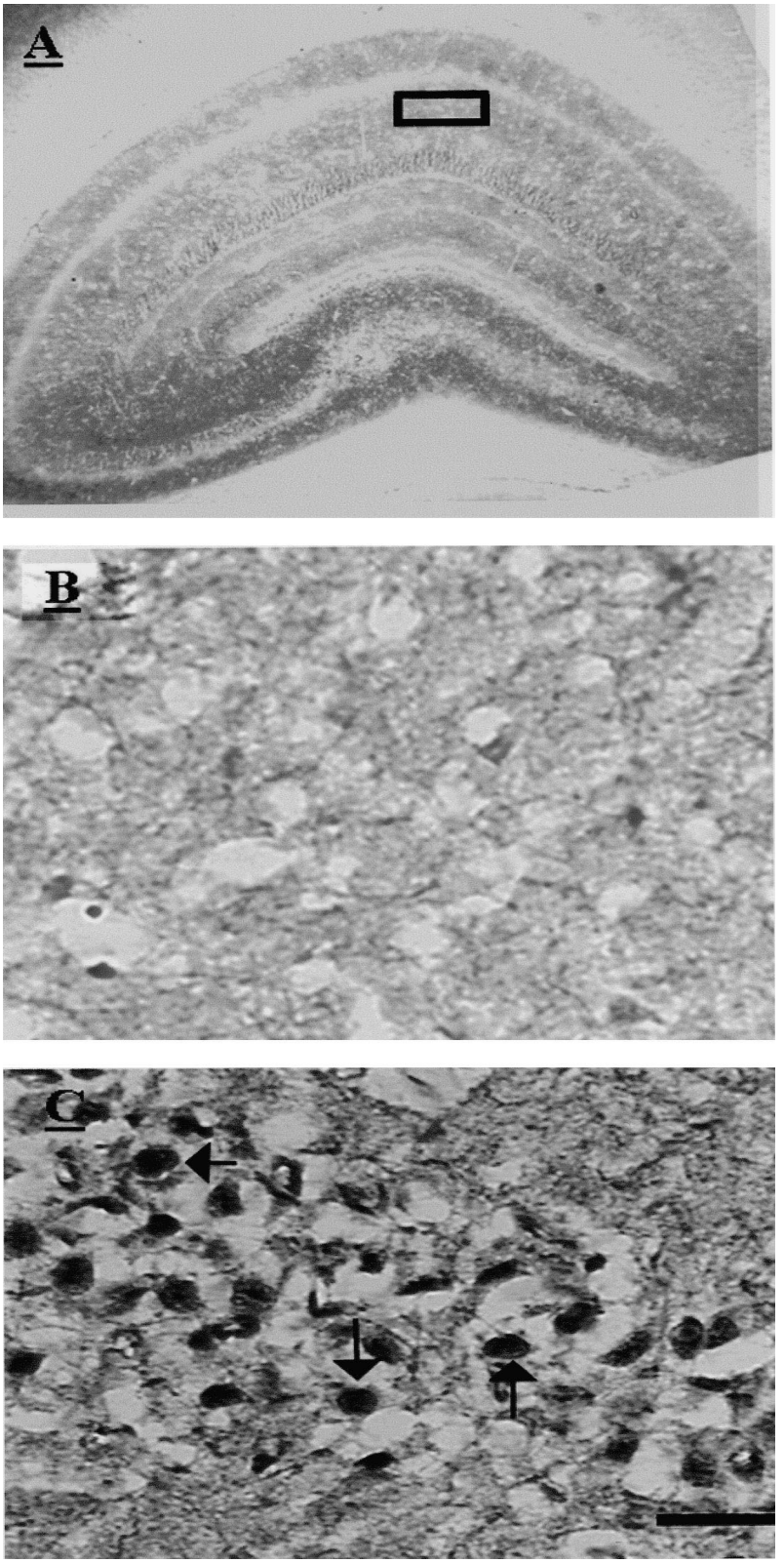

Fig. 4. Immunohistochemical localization of caspase-3 was examined in the pyramidal cell layer of the hippocampus (A, rectangular box). (B) Pyramidal cell layer of controls shows no immunoreactivity for caspase-3 (×400). (C) Positive immunoreactivity for caspase-3 (arrows) in the pyramidal cell layer in Al-treated animals (×400). Bar in (C)=25 μm.

pases [12]. Bcl-2 has also been found to prolong the life of rat neurons subjected to ischemia [3] in haloperidol-induced neuronal death in the hippocampus of mice [16], and in a transgenic mouse model of familial amyotrophic lateral sclerosis [14].

As we report here, the ratio of Bcl-2:Bax could be a key determining factor in the release of cytochrome *c*, the activation of caspase-3 and in the initiation of apoptosis, since these events are accompanied by an up-regulation of the pro-apoptotic Bax and a down-regulation of the anti-apoptotic Bcl-2. Furthermore, as the changes we observed for Bcl-2 and Bax mainly were in the endoplasmic reticulum, we suggest that the Bcl-2:Bax ratio in this cellular organelle regulates the extent of apoptosis. A decrease in this ratio may exacerbate apoptosis, and increasing this ratio may reverse the deleterious effect of cytotoxic stimuli.

The finding of alterations in cytochrome *c*, Bax and Bcl-2 in the present study raises the question as to whether the mitochondria or endoplasmic reticulum trigger apoptosis. Recent evidence now implicates the endoplasmic reticulum in this cell death pathway (for a review see Ref. [23]). In a recent paper, it has been demonstrated that cross-talk between mitochondria and endoplasmic reticulum is controlled by Bcl-2 [8]. Moreover, Bcl-2 is suggested to exert its anti-apoptotic effect by maintaining calcium homeostasis in the endoplasmic reticulum [9].

In conclusion, we have shown that Al maltolate induces cytochrome *c* release and caspase-3 activation in young adult rabbits. These effects are accompanied by a decrease of the anti-apoptotic Bcl-2 and an increase in the pro-apoptotic Bax in the endoplasmic reticulum, but the mechanism by which Al maltolate triggers these changes remains to be determined. Furthermore, whether cytochrome *c* release from mitochondria precedes changes in Bcl-2 and Bax in the endoplasmic reticulum, also remains to be ascertained. Nevertheless, our results demonstrate that in young adult rabbit brain, Al is able to trigger apoptosis via its action as a neurotoxin.

Acknowledgements

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