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GDNF Protects against Aluminum-Induced Apoptosis in Rabbits by Upregulating Bcl-2 and Bcl-X_L and Inhibiting Mitochondrial Bax Translocation

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Direct (intracisternal) injection of aluminum complexes into rabbit brain results in a number of similarities with the neuropathological and biochemical changes observed in Alzheimer's disease and provides the opportunity to assess early events in neurodegeneration. This mode of administration induces cytochrome *c* release from mitochondria, a decrease in Bcl-2 in both mitochondria and endoplasmic reticulum, Bax translocation into mitochondria, activation of caspase-3, and DNA fragmentation. Coadministration of glial cell neuronal-derived factor (GDNF) inhibits these Bcl-2 and Bax changes, upregulates Bcl-X_L, and abolishes the caspase-3 activity. Furthermore, treatment with GDNF dramatically inhibits apoptosis, as assessed by the TUNEL technique for detecting DNA damage. Treatment with GDNF may represent a therapeutic strategy to reverse the neuronal death associated with Alzheimer's disease and may exert its effect on apoptosis-regulatory proteins.

Key Words: **cytochrome *c*; Bax; Bcl-2; Bcl-X_L; caspase-3; GDNF; aluminum; endoplasmic reticulum.**

INTRODUCTION

An understanding of the complex events involved in neuronal injury and protection in neurodegenerative diseases requires the availability of animal model systems. The direct injection of aluminum (Al) compounds into the brain of rabbits has been found to result in conditions that mimic a number of neuropathological and biochemical changes present in Alzheimer's and related human neurodegenerative disorders (Huang *et al.*, 1997). The use of this animal model should not be confused with the ongoing controversy regarding the possible role of Al in the pathogenesis of Alzheimer's disease, a debate which by no means has been concluded (Savory *et al.*, 1996). Here we use this Al/rabbit model system to unravel the events leading to neuronal apoptosis and, most importantly, to demonstrate the anti-apoptotic effect of glial cell line-derived neurotrophic factor (GDNF) against fatal Al neurotoxicity.

Alzheimer's disease is a progressive neurodegenerative disorder characterized by three typical pathological features, namely the extracellular deposition of Aβ, the formation of neurofibrillary tangles, and selective neuronal loss. However, it is unclear which of these pathological features is the primary event in the initiation and progression of this disease. Selective neuronal death involves vulnerable brain regions, in particular the hippocampus and cerebral cortex, and apoptosis may play a role in the process of cell loss (for review, see Honig & Rosenberg, 2000). Besides inducing intraneuronal neurofilamentous changes in the cerebral, cortex, brainstem, and spinal cord, which demonstrate many biochemical features in common with the neurofibrillary tangles seen in Alzheimer's disease (Rao *et al.*, 2000; Huang *et al.*, 1997), the intracisternal administration of Al maltolate to rabbits also leads to biochemical changes suggestive of apoptosis (Savory *et al.*, 1999). Aluminum is highly neurotoxic

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and has been shown to accumulate in neurons following cell depolarization (Suarez Fernandez *et al.*, 1996), where it inhibits Na^{+}/Ca^{+2} exchange and thereby induces an excessive accumulation of mitochondrial Ca^{+2} (Szutowicz *et al.*, 1998). Increases in intramitochondrial Ca^{+2} levels lead to an opening of the mitochondrial transition pore (MTP), with cytochrome *c* release and subsequent apoptosis resulting from activation of the caspase family of proteases. This mitochondrial pathway is now widely considered to be an important step in controlling and initiating apoptosis in neurodegenerative disorders and involves regulating proteins such as the anti-apoptotic Bcl-2 and Bcl-X_L, and the proapoptotic Bax. The present study was initiated to assess the importance of such apoptosis regulation, especially to see whether agents that upregulate Bcl-2 and Bcl-X_L will be protective in the Al/rabbit system. Recently, it has been reported that GDNF provides an antiapoptotic effect on primary cultures of the rat mesencephalon (Sawada *et al.*, 2000), causing an increase in Bcl-2 and Bcl-X_L. Here we demonstrate that GDNF has indeed a markedly antiapoptotic effect in our system, and we have examined factors such as mitochondrial cytochrome *c* release, changes in Bcl-2 in mitochondria and endoplasmic reticulum, Bcl-X_L regulation, Bax translocation into mitochondria, caspase-3 activation, and DNA fragmentation.

MATERIALS AND METHODS

Animals, Treatment Protocol, Clinical Monitoring, 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. Young adult (5–8 months) female New Zealand white rabbits received either intracisternal injections of 100 μl normal saline ($n = 6$; controls), 100 μl 50 mM Al maltolate in saline ($n = 7$; Al-treated group), or 100 μl of 500 ng/ml GDNF in saline plus 100 μl 50 mM Al maltolate ($n = 6$; Al/GDNF-treated group). The GDNF was obtained commercially (R&D systems, Inc., Minneapolis, MN). The injections were carried out slowly over a period of 2 min under ketamine anesthesia as described previously (Savory *et al.*, 1999). All rabbits were monitored daily for clinical symptoms as described by us previously (Huang *et al.*, 1997). Rabbits were euthanized 3 days following the intracisternal injection and perfused with Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY), also as described previously (Savory *et al.*, 1999). 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 and caspase-3-like activity measurements. The respective sides chosen for these studies were alternated between successive animals. Each brain hemisphere intended for tissue sectioning was immediately frozen rapidly 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 and caspase-3-like activity analysis, the hippocampus was rapidly dissected, homogenized, and processed as described below.

Western Blot Analysis

We examined changes of proteins in the subcellular fractions where they are reported to be localized and/or translocated. Bcl-2 is present in mitochondria and the endoplasmic reticulum and does not translocate into the cytoplasm, unlike cytochrome c, which does; Bax also translocates, but from the cytoplasm to mitochondria (Cassarino *et al.*, 1999; Cory, 1995; Eskes *et al.*, 1998). For Bcl-X_L we assessed levels in both the mitochondrial and cytoplasmic fractions.

Proteins from the mitochondrial, cytosolic and endoplasmic reticulum fractions were extracted as described previously (Liu *et al.*, 1996). Tissue from the entire hippocampus was gently homogenized, using a teflon homogenizer (Thomas, Philadelphia, PA), in 7 vol of cold suspension buffer (20 mM Hepes–KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM 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 750*g* at 4°C for 10 min to first isolate the nuclear fraction, and then at 8000*g* for 20 min at 4°C to separate the mitochondrial from the soluble fraction. 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 reticulum fractions were sepa-

rated 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 with mouse monoclonal antibody (mAb) to human cytochrome *c*, Bcl-2 (C-2), Bax (B-9), or Bcl-X_L (H-5), all at a dilution of 1:100. All of these mAbs were obtained from a commercial source (Santa Cruz Biotechnology, Santa Cruz, CA). Cytochrome oxidase subunit IV mAb obtained from a commercial source (Molecular Probes, Eugene, OR) was used as a marker of mitochondrial contamination at 1:1000 dilution, and a calnexin mAb (Transduction Laboratories, Lexington, MD) was applied at 1:500 dilution as an endoplasmic reticulum marker. Following washes with Tris-buffered saline (TBS) containing 0.1% Triton X-100, the blots were developed with enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA). The bands representing cytochrome *c*, Bax, Bcl-2, and Bcl-X_L were developed on radiographic film and analyzed by densitometry with Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

Caspase-3-like Activity Assay

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 at 4°C for 30 min at 160,000*g*, and the protein concentration in the supernatant was determined 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, with and without 10 μL of the caspase-3 inhibitor Ac-DEVD-CHO (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 Inc.). Caspase-3 activity for each sample was calculated as the difference between the rate of cleavage in the absence and presence of the inhibitor.

DNA Fragmentation and Immunohistochemistry

Frozen coronal brain sections (14 μm thick) from the hippocampal level were fixed and permeabilized as described previously (Henshall *et al.*, 2000). Detection of DNA fragmentation was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique. In brief, tissue sections from controls, Al-treated, and Al/GDNF-treated animals were dried for 15 min at room temperature and fixed in 10% formalin for 15 min, followed by 10 min incubation in 1:2 vol/vol ethanol/acetic acid. Sections were washed three times in PBS for 5 min each, permeabilised with 3% Triton X-100 for 20 min and immersed in 3% hydrogen peroxide for 15 min. Sections were then washed three times in PBS buffer for 5 min each and then processed for apoptosis detection using an Apoptosis Detection System, Fluorescein (Promega, Madison, Wisconsin). Positive control fixed sections were incubated with 1 unit/ml of Dnase I for 5 min. All steps were conducted at room temperature.

For dual-labeled caspase-3 immunostaining, brain sections previously stained with the TUNEL from controls, Al-treated, and Al/GDNF-treated animals were blocked with 2% goat serum and incubated for 2 h at 37°C in a 1:200 dilution of the caspase-3/CPP 32 mouse mAb, specific for activated caspase-3 (Transduction Laboratories, Lexington, KY). 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 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Sections were subsequently washed in PBS buffer, mounted in Vectashield, coverslipped, and evaluated and recorded digitally with a fluorescence microscope. TUNEL and caspase-3 positive neurons were counted at a magnification of 400X with an Olympus BH2 microscope (Melville, NY) and Image Pro Plus 4.1 analysis software (Media Cybernetics, Baltimore, MD). Five fields were captured from the CA 1 region of the hippocampus from each animal, and results were compared between the Al-treated and the Al/GDNF-treated animals. Results from the Al-treated rabbits were assigned a value of 100%. The GDNF effect was then expressed as the percent reduction in the number of positive neurons in the Al/GDNF group when compared to the Al-treated rabbits.

Statistical Analysis

Densitometric analysis for cytochrome *c*, Bcl-2, Bax and Bcl-X_L were expressed as mean ± SE values. Differences in densitometric levels of these proteins in mitochondrial, cytoplasmic, and endoplasmic fraction were statistically compared between the controls, Al-treated, or Al/GDNF-treated animals, using ANOVA

with the posthoc Fisher's PLSD test. Changes in caspase-3 protease activity in the different animal groups were compared using one-way ANOVA and the Student's *t* test. A value <0.05 was considered significant.

RESULTS

By day 3 following Al maltolate administration, all animals with this treatment alone develop neurological symptoms characterized by forward head tilt, hemiplegic gait, loss of appetite, splaying of the extremities, and paralysis. These symptoms were so severe at this time that the rabbits had to be sacrificed. Treatment with GDNF protected the animals against this Al-induced toxicity, and no neurological symptoms were evident up to day 3.

Western Blot Analysis

Cytochrome *c* immunoreactivity, as shown by Western blot and densitometric analysis of these blots (Fig. 1A), is faintly detectable in the cytosolic fraction in the control brains and is strongly positive in the mitochondrial fractions. Aluminum treatment induces cytochrome *c* release into the cytoplasm and GDNF administration fails to inhibit the Al-induced translocation of cytochrome *c* into the cytoplasm.

Bcl-2 (Fig. 1B) is detectable in controls in the mitochondrial and endoplasmic reticulum fractions. In the Al-treated group, Bcl-2 reactivity is decreased both in the mitochondria and in the endoplasmic reticulum, but treatment with GDNF maintains the Bcl-2 levels. As shown in Fig. 1B, densitometric analysis of Bcl-2 demonstrates that Al dramatically decreases Bcl-2 levels in both the mitochondrial and endoplasmic reticulum fractions and that this effect is reversed by GDNF.

Bax (Fig. 1C) is distributed with higher intensity in the cytosolic than in the mitochondrial fractions of controls. Following Al treatment, Bax immunoreactivity is markedly increased at the mitochondrial level. Treatment with GDNF significantly decreases Bax intensity in the mitochondria.

Bcl-X_L immunoreactivity (Fig. 1D) in controls is highly positive in the mitochondrial fraction and is present to a lesser extent in the cytoplasmic fraction. Aluminum treatment, while having no significant effect on Bcl-X_L in mitochondria, slightly increases the level in the cytoplasmic fraction. GDNF administration results in an enhancement of Bcl-X_L in both the mitochondrial and cytoplasmic fractions.

Cytochrome *c* oxidase subunit IV and calnexin antibodies, used respectively as specific markers for mitochondrial and endoplasmic reticulum proteins, confirm the purity of the subcellular fractionation carried out in the present experiments. As shown in Fig. 2, cytochrome *c* oxidase subunit IV stains only the mitochondrial fraction and calnexin stains only the endoplasmic reticulum fractions.

Caspase-3-like Activity

The activity of caspase 3-like proteases in hippocampal lysates, assessed by measuring the cleavage of the fluorogenic substrate Ac-DEVD-AMC in the presence and absence of an inhibitor, demonstrates significant elevations in the Al-treated animals. This increase in caspase-3-like activity approaches fivefold greater levels in the Al-treated animals as compared to controls. Treatment with GDNF strongly reduces the caspase-3-like activity (Fig. 3).

DNA Fragmentation and Caspase-3 Immunoreactivity

Sections from control animals treated with DNAse I (positive control) show widespread TUNEL-positive staining in the pyramidal layer (CAl) of the hippocampus (Fig. 4A), while in a similar area in the control untreated group no TUNEL (Fig. 4B) or caspase-3 positive neurons (Fig. 4C) are seen. Aluminum administration induces a large number of TUNEL positive (Fig. 4D) and caspase-3 stained (Fig. 4E) neurons in the same region of the hippocampus. There are also TUNEL and caspase-3-positive neurons in the nearby temporal cortex but to a lesser extent (data not shown). Treatment with GDNF dramatically reduces TUNEL positivity (Fig. 4G), and the caspase-3 staining (Fig. 4H) in these neurons. No positivity for either TUNEL or caspase-3 is observed in the temporal cortex (data not shown). Quantitation of TUNEL positive (Figs. 5A and 5B) and caspase-3 positive (Figs. 5D and 5E) neurons, carried out at a magnification of 400X, shows that the Al-induced TUNEL staining and caspase-3 activity increase are markedly reduced by GDNF treatment (Figs. 5C and 5F, respectively).

DISCUSSION

Hippocampal tissue from Al maltolate-treated rabbits exhibits cytochrome *c* translocation into the cyto-

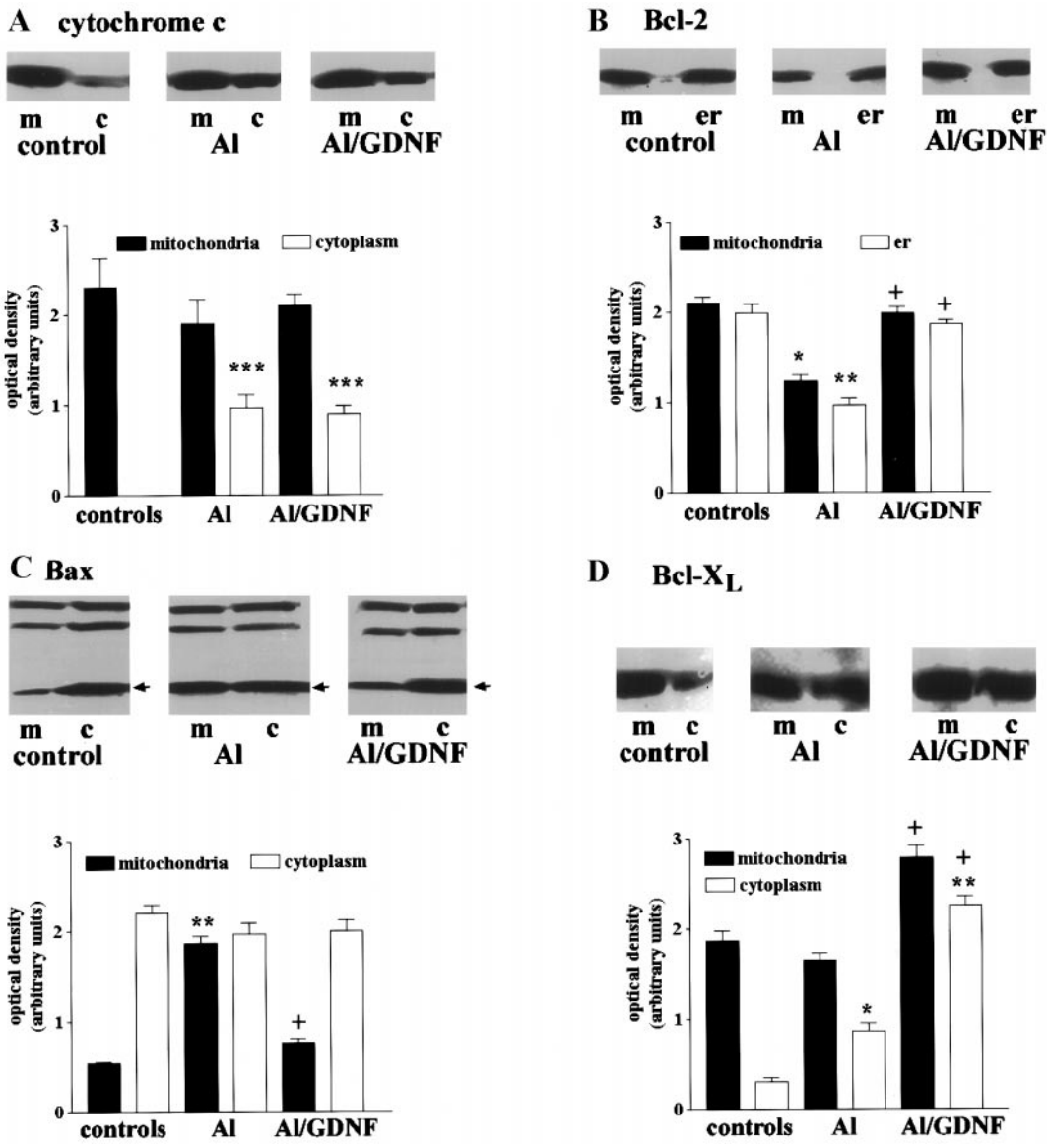

FIG. 1. Representative immunoblot and densitometric analyses for cytochrome *c* Bcl-2, Bax, and Bcl-X_L proteins of mitochondrial (m), cytoplasmic (c), or endoplasmic fractions (er) from hippocampus in controls ($n = 6$), the Al-treated group ($n = 7$), and Al/GDNF-treated group ($n = 6$). (A) Cytochrome *c* (15.5 kDa) is localized in the mitochondria and is barely detectable in the cytoplasm of controls. Aluminum administration induces translocation of cytochrome *c* into the cytoplasm, and coperfusion with GDNF does not inhibit the Al-induced cytochrome *c* translocation (***$P < 0.01$; versus controls). (B) Bcl-2 (27 kDa) in controls is localized in mitochondria and the endoplasmic reticulum. Following Al administration, Bcl-2 levels decrease both in the mitochondria and in the endoplasmic reticulum. Treatment with GDNF maintains Bcl-2 levels at the basal levels observed in the controls, both in the mitochondria and endoplasmic reticulum (*$P < 0.05$, **$P <$ 0.01 versus controls; +$P < 0.05$ versus Al treated). (C) Bax (23 kDa) in controls is present at low levels in mitochondria and highly reactive in the cytoplasm. Aluminum induces an increase in Bax in the mitochondrial fractions, and treatment with GDNF significantly reduces the translocation of Bax into mitochondria (**$P < 0.01$ versus controls; +$P < 0.05$ versus Al treated). Note that Bax immunoblotting results in three distinct bands: the upper band is approximately 54 kDa and the lower band is 23 kDa. (D) Bcl-X_L (31 kDa) immunoreactivity in controls is highly positive in the mitochondrial fraction and is present to a lesser extent in the cytoplasmic fraction. Aluminum induces a slight decrease in mitochondrial levels, with a subsequent increase in the cytoplasmic levels. GDNF leads to a large increase in Bcl-X_L levels in cytoplasm > mitochondria (*$P < 0.05$, **$P < 0.01$ versus controls; +$P < 0.05$ versus Al treated).

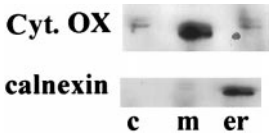

FIG. 2. A representative Western blot analysis for cytochrome *c* oxidase subunit IV (Cyt.OX) and calnexin in cytoplasmic (c), mitochondrial (m), or endoplasmic reticulum (er) fractions. Cytochrome *c* oxidase subunit IV, used as a marker for mitochondrial contamination, is only present in the mitochondrial fraction, and calnexin, applied as a marker for the endoplasmic reticulum, stains only the endoplasmic reticulum fraction.

plasm, accompanied by a decrease in Bcl-2 and an increase in Bax. Furthermore, changes in the levels of these proteins are accompanied by evidence of activation of caspase-3 and DNA fragmentation, suggestive of the initiation of apoptosis. Treatment with GDNF reverses the increase in levels of the proapoptotic protein Bax, and enhances levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L, thus abolishing the caspase-3-like activity and dramatically inhibiting apoptosis.

Mitochondrial cytochrome *c* is considered to play a pivotal role in the initiation of apoptosis when released into the cytoplasm. On the other hand, members of the Bcl-2 family of proteins are believed to determine cell life or death by inhibiting (as in the case of Bcl-2 and Bcl-X_L), or promoting (as in the case of Bax), the release of cytochrome *c* (for a review see Graham *et al.*, 2000). Opening of the MTP has been proposed to lead to cytochrome *c* release; however, it may not represent the unique mechanism for this release (for review, see Gottlieb, 2000). Indeed, it has been suggested that Bax, when translocated into mitochondria following neurotoxic stimuli, can by itself form a channel, or can interact with the voltage-dependent anion channel (VDAC) to form a larger channel that is permeable to cytochrome *c* (Shimizu *et al.*, 2000). In the present work it remains to be determined whether the release of cytochrome *c* induced by Al occurs through opening of the MTP and/or as a result of the increase in mitochondrial Bax. Since Al perturbs Ca^{2+} homeostasis in mitochondria (Szutowicz *et al.*, 1998), it may then open the MTP and release cytochrome *c*. We recently have reported such a mechanism in aged rabbit brains where the Al-induced cytochrome *c* release was supressed by cyclosporin A, a blocker of the MTP (Ghribi *et al.*, 2001).

Treatment with GDNF greatly reduces Bax translocation into mitochondria and increases the levels of the antiapoptotic Bcl-2 and Bcl-X_L, but does not inhibit cytochrome *c* release into the cytoplasm. However, the GDNF-treated animals show no caspase-3-like activity and have a markedly reduced number of apoptotic cells when compared to animals receiving Al. Also, these GDNF-treated rabbits do not demonstrate neurological symptoms for up to the first 3 days, whereas within this time period, those receiving Al alone develop severe symptoms and must be sacrificed. The mechanism by which GDNF exhibits its protective effect in the present study is unknown and, based on present results, it appears that the antiapoptotic effect is independent of the blockade of cytochrome *c* release. It also seems unlikely that the observed cytochrome *c* release originates from a putative Bax channel, since GDNF treatment inhibits mitochondrial Bax translocation and increases mitochondrial Bcl-2 levels, a process normally capable of inhibiting cytochrome *c* release via a Bax-dependent pathway (for review see Tsujimoto and Shimizu, 2000). However, it has been shown recently that during tumor necrosis-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat cells, Bcl-2 overexpression fails to block mitochondrial dysfunction and does not inhibit cytochrome *c* release (Kim *et al.*, 2001). It is known that formation of the cytochrome *c*-Apaf-1 complex (for a review see Gottlieb, 2000), rather than the presence *per se* of cytochrome *c* in the cytoplasm, is the determining factor in the activation of caspases and in the triggering of apoptosis. Indeed, it has been reported that the antiapoptogenic protein Bcl-X_L has the ability, when overexpressed, to sequester Apaf-1, and thereby to

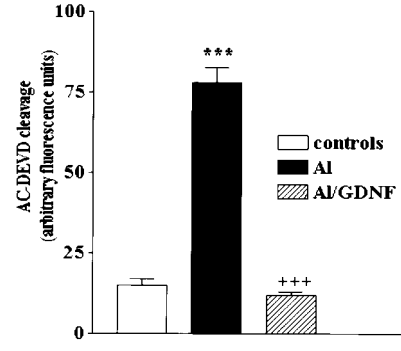

FIG. 3. Caspase-3-like activity in hippocampal lysates of control (white bar), Al-treated (black bar) and Al/GDNF-treated animals (hatched bar). In the controls, the level of caspase-3 activity detected is low. Aluminum treatment induces a large increase (5-fold) in caspase-3 activity; GDNF treatment completely abolishes the Al-induced caspase-3 like activity. Data are presented as mean ± SEM (***$P < 0.001$ versus controls; +++$P < 0.001$ versus Al-treated group). A value of 70 arbitrary fluorescence units corresponds to 50% inhibition of the initial reading when the caspase-3 inhibitor Ac-DEVD-CHO is added.

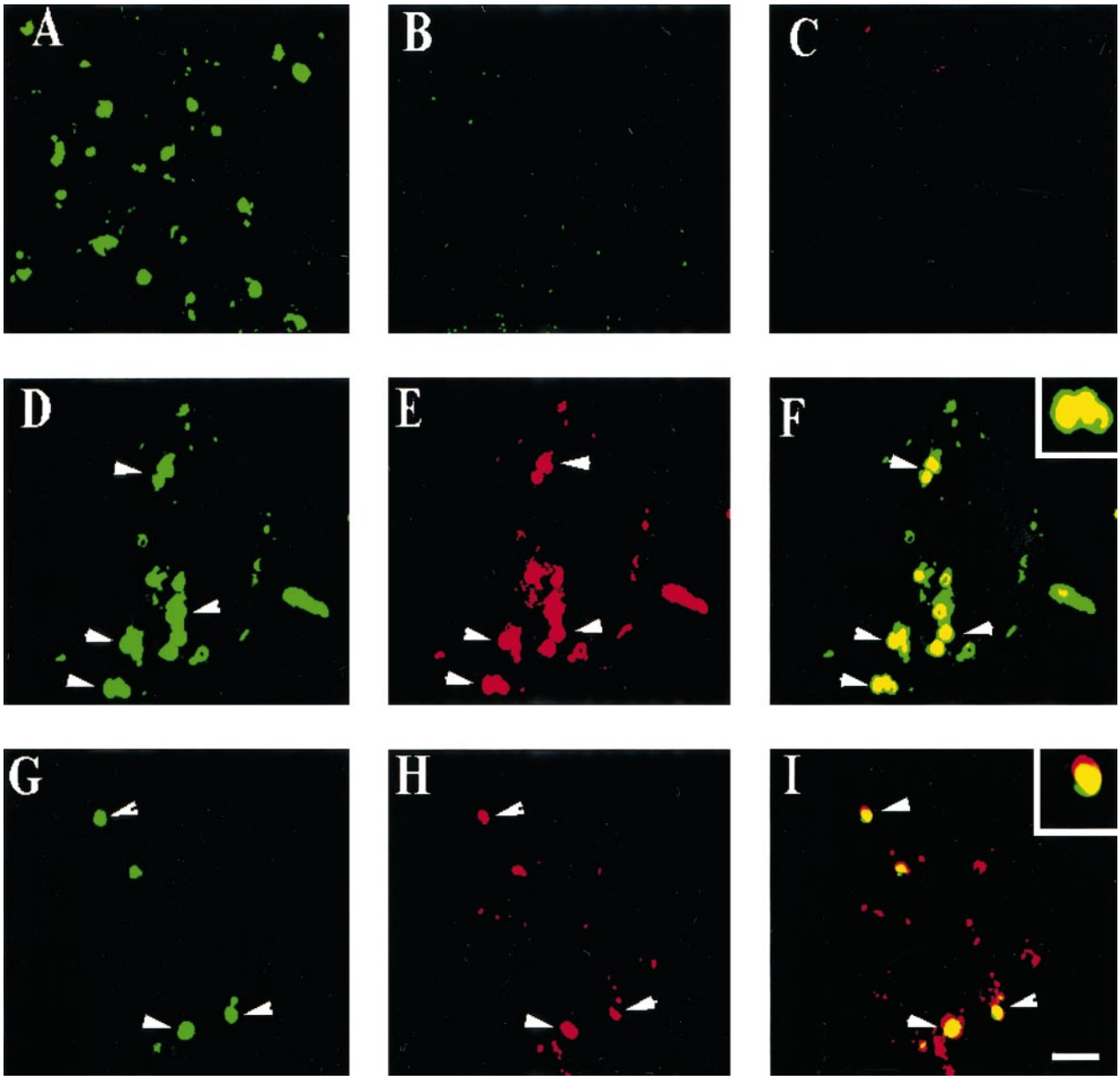

FIG. 4. Immunofluorescence images of TUNEL labeling and caspase-3 protein in the hippocampal CAl area of control, Al-treated, and Al/GDNF-treated animals. Magnification scale bar in I represents 50 μm. (A) TUNEL-positive labeling (positive control) induced by treatment of tissue section from a naive rabbit with Dnase I. (B) TUNEL labeling of DNA fragmentation from control brain with no positively-stained neurons. (C) Caspase-3 immunoreactivity in control. (D) TUNEL labeling demonstrates the emergence of DNA fragmentation following Al administration (arrows). (E) Distinct caspase-3 immunoreactivity is enhanced in the same neurons as in D, from Al-treated rabbits (arrows). (F) Image overlay of D and E showing colocalization of positive TUNEL labeling with caspase-3 immunoreactivity. Box in F: higher power magnification of a neuron double-stained with TUNEL and caspase-3. (G) TUNEL labeling following treatment with GDNF shows a reduced number of neurons exhibiting DNA fragmentation. (H) Caspase-3 immunoreactivity in the CA 1 sector is greatly reduced in rabbits treated with GDNF. (I) overlay image of G and H demonstrates a reduced number of positive neurons, in comparison to F; TUNEL labeling and caspase-3 immunoreactivity are colocalized. Box in I: higher power magnification of a neuron double-stained with TUNEL and caspase-3.

inhibit Apaf-1-dependent caspase-9 activation (Hu *et al.*, 1998; Pan *et al.*, 1998). Interestingly, the GDNF-induced increase in mitochondrial and cytoplasmic Bcl-X_L levels we report here may provide, at least in part, an explanation for the antiapoptotic effect of GDNF. By upregulating Bcl-X_L levels in the cyto-

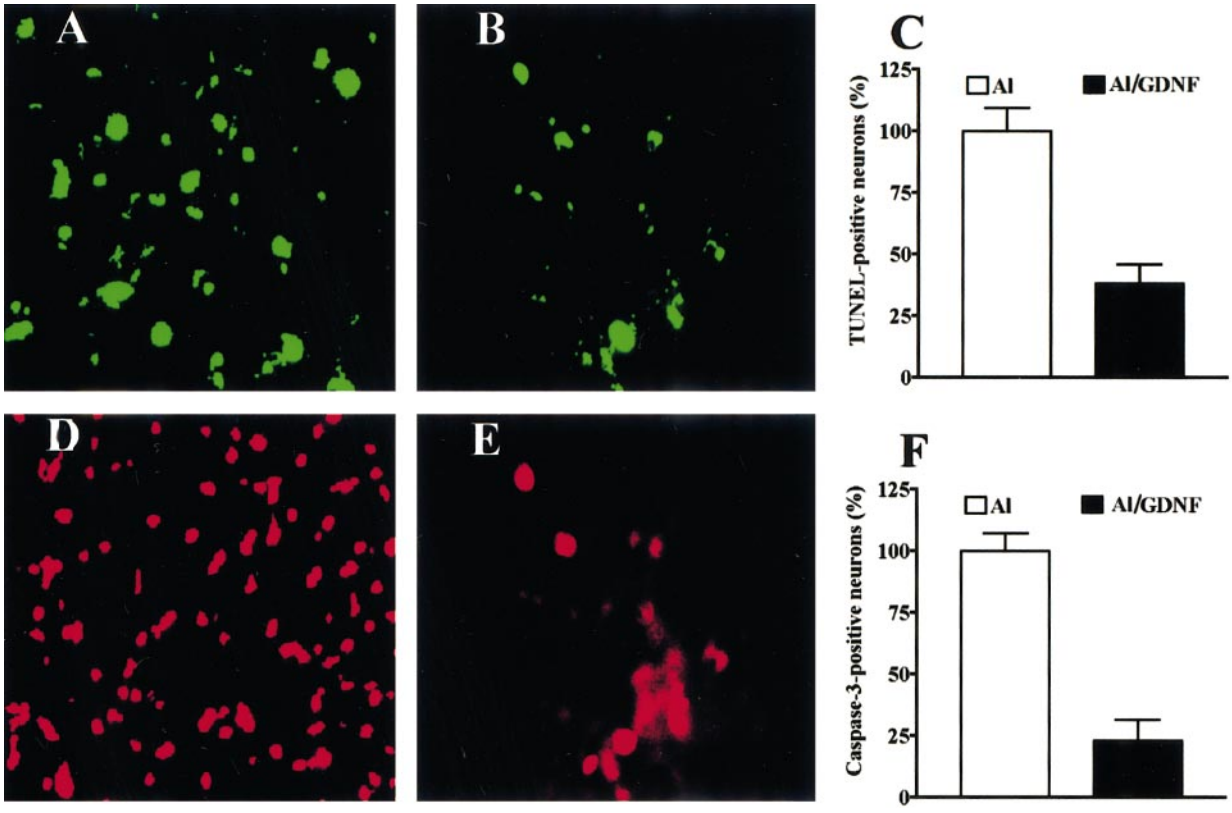

FIG. 5. TUNEL-positive (A and B) and caspase-3 positive (D and E) cells in the CAl region of the hippocampus, with quantitation of the results, in Al treated and in Al/GDNF treated animals. The Al-induced TUNEL labeling (A) and caspase-3 activation (D) are markedly reduced by GDNF treatment, (B) and (E), respectively. (C and F) Quantitative analyses showing the neuroprotective effect of GDNF which reduces the number of TUNEL-positive (C) and caspase-3 positive (F) neurons in comparison to Al-treated animals. (A, B, D, and E, 400×).

plasm, this protein can bind to Apaf-1, leaving cytochrome *c* free but now unable to initiate caspase-9 activation.

Although mitochondrial alterations represent a major step in the initiation of apoptosis, increasing evidence now implicates the endoplasmic reticulum as an important organelle in the regulation of apoptosis. The endoplasmic reticulum-mediated apoptosis occurs either in concert with mitochondria and is controlled by Bcl-2 (Hacki *et al.*, 2000), or occurs by a mechanism independent of the mitochondrial pathway (Nakagawa *et al.*, 2000). Interestingly, our results show that Al administration leads to a decrease in the Bcl-2 levels also in the endoplasmic reticulum, and that these Bcl-2 levels are restored by GDNF treatment. Thus, it appears that the endoplasmic reticulum may play an important role in the initiation or the exacerbation of apoptosis induced by Al, with the possibility that a key event is an increase in Bcl-2 in this organelle, coupled with a concomitant increase in Bcl-X_L, which could sequester Apaf-1 and thereby contribute to the antiapoptotic effect of GDNF. Such findings have been reported, suggesting that Bcl-2 targeted to the endoplasmic reticulum is capable of blocking certain types of apoptosis (Lee *et al.*, 1999; Zhu *et al.*, 1996). However, it remains unclear how Bcl-2 in the endoplasmic reticulum exerts such an antiapoptotic effect. More recently it has been demonstrated that stress in the endoplasmic reticulum activates a specific apoptosis pathway mediated by caspase-12 that is independent from the mitochondrial apoptosis pathway (Nakagawa *et al.*, 2000). Investigation of this caspase-12-mediated endoplasmic reticulum apoptosis in our Al-model of neurodegeneration is important, and studies are underway in our laboratory.

The activity most often attributed to GDNF is that of promoting neuronal survival. GDNF binds to the GDNF family receptor α (GFRα), with a higher affinity to GFRα1 than to GFRα2 (Klein *et al.*, 1997; Sanicola *et al.*, 1997) to form a complex that subsequently acti-

vates the receptor tyrosine kinase, c-ret (Jing *et al.*, 1996). Upregulation of GDNF mRNA in rat brains has been shown to occur following excitotoxicity induced by glutamate (Ho *et al.*, 1995) or kainate (Humpel *et al.*, 1994). GDNF has been shown to protect neurons against oxidative stress in cultured mesencephalic neurons and glial cells (Iwata-Ichikawa *et al.*, 1999), against ischemic/hypoxic-induced brain injury in neonatal rats (Ikeda *et al.*, 2000), after brain injury following permanent middle cerebral artery occlusion in rats (Kitagawa *et al.*, 1998), and in primate models of Parkinson's disease (Kordower *et al.*, 2000). The mechanisms by which GDNF exerts its neuroprotective effect are diverse, and recent evidence demonstrates that the rescue and repair of injured neurons is a consequence of an antiapoptotic action of GDNF. Indeed, GDNF has been shown to up-regulate Bcl-2 and Bcl-X_L levels in rat mesencephalic neurons subjected to apoptosis, resulting in a reduction of caspase activation (Kitagawa *et al.*, 1998; Sawada *et al.*, 2000). The present investigation not only confirms these antiapoptotic properties of GDNF, but further elucidates the cell organelles in which the apoptosis-regulatory proteins are controlled.

Apoptosis in neurodegenerative disorders, including Alzheimer's disease, may be responsible for the neuronal losses associated with these diseases. Therefore, the elucidation of the mechanisms of cell death could culminate in the development of novel therapeutic strategies for prevention and therapy. We conclude that the intracisternal administration of the neurotoxin, Al maltolate, induces changes in the level of proteins regulating apoptosis, and that GDNF regulates the increases in Bcl-2 and Bcl-X_L, both of which are antiapoptotic. This regulation, coupled with decreases in the proapoptotic Bax, serves to inhibit apoptosis. Most importantly, these changes dramatically protect against the development of the acute, severe, and invariably fatal neurological symptoms associated with Al administration. Further experiments are needed to examine the long-term effects of GDNF treatment on Al-induced fatal neurological symptoms. We have performed a preliminary experiment with nine animals treated with Al maltolate and GDNF and allowed to survive longer than the 3 days reported in the present study. Five of these rabbits developed neurological symptoms after 8–9 days, and the remaining four rabbits were asymptomatic up to 2 months. Because experimentally induced neurofibrillary degeneration resulting from Al administration shares many features of the neurofibrillary pathology in a number of human neurodegenerative disorders, particularly in Alzheimer's disease, our results support proposed treatments with GDNF or related compounds as approaches to provide protection against the neuronal loss associated with these conditions.

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