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Research report

Aβ(1-42) and aluminum induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of *gadd 153* and NF-κB

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Abstract

Apoptosis may represent a prominent form of neuronal death in chronic neurodegenerative disorders, such as Alzheimer's disease. Although apoptosis under mitochondrial control has received considerable attention, mechanisms used within the endoplasmic reticulum (ER) and nucleus in mediating apoptotic signals are not well understood. A growing body of evidence is emerging from different studies which suggests an active role for the ER in regulating apoptosis. Disturbances of ER function have been shown to trigger two different apoptotic pathways; one involves cross-talk with mitochondria and is regulated by the antiapoptotic Bcl-2, and the second is characterized by the activation of caspase-12. Also, stress in the ER has been suggested to result in the activation of a number of proteins, such as *gadd 153* and NF-κ, and in the downregulation of the antiapoptotic protein, Bcl-2. In the present study, the intracisternal injection in aged rabbits of either the neurotoxin aluminum maltolate or of Aβ(1-42), has been found to induce nuclear translocation of *gadd 153* and the inducible transcription factor, NF-κB. Translocation of these two proteins is accompanied by decreased levels of Bcl-2 in both the ER and the nucleus. Aluminum maltolate, but not Aβ, induces caspase-12 activation which is a mediator of ER-specific apoptosis; this is the first report of the in vivo activation of caspase-12. These findings indicate that the ER may play a role in regulating apoptosis in vivo, and could be of significance in the pathology of neurodegeneration and related disorders.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Endoplasmic reticulum; Aβ(1-42); Aluminum; *gadd 153*; Caspase-12; NF-κB

1. Introduction

Evidence is accumulating that apoptotic cell death is a major factor in human neurodegenerative disorders, including Alzheimer's disease, and that this event may precede the formation of intraneuronal neurofibrillary tangles and neuritic plaques [38]. Although mitochondrial alterations may represent an important step in the mechanisms underlying this neuronal cell death, mitochondria may not be the only cellular organelle which control neuronal loss in neurodegeneration. There is now evidence suggesting that the endoplasmic reticulum (ER) also may play an important role in regulating neuronal cell death, thereby raising the question of whether the cell death pathway in neurodegenerative disorders is triggered by mitochondria or by the ER [29,37]. However, studies of involvement of the ER in neuronal death have lagged behind those of mitochondria, and the role of the ER in the pathogenesis of neurodegenerative disorders should be considered. Indeed, in addition to its physiological role as a calcium and protein store, the ER is the site of localization for the presenilin-1 mutation which has been linked to the early

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onset of familial Alzheimer's disease [1]. The ER has also been identified as the site of formation of the peptide Aβ(1-42), which may be the earliest event to take place in Alzheimer's disease [11]. In addition, ER stress-inducing agents have been shown to activate the expression of various genes, such as those coding for the gene *gadd 153*, important in growth arrest and DNA damage-induction [30], and the inducible transcription factor, NF-κB [4,36]. Several lines of evidence suggest that NF-κB plays an important role in the survival of neurons by its translocation from the cytoplasm into the nucleus [3,24,27]. Moreover, members of the *Bcl-2* family of proteins, Bcl-2 and Bcl-X_L, reside in the ER, and it has been established that they are also important in determining cellular and neurological outcomes in neurodegenerative disorders (for review, see Ref. [8]). Recently, stress in the ER has been shown to result in a specific type of apoptosis, independent of mitochondrial-targeted apoptotic signals; this novel pathway is mediated by caspase-12 [23]. Procaspase-12 resides in the ER, and upon its activation by ER stress, is released into the cytoplasm in an active form (caspase-12), thus initiating apoptosis.

Previous studies in our laboratory have used the neurotoxin, aluminum (Al) maltolate, injected into rabbit brain as a means for investigating the mechanisms of neurodegeneration, since this system demonstrates cytoskeletal changes that share a number of biochemical similarities with those found in Alzheimer's disease [12,33]. Having observed these results with the highly neurotoxic Al maltolate, we now extend our studies to Aβ, which also has been demonstrated to be neurotoxic and is now considered to play a central role in the pathogenesis of Alzheimer's disease, a hypothesis supported by genetic, biochemical, histopathological and animal modeling data [35]. In the present experiments, carried out on aged male New Zealand White rabbits, we have examined Al maltolate-induced stress in the ER, as assessed by the activation of *gadd 153* and its translocation into the nucleus, and have compared the results to changes produced by the administration of Aβ(1-42). We have also examined the effect of these two treatment regimens on caspase-12 activation, Bcl-2 protein levels in ER and in the nuclear fractions, and on NF-κB translocation. Since the hippocampus is frequently involved in Alzheimer's disease, we have focused our studies on this area of the brain.

2. Materials and methods

2.1. Materials

Aβ(1-42) was obtained from American Peptide Company (Sunnyvale, CA). Mouse monoclonal antibody (mAb) specific to human *gadd 153* (B-3), Bcl-2 (C-2), and NF-κB (C-5), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-12 mAb was a gift from Dr Junying Yuan, Harvard Medical School, Boston, MA; β-actin and α-mouse IgG FITC were obtained from Sigma (St Louis, MO); and Cy3-conjugated goat anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Animals, treatment protocol and tissue collection

All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the 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. Aged (4.5–5 years old) male New Zealand White rabbits received either intracisternal injections of 100 μl normal saline ($n=5$; controls), 100 μl of 25 mM Al maltolate in saline ($n=6$; Al-treated group), or 100 μl of 1 mg/ml Aβ(1-42) in saline ($n=7$; Aβ-treated group). Aggregate Aβ(1-42) was prepared by incubating freshly solubilized Aβ(1-42) at a concentration of 1 mg/ml in saline at 37°C for 3 days. The injections were carried out under ketamine anesthesia as described previously [34]. All rabbits were euthanized after 15 days, at which time the Al-treated animals had developed severe neurological symptoms, requiring their sacrifice, including forward head tilt, hemiplegic gait, loss of appetite, splaying of the extremities and paralysis. The controls and Aβ(1-42) animals did not display clinical symptoms. At the time of sacrifice the rabbits were perfused with Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY), also as described previously [34]. Brains were immediately removed, and a coronal section cut and bisected to yield two symmetrical hippocampal segments, one for immunohistochemistry and the other for immunoblot analysis. Each brain hemisphere intended for histochemistry was immediately frozen rapidly on a liquid nitrogen-cooled surface, placed into a zipper-closure plastic bag, and buried in dry ice pellets until transfer to −80°C for storage before sectioning. For immunoblot analysis, tissue from the hippocampus was rapidly dissected, homogenized and processed as described below.

2.3. Western blot analysis

Proteins from the subcellular fractions were extracted as described previously [19]. Approximately 50 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 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin and 12.5 mg/ml of *N*-acetyl-Leu-Leu-Norleu-Al). The homogenates were centrifuged at 900×*g* at 4°C for 10 min to isolate the nuclear fraction, and then at 8000×*g* for 20

min at 4°C to separate the mitochondrial fraction from the soluble fraction. The supernatant was further centrifuged at 100 000×*g* for 60 min at 4°C to separate the cytoplasmic from the ER fractions. We examined changes of proteins in the subcellular fractions where they are reported to be localized and/or translocated; *gadd 153* and NF-κB in the cytoplasmic and nuclear fractions, Bcl-2 in the endoplasmic reticulum and nuclear fractions, and caspase-12 in cytoplasmic fractions. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, IL). Proteins (7.5 μg) were separated by SDS–PAGE (15% gel) under reducing conditions, followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) at 300 mA for 210 min in transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). Following transfer, membranes were incubated overnight at 4°C with mouse mAb to human *gadd 153* and NF-κB at a 1:250 dilution, caspase-12 at 1:10, and Bcl-2 at 1:100. β-Actin was used at a 1:500 dilution as a gel-loading control. The blots were developed with enhanced chemiluminescence (Immun-Star detection kit, Bio-Rad, Hercules, CA). The bands of *gadd 153*, caspase-12, Bcl-2 and NF-κB 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. The unpaired Student's *t*-test was used to compare levels of each protein between the controls and Al-treated or Aβ(1-42)-treated animals in the same subcellular fraction.

2.4. Immunohistochemistry

Serial 14-μm-thick coronal frozen sections from control, Al-treated, and Aβ(1-42)-treated animals were cut at the level of the mid-hippocampus and stored under dessicant 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 at a 1:250 dilution against *gadd 153* or NF-κB. 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). Immunostaining for *gadd 153* was visualized by the use of Vector SG substrate and counterstained with a Nuclear Fast Red counterstain kit (Vector Laboratories, Burlingame, CA). Sections incubated with NF-κB antibody were visualized with DAB Chromogen (Dako Corporation, Carpinteria, CA) and counterstained with methyl green (Sigma, St Louis, MO). For negative controls, using similar sections, normal saline was substituted for the monoclonal antibody.

For double labeling of NF-kB and *gadd 153*, frozen coronal brain sections (14 μm thick) from the hippocampal level of controls, Al-treated and Aβ(1-42)-treated animals were dried for 15 min at room temperature (RT) and fixed in 10% formalin for 15 min, followed by a 10 min incubation in 1:2 vol/vol ethanol/acetic acid. Sections were washed three times in PBS for 5 min each and permeabilised with 0.3% Triton X-100 for 20 min at RT. Sections were then washed three times in PBS buffer for 5 min each, blocked with 2% goat serum and incubated for 2 h at 37°C in a 1:200 dilution of the *gadd 153* mouse mAb. Sections were then washed three times in PBS for 5 min and incubated for 2 h at 37°C in a 1:500 dilution of the Cy3-conjugated goat anti-mouse IgG. Sections were washed in PBS buffer and then in distilled H_2O, blocked with 2% goat serum and incubated for 2 h at 37°C in a 1:250 dilution of NF-κB mouse mAb, then incubated for 2 h at 37°C in a 1:250 dilution of α-mouse IgG FITC. After 3×5 min washes in PBS and a 5-min wash in distilled H_2O, sections were coverslipped and examined with a fluorescence Olympus BH2 microscope (Melville, NY), using Image Pro Plus 4.1 analysis software (Media Cybernetics, Baltimore, MD).

3. Results

3.1. Western blot analysis

As shown in Fig. 1A and Table 1, *gadd 153* (~30 kDa) is found only in the cytoplasmic fraction in control animals, but is present in the cytoplasmic and nuclear fractions in the Al- and Aβ(1-42)-treated groups. Caspase-12 (~60 kDa) is detected in the cytoplasmic fractions from Al-treated rabbits but is not expressed in the cytoplasmic fractions from controls (Fig. 1B and Table 1). A very faint band corresponding to caspase-12 is seen in the Aβ-treated animals (Fig. 1B), but its significance is not conclusive. Bcl-2 can be identified by a protein band having an apparent molecular weight of 27 kDa, both in the ER and nuclear fractions in controls (Fig. 1C). In comparison to these control animals, Al maltolate and Aβ(1-42) administration induce a decrease in Bcl-2 levels, both in the ER and nuclear fractions (Fig. 1C and Table 1). Two bands are detected for NF-κB (Fig. 1D), corresponding to p52 and p100; these are observed only in the cytoplasmic fraction of controls, with no bands being detected in the nuclear fraction. In the Al maltolate and Aβ(1-42)-treated animals, the two bands corresponding to p52 and p100 are also detected in the cytoplasmic fractions; in these animals there is an intense band in the nuclear fraction, corresponding to p52, and a lighter band for p100 (Table 1).

3.2. Immunohistochemistry

The immunohistochemical localization of NF-κB and

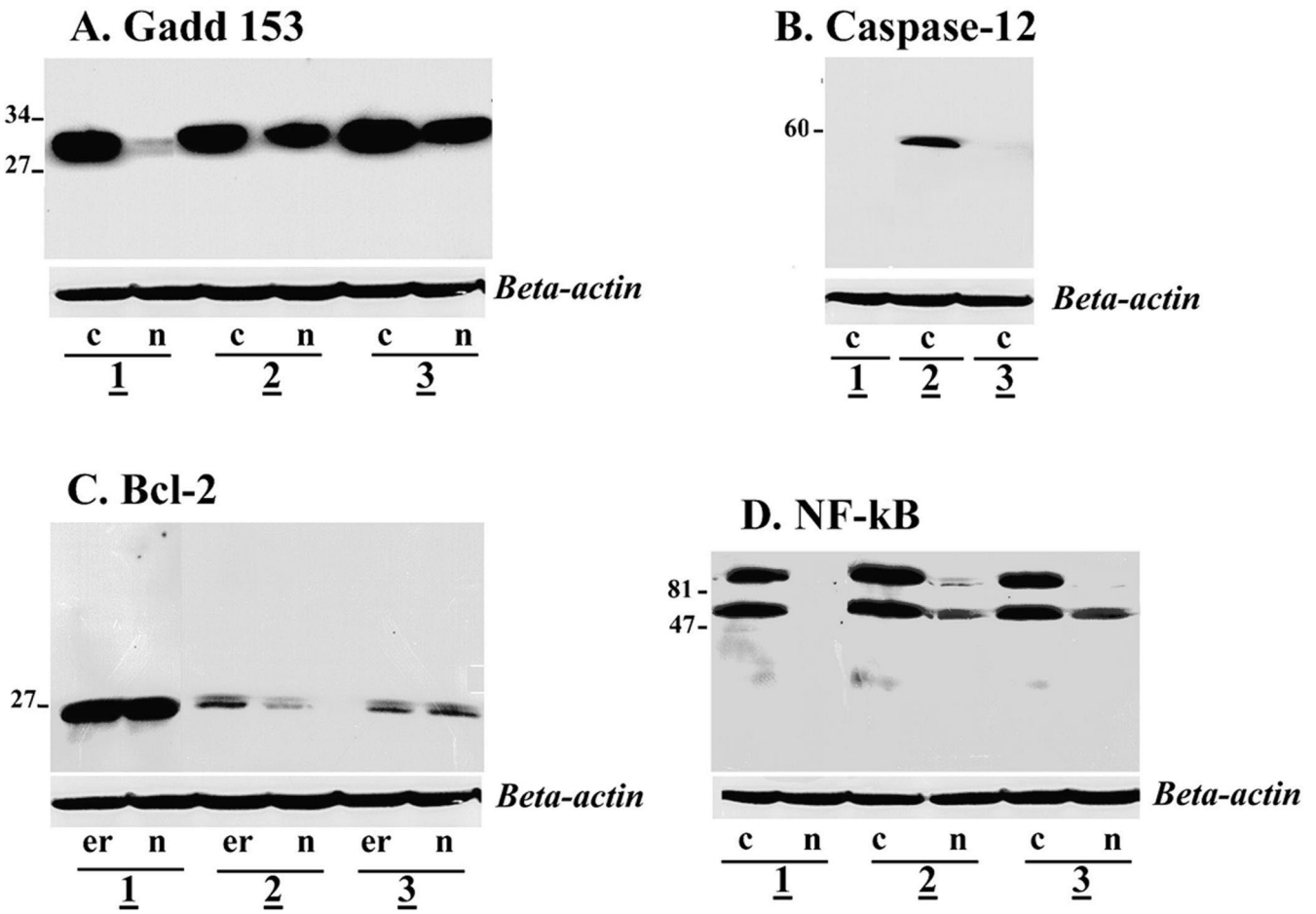

Fig. 1. Western blot of *gadd 153* (A), caspase-12 (B), Bcl-2 (C) and NF-κB (D), in cytoplasmic (c), nuclear (n), or endoplasmic reticulum (er), fractions from controls (1), and Al-treated (2) or Aβ(1-42)-treated (3) animals. (A) In controls (1), *gadd 153* is detected in the cytoplasmic, but not in the nuclear, fraction. Following Al (2) or Aβ(1-42) (3) administration, *gadd 153* is present in the cytoplasmic fractions and also in the nuclear fractions. (B) Caspase-12 is expressed only in the cytoplasmic fraction of Al-treated animal (2), and not in control (1) or Aβ(1-42)-treated (3) animals. (C) Bcl-2 staining is intense in both the endoplasmic reticulum and nuclear fractions from controls (1). The staining for Bcl-2 is strongly reduced in both the endoplasmic reticulum and nucleus with Al (2) or Aβ (3). (D) In the cytoplasmic fraction of controls (1), NF-κB is present as a dimer, the two bands corresponding to p52 and p100. Following Al (2) or Aβ(1-42) (3) administration, respectively, p52 and p100 are detectable in the cytoplasmic fractions; in the nuclear fraction, while p100 is barely detectable, p52 is present in abundance.

Table 1
Densitometric analysis of *gadd 153*, caspase-12, Bcl-2, and NF-κB blots in control ($n=5$), Al-treated ($n=6$), and Aβ(1-42) ($n=7$)-treated rabbits

		Cytoplasm		ER	Nucleus	
gadd 153	Controls	3.17±0.23			–	
	Aluminium	2.98±0.33			1.60±0.18	
	A-β(1-42)	2.85±0.11			1.75±0.16	
Caspase-12	Controls	–				
	Aluminium	1.12±0.12				
	A-β(1-42)	±				
Bcl-2	Controls			2.50±0.11	2.29±0.22	
	Aluminium			0.82±0.17**	0.75±0.12**	
	A-β(1-42)			0.91±0.18**	0.78±0.26**	
		p52	p100		p52	p100
NF-κB	Controls	1.61±0.22	1.48±0.22		–	–
	Aluminium	1.78±0.10	1.88±0.13		0.66±0.11	0.24±0.09
	A-β(1-42)	1.66±0.18	1.60±0.18		0.72±0.13	0.16±0.08

–, undetectable levels; ±, barely detectable; empty columns, not measured.
**$P<0.01$ (Student's *t*-test, in comparison to controls).

gadd 153 in the pyramidal cell layer of the hippocampus (CA1) of all animals has been examined (Fig. 2A).

In sections from control rabbits, no staining for NF-κB is present (Fig. 2B) but minimal cytoplasmic staining for *gadd 153* is noted (Fig. 2C). However, in sections from Al-treated (Fig. 2D,F) and Aβ(1-42)-treated animals (Fig.

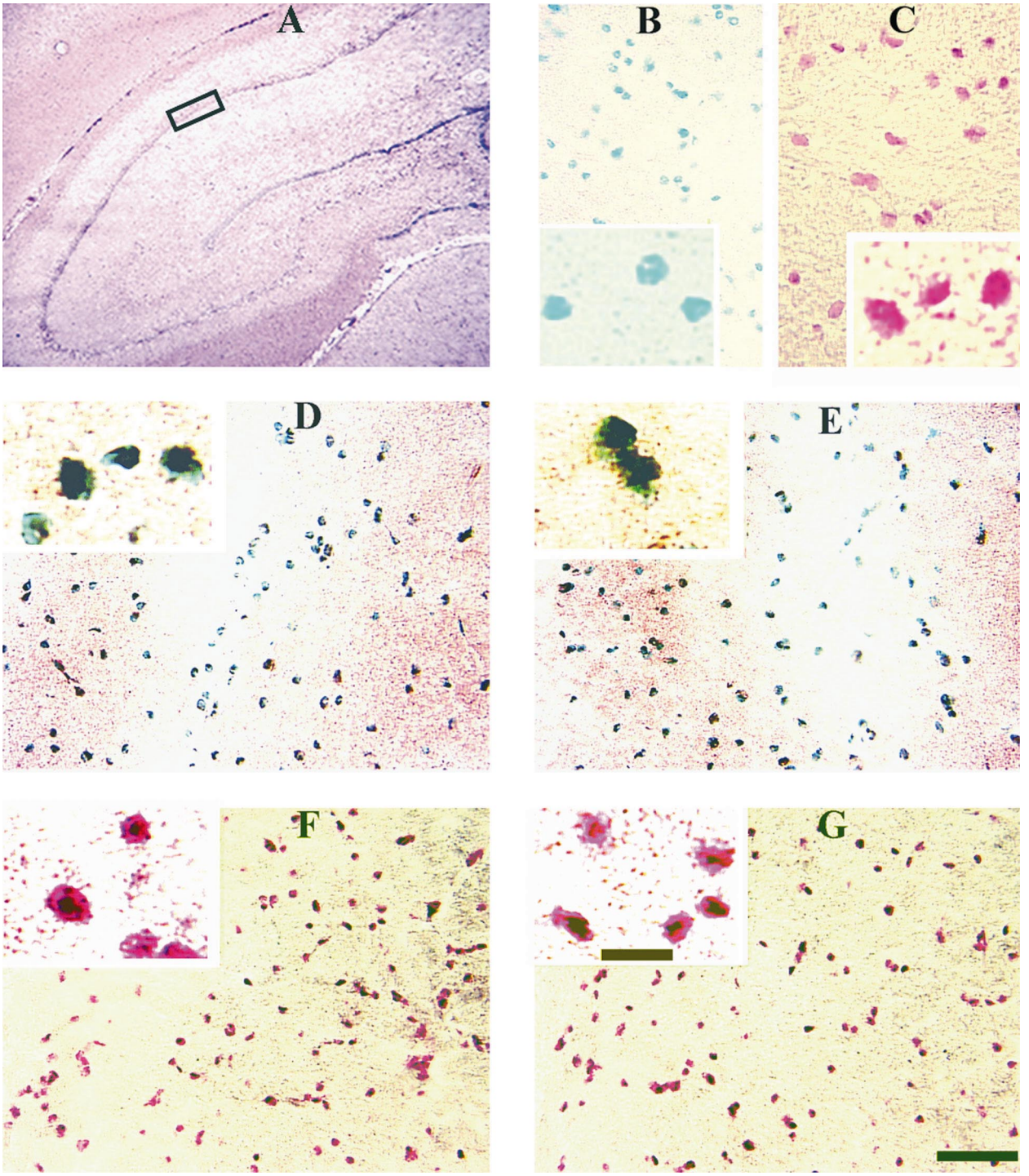

Fig. 2. Photomicrographs of labeling in the CA1 pyramidal cell layer of the hippocampus (A) for NF-κB (B, D and E) and *gadd 153* (C, F and G) in controls, Al-treated and Aβ(1-42)-treated animals. Control sections are immunostained with NF-κB (B) or *gadd 153* (C) and demonstrate negativity for NF-κB but scanty cytoplasmic positivity for *gadd 153*. Positive (brown-black) reactivity for NF-κB is found in the nucleus in sections from Al-treated (D) or Aβ(1-42)-treated (E) animals (methyl green nuclear counterstain), with some cytoplasmic positivity in both. *gadd 153* antibody also demonstrates nuclear positivity (deep-purple staining) in Al-treated (F) and Aβ(1-42)-treated (G) animals (nuclear fast red counterstain), with lesser cytoplasmic staining in both groups. Insets in B–G are of cells at higher magnification but from their respective low magnification fields. Scale bars: in B–G, 50 μm; in B–G insets, 10 μm.

2E,G), enhanced positive immunostaining for *gadd 153* (brown-black reaction product) and NF-κB (purple reaction product) are found in association with the nuclei of most neurons, as well as variable cytoplasmic positivity.

Using fluorescent microscopy, sections from control animals labeled for NF-κB (Fig. 3A) and *gadd 153* (Fig. 3B) and colabelled for both proteins (Fig. 3C) demonstrate a very few scattered, labelled neurons in the pyramidal layer (CA1) of the hippocampus, while Al maltolate administration induces numerous NF-κB-positive (Fig. 3D) and *gadd 153*-positive (Fig. 3E) neurons in the same region. As shown in Fig. 3F, NF-κB is occasionally colocalized with *gadd 153*. Treatment with A-β(1-42) also induces fluorescent labelling for both NF-κB (Fig. 3G) and *gadd 153* (Fig. 3H) and, as with Al maltolate treatment, several neurons exhibit both NF-κB and *gadd 153* positivity (Fig. 3I).

4. Discussion

Disturbances of ER function have been shown to trigger different apoptotic pathways. The first involves cross-talk between the ER and mitochondria, and is regulated by the antiapoptotic protein, Bcl-2 [10]. A second pathway, distinct from that involving mitochondria, has recently been demonstrated and involves activation of caspase-12 [22,23]. In these studies, it has been shown in mouse cortical neurons that Aβ(1-40) triggers an ER-specific apoptosis mediated by active caspase-12, and that a

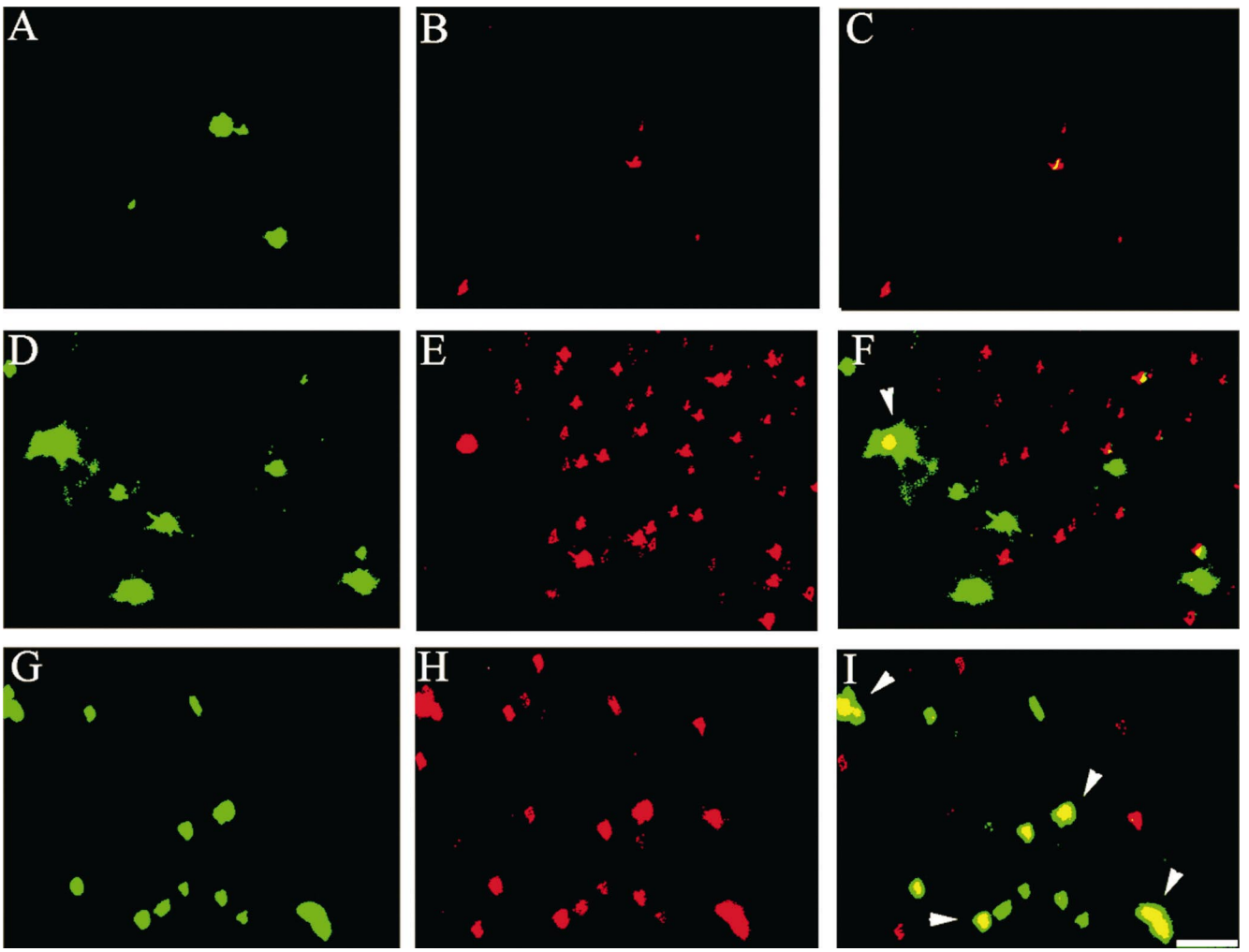

Fig. 3. Immunofluorescence images of NF-κB (green) and *gadd 153* (red) labeling in the hippocampal CA1 area of control (top column), Al-treated (middle column), and Aβ(1-42)-treated (lower column) animals; columns proceed left to right. NF-κB (A) and *gadd 153* (B) demonstrate labeling in controls; (C) is an overlay of (A) and (B). NF-κB labeling shows more numerous immunopositive cells following Al administration (D) or following Aβ(1-42)-treatment (G), compared to the sparsely-positive cells in the control (A). *Gadd 153* immunoreactivity is also greatly increased in neurons from an Al-treated (E) or Aβ(1-42)-treated animal (H) compared to a control (B). (F) Image overlay of (D) and (E), showing co-localization of positive NF-κB labeling with *gadd 153* in an Al-treated animal. (I) Image overlay of (G) and (H) demonstrates a number of positive neurons double-labelled (arrows) with NF-κB and *gadd 153* in an Aβ(1-42)-treated animal. Scale bars represent 20 μm.

reduction in caspase-12 provides protection from apoptosis. The authors suggest that cleavage of procaspase-12 (which resides in the ER) to active cytoplasmic caspase-12 is accomplished by a cysteine family member, *m*-calpain [22]. In addition, factors that induce ER stress activate the expression of various genes that code for ER resident proteins; examples include *gadd 153* [30] and activation of the inducible transcription factor, NF-κB [26]. Activation of *gadd 153* or NF-κB leads to their translocation into the nucleus, where they may play a role in neuronal survival or death. It has been demonstrated that the *gadd 153* gene is specifically activated by agents that disturb ER function. mRNA levels for *gadd 153* are increased, both during hypoxia and after exposure of cells to agents that elevate the levels of glucose-regulated proteins [31]. Activation of *gadd 153* expression has also been confirmed following transient cerebral ischemia in the rat [30]. Furthermore, the magnitude of *gadd 153* expression appears to be proportional to the extent of damage, as in homocysteine-induced death in neuronal cell cultures [2].

In the present report, we show that both Al and Aβ(1-42) induce stress in the ER, as demonstrated by the activation of *gadd 153* and its translocation into the nucleus, which we have confirmed both by immunohistochemistry and Western blot analysis. We also report for the first time the in vivo activation of caspase-12; this is clearly seen following Al treatment, but is inconclusive when we administer Aβ(1-42).

Bcl-2 possesses an antiapoptotic function and is located in membranes of the mitochondria, ER, and nucleus of different cell types (for review, see Ref. [6]). Overexpression of Bcl-2 has been demonstrated to prevent the efflux of cytochrome *c* from the mitochondria and the subsequent initiation of apoptosis in staurosporine-treated cells [39]; it prolongs the life of neurons in rats subjected to ischemia [5] or in a transgenic mouse model of familial amyotrophic lateral sclerosis [16]. Bcl-2 targeted to the endoplasmic reticulum has been shown to block certain types of apoptosis [18,40]. We have recently demonstrated that in mitochondrial and ER fractions derived from brain, levels of Bcl-2 are decreased following Al maltolate administration to young adult rabbits [7]. Recent reports have shown that neuronal apoptosis induced by the Alzheimer's disease Aβ peptide is related to an alteration of the proapoptotic Bax/antiapoptotic Bcl-2 ratio [28], and that transgenic murine cortical neurons expressing human Bcl-2 exhibit increased resistance to Aβ(1-42) [32]. Treatment of primary cultures of human neurons with Aβ(1-40) provokes a down-regulation of Bcl-2 expression [28]. In the present experiments, we have measured levels of Bcl-2 protein in the ER and in the nucleus, and our results show that treatment with either Al or Aβ(1-42) induces a marked decrease in Bcl-2 levels in both organelles.

The inducible transcription factor, NF-κB, is an important mediator of the human immune and inflammatory response [3]. Exposure of cells to various pathological stimuli activates NF-κB; activated NF-κB dimer is rapidly released from the cytoplasm, where it is normally sequestered by the inhibitory unit IκB, and then translocates to the nucleus, where it activates transcription of different genes (for a review, see Ref. [26]). Various agents that induce stress in the ER have been shown to activate NF-κB [9,25,27]. The role of NF-κB in regulating neuronal death is complex. In some cases it has been demonstrated to promote neuronal survival, and in other cases to promote neuronal death. NF-κB, activated by low doses of Aβ(1-40), has been shown to be neuroprotective in cerebellar granule cells [15]. NF-κB has also been reported to be involved in the survival of cerebellar granule neurons subjected to different potassium concentrations [17]. In global ischemia and traumatic spinal cord injury, however, NF-κB promotes neuronal death (for review, see Ref. [20]). Moreover, increased levels of NF-κB activity have been observed in the brain of patients with various neurodegenerative disorders, including Alzheimer's disease [14], Parkinson's disease [13], and amyotrophic lateral sclerosis [21]. Thus, it appears that whether NF-κB acts as a promoter or inhibitor of neuronal loss depends on the cell type and the nature of the toxic stimuli. In our study, we demonstrate that NF-κB is activated in response to the administration of either Al or Aβ (1-42). Whether the translocation of NF-κB into the nucleus represents a cellular defensive mechanism, or represents an event facilitating neuronal injury remains unclear.

In summary, our data show that both Al maltolate and Aβ(1-42) induce stress in the ER of rabbit brain. In response to the induced stress, the ER-resident protein, *gadd 153*, and the inducible transcriptional factor, NF-κB, are both translocated into the nucleus. Furthermore, Al- and Aβ(1-42)-induced stress is also accompanied by a decrease in the antiapoptotic protein, Bcl-2, both in the ER and in the nucleus. In addition, Al activates caspase-12, a process specific to the ER-mediated apoptosis pathway. We propose that, although mitochondrial apoptotic signals are important in regulating apoptosis, the ER and nuclear organelles may also participate in the molecular mechanisms of apoptosis following neurotoxic stimuli.

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