

CAS role in the brain apoptosis of *Bufo arenarum* induced by cypermethrin

M. F. IZAGUIRRE, M. N. VERGARA, AND V. H. CASCO.

Laboratorio de Microscopía, FI-Bioingeniería, UNER, Ruta 11, CC 47, suc. 3, (3100), Paraná, Entre Ríos, Argentina.

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ABSTRACT: CAS might have a key role in the apoptosis induced by toxins, acting as anti-apoptotic factor, stimulating the cellular proliferation and the cell contact stabilization. To start to elucidate their role in the brain apoptosis of *Bufo arenarum* induced by cypermethrin (CY), the expression patterns of CAS and several cell adhesion molecules (CAMs) were established.

Bufo arenarum tadpoles of the control and acute bioassay survival at different doses (39, 156, 625 and 2,500 µg CY/L) and times (24, 48, 72 and 96 h) of CY treatment were fixed in Carnoy, embedded in paraffin and sectioned. CAS and CAMs expression was determined by immunofluorescence and immunohistochemistry, respectively.

When the bioassay starts, CAS increases suggesting a proliferative or regenerative effect, but decreases when the doses and/or the biocide exposure time increases, suggesting compromise of the cellular cycle control and trigger of an apoptotic wave. However, these neurotoxic mechanisms should not involve degradation of N-cadherin and α -catenin, in contrast of β -catenin and axonal N-CAM180, at least in the initial apoptotic phase. Additionally, an adhesion compensatory mechanism by N-CAM180 is observed in the neuron cell body.

These results suggest a dual role of CAS in the cellular cycle control during the CY-induced apoptosis: induction of cell proliferation and stabilization of the cell-cell junctions by modulating CAMs expression.

Introduction

The CAS (cellular apoptosis susceptibility) gene is the human homolog of the yeast chromosome segregation gene CSE1 (Brinkmann *et al.*, 1996a; Behrens *et al.*, 2003). The CSE1L/CAS protein is a Ran-binding protein which acts as nuclear transport (export) factor, and simultaneously plays a role in the mitotic

spindle checkpoint, which assures genomic stability during cell division. This checkpoint is frequently disturbed in neoplasias of various origins (Wellmann *et al.*, 2001). CAS is also implicated in the nuclear to cytoplasmic reshuffling of importin alpha, which itself is necessary for the nuclear transport of several proliferation activating proteins, transcription factors, oncogene and tumor suppressor gene products such as p53 and BRCA1 (Behrens *et al.*, 2003). Furthermore, CAS may have a dual function in mammalian cells, one in cell proliferation and another in apoptosis (Brinkmann *et al.*, 1996a).

CAS (p130^{CAS}) was first identified as a highly tyrosine-phosphorylated 130 kilodalton protein associated with the v-Src and v-Crk-oncoproteins (Matsuda *et al.*, 1990; Kanner *et al.*, 1990, 1991; Birge *et al.*,

Address correspondence to: Dr. Victor Hugo Casco. Laboratorio de Microscopía. FI-Bioingeniería, UNER. C.C. 47 Suc. 3. (3100) Paraná, Entre Ríos, ARGENTINA.
Fax: (+54-343) 475077. E-mail: vcasco@bioingenieria.edu.ar
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1992). Structurally, p130^{CAS} is a scaffolding molecule that resembles a “docking protein” because it contains SH3 domain followed by multiple SH2 binding motifs (Sakai *et al.*, 1994). The SH3 domain of p130^{CAS} is essential for focal adhesion targeting, a function that is independent of FAK binding (Nakamoto *et al.*, 1997). A pool of p130^{CAS} is associated constitutively with FAK, but upon adhesion, Src kinase activity may be responsible for stabilizing the FAK- p130^{CAS} complex (Nakamoto *et al.*, 1996, 1997; Polte and Hanks, 1997). The CAS proteins (p130^{CAS}, HEF1/CAS-L and Efs/Sin), a family of docking proteins, are important components of integrin receptor signaling and have been implicated in cell-matrix adhesion, cellular migration, proliferation, transformation and apoptosis (O’Neill *et al.*, 2000). In humans, CAS gene is localized in the chromosome 20q13. This region is known to harbor amplifications in several cancers (Brinkmann *et al.*, 1996a; Wellmann *et al.*, 2001; Behrens *et al.*, 2003). Wellmann *et al.* (2001) found that the degree of CAS-expression correlates with the grade of tumor dedifferentiation, being a prognostic marker for neoplasms.

Normal hepatocytes reveal no CAS expression, while embryonic liver shows strong expression in all parenchymal cells. Also, bile ducts express CAS, and the interface between bile ducts and hepatocytes under conditions associated with regenerative proliferation. The localization of these CAS expressing cells is correlated with the distribution of putative liver stem-cells (Wellmann *et al.*, 2001). CAS is highly expressed in human tumor cell lines, in human testis and fetal liver, tissues containing actively dividing cells. Furthermore, CAS expression increases when resting human fibroblasts are induced to proliferate and decreases when they are growth-arrested. Additionally at their proliferative role, CAS appears to play an important role in both toxins and tumor necrosis factor-mediated cell death (Brinkmann *et al.*, 1995). For that reason, to investigate their role in the *Bufo arenarum* brain apoptosis induced by CY (Izaguirre *et al.*, 2000a, 2001a, Casco *et al.*, in press), the CAS expression pattern was established by immunohistochemistry.

Additionally, it is postulated that CAS could influence the cell adhesion molecules (CAMs) stabilization of cell-cell contacts (Jiang *et al.*, 2002). Therefore, in order to know the CAS role in the modulation of CAMs expression, and find the relation with the loss of cell-cell contacts induced by CY (Casco *et al.*, in press), their expression levels were quantified by immunohistochemistry and morphometry.

Materials and Methods

Animals

Premetamorphic tadpoles of stages 28 (Gosner, 1960) control and survival of the 96-hour cypermethrin (CY)-toxicity acute test (Izaguirre *et al.*, 2000a, 2001a; Casco *et al.*, in press) were used to evaluate the CAS role in the brain apoptosis induced by CY.

The tadpoles were obtained by artificial fertilization (Izaguirre *et al.*, 2000b).

Bioassay

The CY toxicity test was conducted according to Casco *et al.* (in press). The assayed product was a commercial formulation containing 25% of CY in xylene (Sherpa, Rhône Poulenc Argentina Fomental, Buenos Aires, Argentina). The concentrations used were 39, 156, 625, 2,500 and 10,000 µg CY/L and the mortality was registered at 24, 48, 72 and 96 h. Controls were conducted with or without vehicle (xylene) during the same periods. The lethal concentration 50 (LC-50) with confidence limits ($p < 0.05$) was estimated by using a Probit Analysis Program (Finney, 1971). Data from control and experimental groups were analyzed by one-way analysis of variance in conjunction with LSD test (Sokal and Rohlf, 1979). Neurotoxicity was evaluated as described previously Salibián (1992).

Immunohistochemistry

Control and survival tadpoles from 24, 48, 72 and 96 hs of the CY concentration exposition (39, 156, 625, 2,500 µg CY/L) were fixed in Carnoy’s solution. The tissues were washed in phosphate buffer saline (PBS) and dehydrated in a series of increasing concentrations of ethanol, cleared by xylene and paraffin embedded (Cicarelli, San Lorenzo, Sta. Fe, Argentina). Sagittal 5 µm-thick sections were obtained with a Reichert Hn 40 microtome and placed onto 1% gelatin-coated glass slides.

Reagents. Mouse monoclonal antibodies to CAS, to E-cadherin (clone 36 mouse IgG2a) and to α -catenin (clone 5 mouse IgG1) (Transduction Labs, Lexington, KY) were used at a 1:25 and 1:50 dilution in PBS. Rabbit polyclonal IgG to β -catenin was a gift from Dr. P. McCrea (MD Anderson Cancer Center, University of Texas), and was used in a 1:100 dilution in PBS. Mouse monoclonal antibody (IgG1, chicken, frog) directed to a 180 kDa N-CAM polypeptide (cytoplasmic domain) was purchased from the Developmental Studies Hybri-

doma Bank (Dept. of Pharmacology and Molecular Sciences, Johns Hopkins Univ. School of Medicine, Baltimore, MD, and Dept. of Biological Sciences, Univ. of Iowa, Iowa City IA, USA) and used at a 1:50 dilution in PBS. Monoclonal antibody GC-4, a mouse IgG1 to chicken N-cadherin was purchased from Sigma Chemical Co. (St. Louis, MO), and was used in a 1:50 dilution in PBS. Normal goat serum (Vector Laboratories, Burlingame CA, USA) was used as negative control, diluted 1:20 in PBS. Goat anti-mouse and anti-rabbit biotinylated secondary antibodies (Vector Laboratories, Burlingame CA, USA) were used at a 1:13 dilution. Avidin-fluorescein complex (Vectastain Elite, Vector Laboratories, Burlingame CA, USA) was used at a 1:100 dilution. Avidin-biotin complex (ABC kit Vectastain Elite, Vector Burlingame, CA).

Cas and CAMs expression. Sagittal sections at cerebral level from *Bufo arenarum* tadpoles were dewaxed in xylene, re-hydrated and permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO.) in PBS for 15 min. The sections were incubated in goat normal serum for 30 min followed by overnight incubations with the primary antibodies at 4°C, in a wet chamber. The sections were rinsed in PBS and incubated with species-specific biotinylated secondary antibodies containing goat normal serum at 1:20 dilution. Sections were pre-treated with 0.3% H₂O₂-methanol for 1 h, washed in

PBS for 15 min. After, to reveal CAS expression, the sections were incubated with avidin-fluorescein complex for 30 min, rinsed two times in PBS for 5 min and mounted with Vectashield (Vector Laboratories, Burlingame CA, USA). In contrast, to reveal CAMs expression, the sections were incubated with avidin-biotin complex for 30 min, rinsed in PBS for 5 min and color developed with 3,3'diaminobenzidine substrate (Sigma, St. Louis, MO.). Slides were mounted with Canada balsam (Biopack, Buenos Aires, Argentina).

Negative controls were done replacing the primary antibodies with goat normal serum diluted 1:20. Sections were examined and photographed in a BX50 Olympus microscope.

Cell adhesion molecule quantification

For cell adhesion molecule expression quantification, images of brain sagittal sections from three surviving *Bufo arenarum* tadpoles of both control and 96-h CY treated (2,500 µg CY/L) ($n=3$) were registered using an Apogee CCD camera.

Data of immunolabelling intensity for each molecule were registered in the preestablished random areas of a squared grid applied on the right ocular of microscope. The squared grid was superposed successively through the sections brain using a X50 lens. The

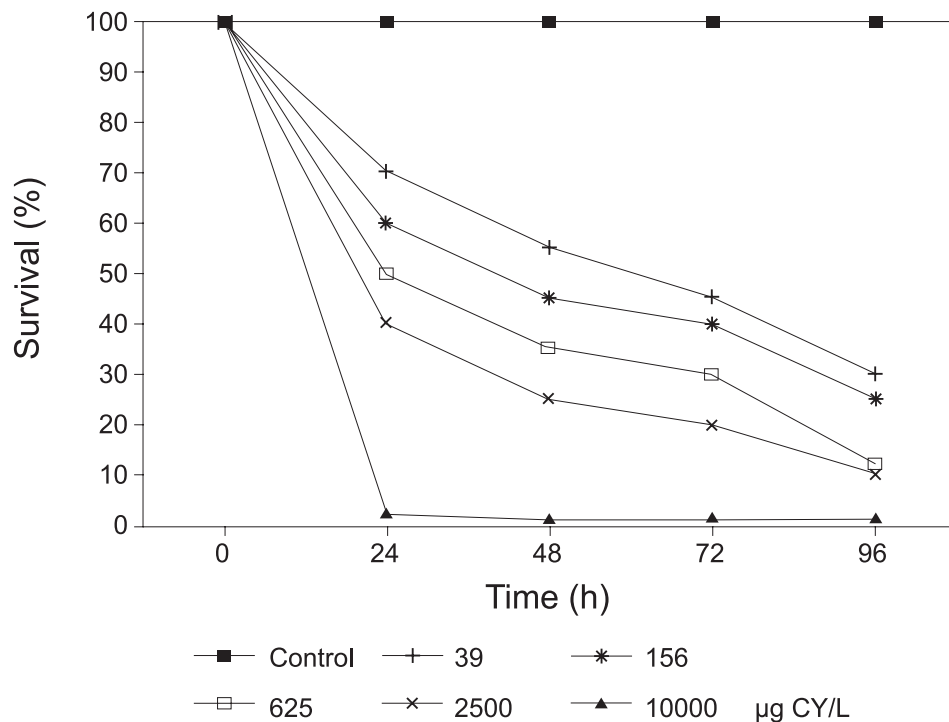


FIGURE 1. Survival curves for *Bufo arenarum* tadpoles exposed to cypermethrin, number of independent experiments $n=3$, ten larvae each.

immunolabel intensity of the selected areas was analyzed with Image ProPlus 4.5.0.29 (Image ProPlus Software, Media Cybernetics, Inc.).

Data were expressed as mean (M) \pm standard deviation (SD) and analyzed with GraphPad InStat 3.06 (GraphPad Software, San Diego, California, USA). Mean analysis of the results were performed by the t test unpaired with and without Welch's correction conform SD and normality of the curves, or non parametric Mann-Whitney test by non normal curves. Differences were considered significant with $p < 0.05$ values.

Results

Bioassay

High death rates (~65-70%) were observed exposing to cypermethrin at concentrations between 39 and 156 μg CY/L for 96 h (Fig. 1). There were no deaths among tadpoles treated with vehicle solution. Between 625 and 2,500 μg CY/L, the survival rate was approximately 15% at 96 h. At 24 h, practically all the tadpoles treated with 10,000 μg CY/L died (Fig. 1).

LC50 values found at different times of exposure to CY are shown in Table 1. The LC50 values after 24 h of treatment were 6, 13 and 30 times less at 48, 72, and 96 h respectively, indicating a linear increase in toxicity with increased exposure time.

Moreover, a dose-dependent hyperactivity syndrome and abnormal behavior of the tadpoles was observed, including lateral curving of the tails, arching of the cephalic-caudal axis, twisting and sinuous writhing of the body, and circular movement activity.

TABLE 1.

Acute toxicity response (LC50) of *Bufo arenarum* tadpoles exposed to cypermethrin, $n=3$.

Time (h)	LC ₅₀ mg CY/L	Confidence limits	
		Lower	Upper
24	3274	428	9550
48	488	47	2350
72	249	12	1104
96	110	3	476

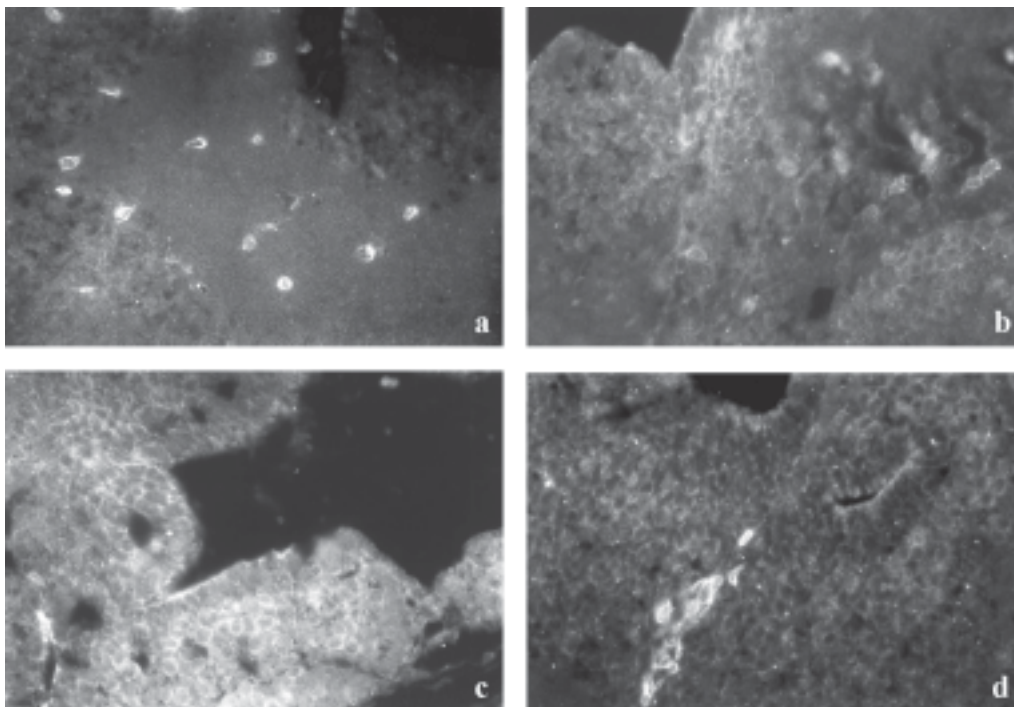


FIGURE 2. CAS expression in the brain of stage 28 *Bufo arenarum* tadpoles (a) control and treated with CY during 24 h (b-d) (133X). Cas expression increases from 39 μg CY/L (b) to 156 μg CY/L (c), correlated with the increase of cellular apoptosis. However, from 2,500 μg CY/L (d) Cas decrease exhibiting high immunolabel on specific cellular groups.

CAS expression in Bufo arenarum brain

Control tadpoles of stage 28 *Bufo arenarum* showed CAS expression in specific cellular population of the brain (Fig. 2a), suggesting cerebral stem cells. At 24 h of the bioassay, the tadpoles treated with 39 μg CY/L showed a generalized cerebral expression (Fig. 2b). When the CY concentration increases, the CAS cellular expression also increases, which is concordant with the generalization of the programmed cellular death process (Figs. 2b-c). However, the animals treated with 2,500 μg CY/L showed a decrease in the CAS expression at the specific cellular groups (Fig. 2d).

At 48 h of CY treatment, the same behavior is verified in the CAS expression than that observed at 24 h (Figs. 3a-c). In contrast, at 72 h only the 39 μg CY/L concentration displays high levels of CAS, decreasing suddenly its expression at higher concentrations (Fig. 3d), whereas at 96 h no signal was detected for all concentrations used.

In all cases, CAS was expressed in the cellular cytoplasm, and was not detected at nuclear level. The CAS regional expression does not exhibits a specific pattern.

CAMs expression in Bufo arenarum brain

The control tadpole central nervous system exhibits N-CAM180 and N-cadherin expression in neuronal soma and axons. The expression of N-CAM180 in neurons was significantly altered by CY treatment. In contrast to the relatively homogeneous expression of N-CAM180 observed in the neurons of control tadpoles, CY-treated tadpoles exhibit a significant increase of N-CAM180 in the neuronal cell body (116.5973 vs. 123.5720, $p \leq 0.005$) (Figs. 4a-b and 5) and a significant decrease in the axons regions (144.4598 vs. 141.3270, $p < 0.005$) (Figs. 4a-b and 5).

A discernible expression of β -catenin was detected on immature neurons and choroid plexus in the central nervous system (Fig. 4c). In CY-treated animals, β -catenin showed a significantly decreased expression at axons compared with vehicle-treated controls (156.4825 vs. 165.3559, $p < 0.005$) (Figs. 4c-d and 6). However, neuronal cell body, exhibit not significantly differences (136.3876 vs. 130.7324) (Figs. 4c-d and 6). The levels of α -catenin remained unchanged after cypermethrin treatment (not shown).

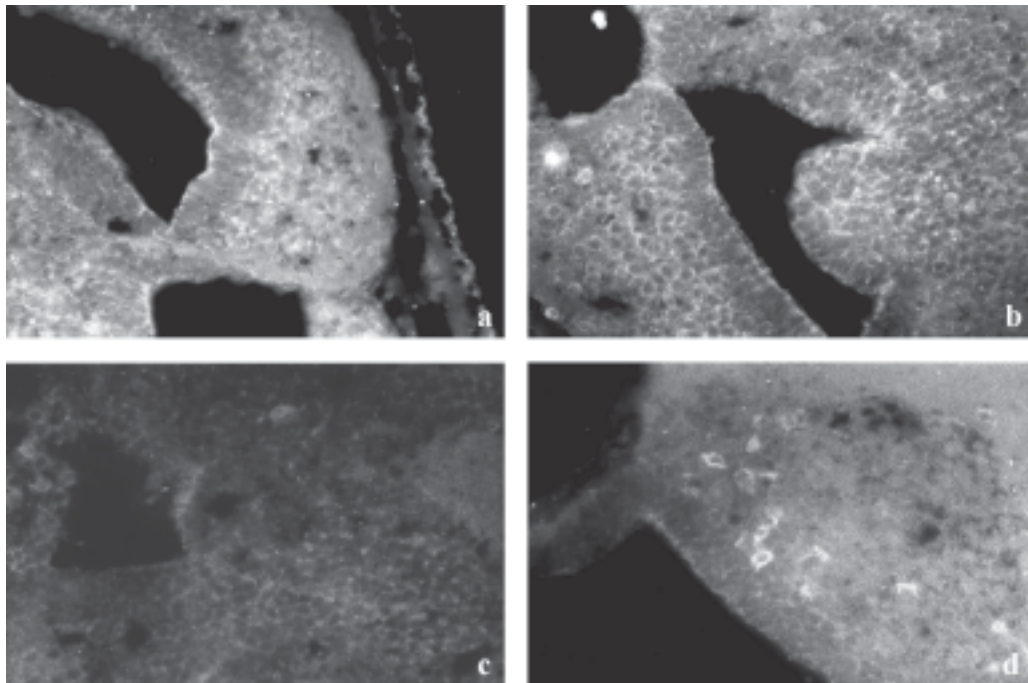


FIGURE 3. CAS expression in the brain of stage 28 *Bufo arenarum* tadpoles treated with CY during 48 h (a-c) and 72 h (d) (133X). The time increase (48 h) of the CY treatment produces a Cas cellular expression decrease with the increase of the CY dosis from 39 μg CY/L (a) to 156 μg CY/L (b) and to 2,500 μg CY/L (c). This decrease is pronounced from 72 h treatment (d) where the tadpoles treated with 39 μg CY/L exhibit high immunolabel on specific cellular groups, similar that occurs with high CY concentration at 24 h. At 96 h no CAS signal was detected for none CY doses.

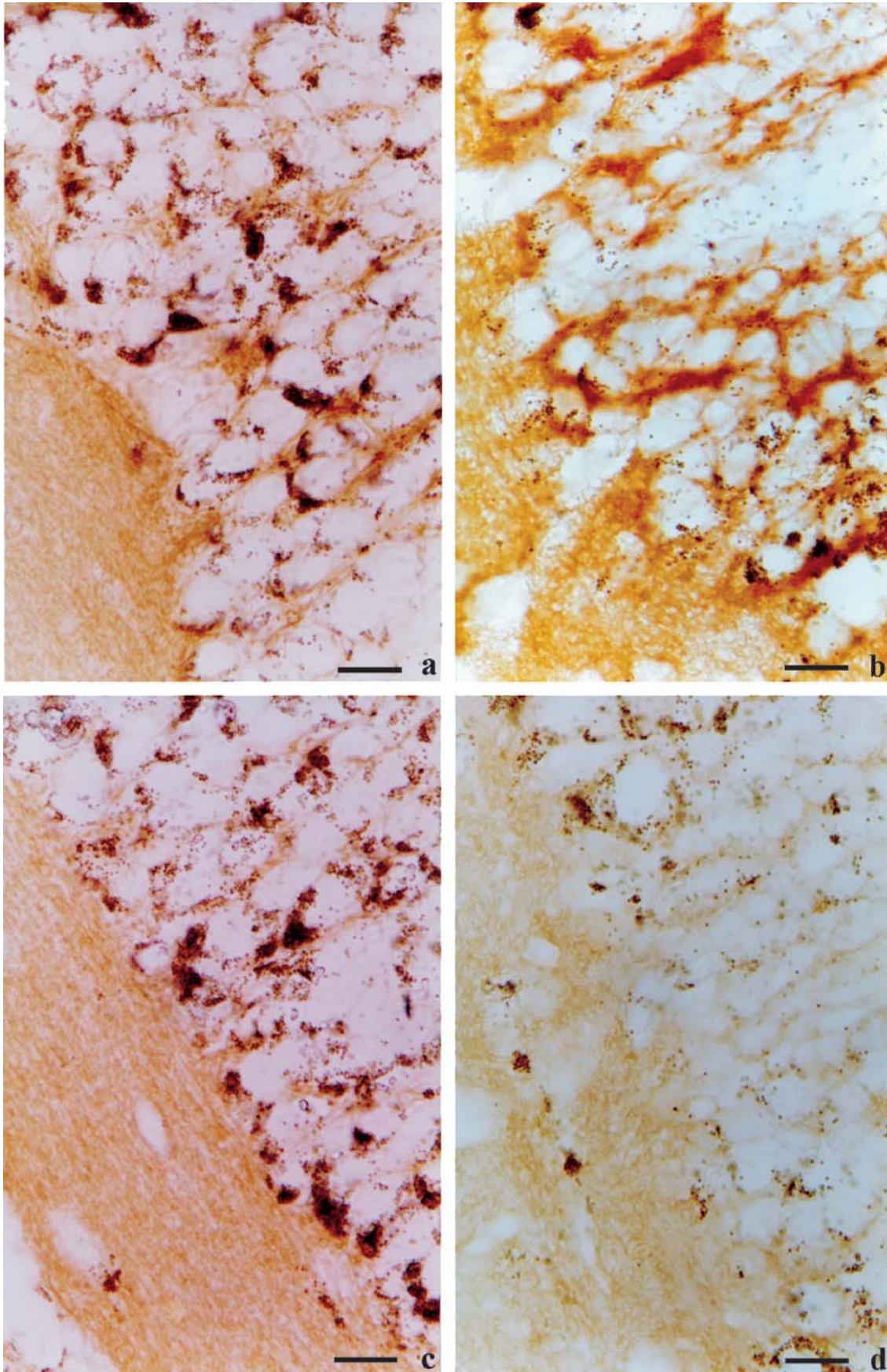


FIGURE 4. Sagittal sections of control and 2,500 µgCY/L-treated at 96 h *Bufo arenarum* tadpoles at central nervous system level. (a) N-CAM180 expression in control tadpoles, X333 and (b) CY-treated tadpoles, X333. (c) β-catenin expression in control tadpoles, X333 and (d) CY-treated tadpoles, X333.

Corroborating our previous studies of normal larval development (Izaguirre *et al.*, 2001b), E-cadherin was detected only in the peripheral nervous system level and did not exhibit changes of its expression pattern in CY-treated tadpoles (not shown).

Discussion

The CAS gene is highly conserved in eukaryotes, it consists of 25 exons. The mRNA homologous over its entire length to the yeast homologue CSE1 is the predominant transcript in proliferating tissues. Additional mRNAs are generated by alternative splicing in tissue-specific manner. An extended 3'-end is found in fetal and adult brain. mRNA containing the 5'-end of CAS up to position 690 and an alternative 3'-end is expressed in trachea and encodes a truncated Ran-binding domain. Fetal liver expresses mRNA with deletions of a central portion of CAS and additional sequences encoded by the last intron. SW480 colon cancer cells express another approximately 1500-base mRNA. It is known that

there is a correlation of CAS transcripts and CAS protein in different tissues (Brinkmann *et al.*, 1999). Thus, CAS isoforms may control nuclear transport of tissue-specific proteins. Therefore, hCSE1/CAS can play a role in several and oppose processes as the cellular proliferation and apoptosis (Brinkmann *et al.*, 1996a; Behrens *et al.*, 2003).

In order to determine if the protein CAS act as an anti-apoptotic factor in the *Bufo arenarum* cerebral apoptosis induced by the CY toxin, stimulating cellular proliferation, the CAS expression pattern was established by immunohistochemistry techniques.

Cypermethrin (RS)-alpha-cyano-3-phenoxybenzyl (1RS)-cis-, trans-3-(2, 2, -dichlorovinyl)-2, 2-dimethylcyclopropane carboxylate) is a highly active synthetic pyrethroid type II. These are among the most active insecticides and at concentrations as low as 0.02 to 0.05% (5 to 200 g/L ha) can be as efficient as organophosphorous compounds (Kaloyanova *et al.*, 1991).

Pyrethroids are characterized by strong, broad-spectrum insecticidal activity, based on their neurotoxicity

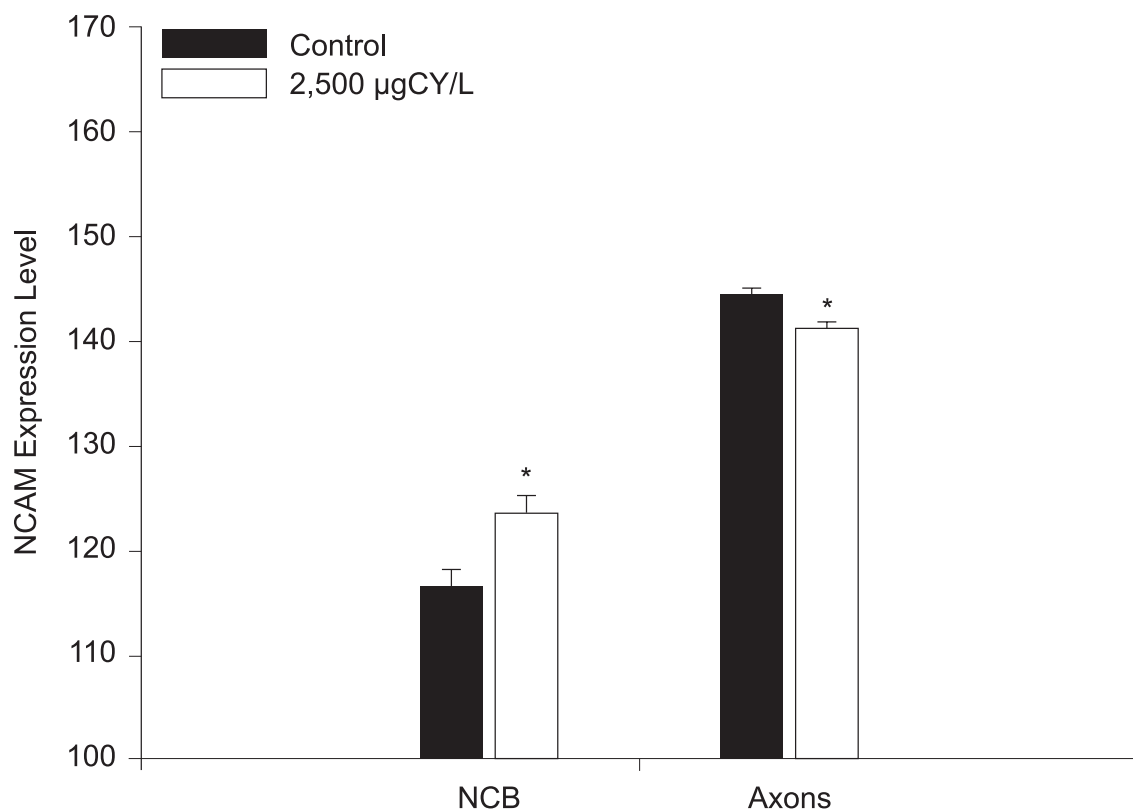


FIGURE 5. N-CAM180 expression on the neuronal cell bodies (NCB) and axons of *Bufo arenarum* tadpole brains treated with 2,500 µg CY/L at 96 h. NCB CY-treated vs. control: * $P < 0.005$; Axons CY-treated vs. control: * $P < 0.005$.

(Cole and Casida, 1983; Kaloyanova *et al.*, 1991; Salibián, 1992; Izaguirre *et al.*, 2000a; 2001a; Casco *et al.*, in press). They act on the axons of the peripheral and central nervous system by interacting with sodium channels gating mechanisms. Synthetic pyrethroids induce delaying in closing of sodium channels (Kaloyanova *et al.*, 1991) producing alterations in the ion conductance of nerve cell membranes, which may be one of the mechanisms of pyrethroid toxicity (Salibián and Marazzo, 1995). This in turn results in increased transmembrane sodium influx and inhibition of ion-dependent ATPases in the nervous systems of insects, squids and toads (Berlin *et al.*, 1984). One by changes in the flow of K^+ and Na^+ are known to play a critical role in the activation of neuronal apoptosis. Our studies have demonstrated that lower doses than those used in routine applications can cause massive apoptosis in the central nervous system of anuran larvae (Izaguirre *et al.*, 2000a, 2001a; Casco *et al.*, in press).

The present work demonstrate that during the first hours of the CY intoxication, the CAS expression is increased in the brain when are used low doses of CY

(39 - 156 μg CY/L). These results suggest that the survival brain cells increase CAS synthesis and /or brain stem cells could proliferate opposing the apoptosis induced by cypermethrin. Recently, Wellmann *et al.* (2001) have found that the localization of CAS expressing cells is correlated with the distribution of putative stem-cells. However, when the CY doses increase (up to 2,500 μg CY/L) or the CY exposition times reach at 96 h, CAS expression became restricted to specific cellular groups, like in the normal brain. Present results allow us to speculate that, under these conditions, CAS expression may be unable to act as a survival signal.

Scherf *et al.* (1996) found that CAS is present in the cytoplasm of proliferating cells at levels between 2×10^5 and 1×10^6 molecules *per* cell. The intracellular distribution of CAS resembles that of tubulin. Thus, in interphase cells, CAS shows microtubule-like patterns and in mitotic cells it labels the mitotic spindle. However, CAS appears to be associated with, but not to be an integral part of microtubules. Elevated amounts of CAS are found in proliferating cells such as testicular spermatogonia and cells in the basal layer of the colon.

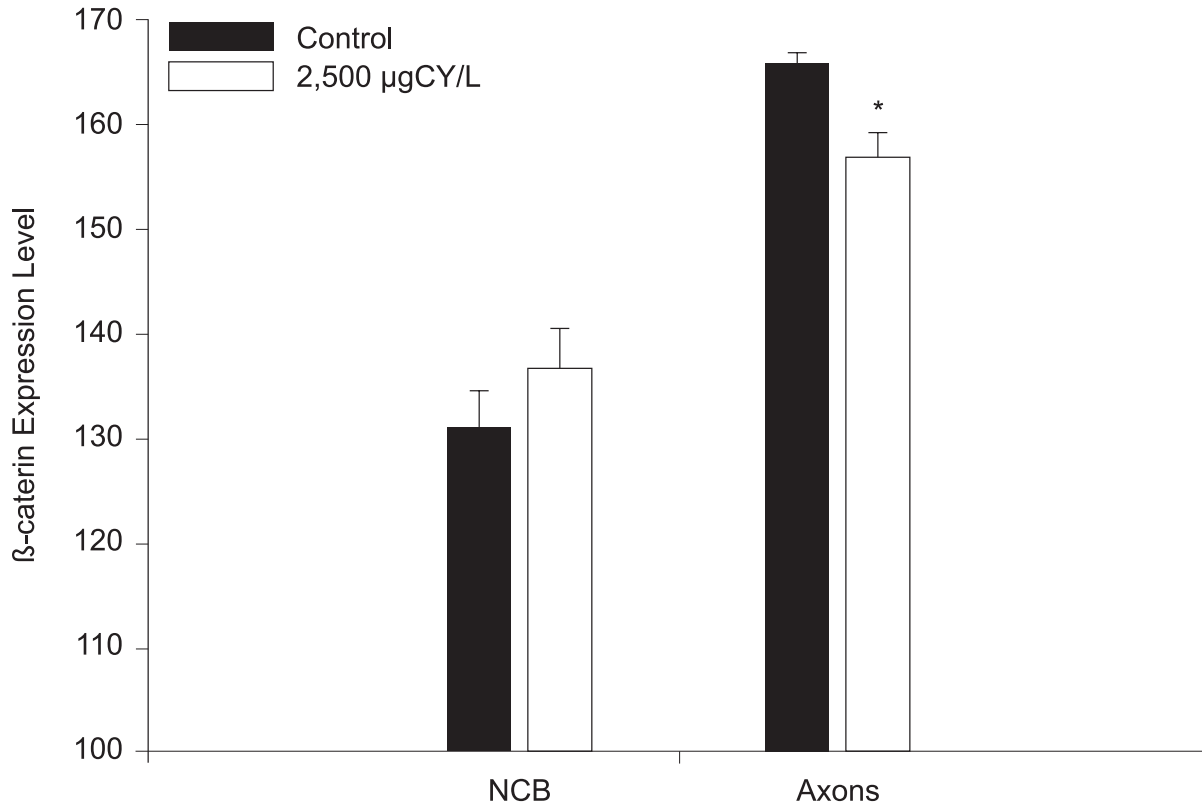


FIGURE 6. β -catenin expression on the neuronal cell bodies and axons of *Bufo arenarum* tadpole brains treated with 2,500 μg CY/L at 96 h. NCB CY-treated vs. control: no significant; Axons CY-treated vs. control: * $P < 0.005$.

CAS was also concentrated in the respiratory epithelium of the trachea and in axons and Purkinje cells in the cerebellum. These cells contain many microtubules. The cellular location of CAS is consistent with an important role in cell division as well as in ciliary movement and vesicular transport (Scherf *et al.*, 1996).

In the other hand, it has been reported that the expression of CAS is up regulated in a variety of human tumor cells, and such expression correlates with the development of tumors, and with the susceptibility of tumor cells to IFN-gamma-induced apoptosis. The IRF-1 transcriptional factor mediates IFN-gamma-induced CAS expression. CAS over expression *via* IFN-gamma enhances the CPP32 cellular expression and apoptosis. Because of CPP32 is regarded as one of the central apoptosis executioner molecules, it is possible that tumor cells highly expressing CAS may be more susceptible to apoptosis induced by reagents that are capable of inducing CAS expression. Thus, CAS may be a target for the elimination of tumors (Jiang *et al.*, 2001). Therefore, CAS is not only up regulated in proliferate cells but also in apoptotic cells induced by IFN-gamma. However, our previous results (Casco *et al.*, in press) suggest that the CAS expression increases is doubt at the protective factor to the apoptosis and non for cellular increase of apoptosis susceptibility, as is demonstrated for the CY dose- and time-dependent apoptosis increase, and the Cy doses- and times-dependent CAS expression decrease.

These results show that a single molecule can participate in proliferation and apoptosis through of the CAS expression increase. In support at these results, Brinkmann *et al.* (1996b), working with an antisense cDNA fragment homologous to the yeast chromosome segregation gene CSE1, found that those reduces the intracellular content of the human CSE1 homologue CAS protein. CAS reduction confers resistance not only to the ADP-ribosylating toxins PE and DT, but also to tumor necrosis factor alpha and beta. The resistance was observed as reduced apoptosis. CAS antisense did not affect the cell death induced by staurosporine, cycloheximide, or etoposide. The observation that CAS antisense can interfere with apoptosis mediated by TNF and ADP-ribosylating toxins suggests that CAS may play a role in selected pathways of apoptosis.

It is known that SRC family kinases play essential roles in a variety of cellular functions, including proliferation, survival, differentiation, and apoptosis. The activities of these kinases are regulated by intramolecular interactions and by heterologous binding partners that modulate the transition between active and inac-

tive structural conformations. p130^{CAS} binds directly to both the SH2 and SH3 domains of c-SRC and therefore has the potential to structurally alter and activate this kinase. The c-SRC activation induces c-SRC-dependent tyrosine phosphorylation of multiple endogenous cellular proteins, acting as an adaptor protein. These events suggest that CAS plays an important role in regulating specific signaling pathways governing cell growth and/or survival, in part through its ability to interact with and modulate the activity of c-SRC (Burnham *et al.*, 2000). Therefore, through its ability to undergo rapid changes in phosphorylation, subcellular localization and association with heterologous proteins, CAS may spatially and temporally regulate the function of its binding partners. Numerous proteins have been identified that bind to CAS *in vitro* and/or *in vivo*, but in only a few cases there is an understanding of how CAS may function in these protein complexes. To date, CAS-Crk and CAS-Src complexes have been most frequently implicated in CAS function, particularly in regards to processes involving regulation of the actin cytoskeleton and proliferation.

It is well known that in normal cells, the p130^{CAS}-associated tyrosine kinase activities are anchorage-dependent. Thus, cell detachment triggers rapid dephosphorylation of p130^{CAS} in the anoikis-sensitive normal epithelial cells. FAK and Src are p130^{CAS}-associated tyrosine kinases, which tyrosine phosphorylating mechanisms are altered in the tumor cells. Inhibition of Src specifically abolished phosphorylation of p130^{CAS} and induced anoikis. Furthermore, over expression of dominant-negative forms of p130^{CAS} also induced apoptosis. Taken together, these data suggest that p130^{CAS} mediates a cell survival signal from cell-matrix interaction. Alterations in tumor cells that lead to constitutive phosphorylation of p130^{CAS} can prevent cells from anoikis; hence contribute to tumor cell anchorage independence and metastasis (Wei *et al.*, 2002). Furthermore, Jiang *et al.* (2002) found that the interaction of CAS with E-cadherin, a cell-cell adhesion molecule, enhances the formation of E-cadherin/beta-catenin cell-cell adhesive complex. Their results indicate that CAS cooperates with E-cadherin and plays a role in the establishment of cell-cell interactions and epithelial cell polarity.

Our results suggest that exists a relation between neuronal apoptosis and survival and cell adhesion mediated by CAMs. Cell adhesion is a multifactorial mechanism that depends on the expression of tissue-specific isoforms of cell adhesion proteins, surface density of individual molecules and regulatory factors (Edelman, 1988). N-cadherin and N-CAM both play a

key role in the central nervous system morphogenesis and function in vertebrates (Rutishauser, 1984; Hatta and Takeichi, 1986; Cunningham *et al.*, 1987; Rutishauser and Jessell, 1988; Neugebauer *et al.*, 1988; Matsunaga *et al.*, 1988; Drazba and Lemmon, 1990; Bixby and Zhang, 1990; Takeichi *et al.*, 1991; Doherty *et al.*, 1991; Paz *et al.*, 1995; Izaguirre *et al.*, 2000b). Catenins are part of a sub-membranous protein network by which cadherins are connected to other integral membrane proteins (McNeill *et al.*, 1990). Catenins regulate the extracellular adhesive properties of cadherins as well as their interaction with the cytoskeleton (Kemler *et al.*, 1990). Catenins also mediate signals for gene expression during development and tissue morphogenesis (Matsuyoshi *et al.*, 1992; Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Hamaguchi *et al.*, 1993; Behrens *et al.*, 1993; Shibamoto *et al.*, 1994; Hoschuetzky *et al.*, 1994). In this study, we did not detect changes in the expression pattern of N-cadherin, E-cadherin and α -catenin due to CY treatment. These results suggest that the loss of cellular contacts of CY-induced apoptotic cells in the central nervous system may not completely depend on down regulation of cell adhesion molecules expression but possibly on loss of functionality. Additionally, we verified that N-CAM180 increased in neuronal cell body, likely as a compensatory mechanism against the loss of cellular contact and also as a protective factor inhibiting apoptosis. It is known that neural cell adhesion molecule plays a pivotal role in neural development, regeneration, and plasticity. N-CAM mediates cell adhesion and subsequent signal transduction through N-CAM - N-CAM binding. Pedersen *et al.* (2004) demonstrated that a small peptide mimicking homophilic N-CAM interaction is capable of inducing differentiation in several neuronal cell types and inhibiting apoptosis in cerebellar granule neurons. However, N-CAM expression decreased in the axons. In support of this result it is known that in a mature neuron, polarized targeting of proteins from the cell body to the axonal and dendritic processes is essential for its proper function, especially, for the maintenance of synaptic function. Any disruption of this protein distribution can lead to a disturbance in synaptic transmission and produce neurodegeneration.

The expression of β -catenin expression was also significantly decreased in axons suggesting catenin cleavage during the apoptosis. In agreement with these results, other studies have showed loss of cell-cell interactions and catenin cleavage by caspases during apoptosis (Brancolini *et al.*, 1998). The caspase fam-

ily of cystein proteases plays a key role during the execution phase of the apoptotic program. These proteolytic enzymes, once activated, cleave cellular proteins which are important for the maintenance of cell integrity. Multiple proteolytic events regulate the dismantling of the cell-cell junctional complexes during apoptosis (Brancolini *et al.*, 1998). However, other studies demonstrate that the proteolysis and changes of many cytoskeletal proteins associated with apoptosis precede degradation of catenins and cadherins by several hours (Schmeiser and Grand, 1999). Apparently, caspase-mediated degradation of cytoskeletal components, or disruption of adherens junction protein-protein interactions are not required for the morphological changes that occur during the apoptosis process (Schmeiser and Grand, 1999). Furthermore, it is known that in Alzheimer's disease phospho-beta-catenin accumulates in cytoplasmic inclusions that coalesce into granulovacuolar degeneration bodies and colocalize with gamma-tubulin and vimentin. These aggregates that might result from impaired proteasome function were associated with apoptotic cell death and with activation of caspase-3, c-Jun-N-terminal kinases, and c-Jun (Ghanevati and Miller, 2005).

The present work demonstrates the key role of CAS in the apoptotic process induced by CY, but additional experiments will be necessary to establish the intricate signaling mechanisms involved. Additionally, it has been demonstrated that whereas some cell adhesion molecules (CAMs) increase during apoptosis others are unaltered or decreased, suggesting different roles of adhesion proteins in this process. The loss of cellular contacts that is produced can occur without degradation of N-cadherin and α -catenin at least in the initial phases of the apoptotic mechanism or with decrease of β -catenin suggesting their cleavage. In contrast, N-CAM180 shows selective cell-cell adhesion mechanisms. Increased N-CAM180 in the neuronal body may reflect activation of anti-apoptotic mechanisms, whereas decrease in the axons may be the result of protein cleavage during apoptosis, or an effect of redistribution of molecules into plasmatic membrane from axons to soma. These results demonstrate the alterations on cell-cell adhesion induced by CY. Low doses and short time of CY treatment are associated with CAS expression increase and cell-cell adhesion molecules expression control.

These results suggest a dual role of CAS in the cellular cycle control during the CY-induced apoptosis, by inducing cell proliferation and by stabilization of the cell-cell junctions by modulating cell-cell adhesion molecule expression.

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