Chromaffin cell death induced by 6-hydroxydopamine is independent of mitochondrial swelling and caspase activation

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Abstract

Our results provide evidence that 6-hydroxydopamine induced, after auto-oxidation, toxic levels of hydrogen peroxide (H₂O₂) that caused bovine chromaffin cell toxicity and death. 6-Hydroxydopamine (6-OHDA) treatment markedly reduced, in a dose–response fashion, chromaffin cell viability. Cell death was accompanied by cell shrinkage, nuclear condensation and DNA degradation. Under our experimental conditions, 6-OHDA auto-oxidation formed quinones and reactive oxygen species (ROS) that mainly contributed to 6-OHDA-induced cytotoxicity in bovine chromaffin cells. Accordingly, different antioxidants, including catalase, vitamin E, Mn(IIItetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) or ascorbic acid, provided protection against 6-OHDA-induced toxicity. Further evidence that 6-OHDA induces oxidative stress is provided by the fact that this compound decreased total mitochondrial reduced NAD(P)H levels. Our results also suggest that mitochondrial swelling and caspase activation do not play a direct role in 6-OHDA-induced death in bovine chromaffin cells.

Keywords: apoptosis, cell death, mitochondria, neurodegeneration, oxidative stress, reactive oxygen species.


Bovine chromaffin cells are considered a good model to study neuronal function. These cells are of neuronal origin (Le Douarin et al. 1981), have sodium and calcium channels (Fenwick et al. 1982), release catecholamines in response to different stimuli (Calvo et al. 1995) and possess a catecholamine uptake system that is blocked by cocaine and desipramine (Ceña et al. 1984).

6-Hydroxydopamine (6-OHDA) is a neurotoxin widely used to selectively destroy catecholaminergic systems in either in vivo (Sauer and Oertel 1994; Crocker et al. 2001) or in vitro studies. Different cell types have been described as susceptible to 6-OHDA treatment, including primary rat striatal neurons (Shinkai et al. 1997; Cheng et al. 1996), chick sympathetic neurons (Ziv et al. 1994), human neuroblastoma cells (Simantov et al. 1996), rat pheochromocytoma (PC12) cells (Walkinshaw and Waters 1994), mouse thymocytes (Ofen et al. 1997) and chromaffin cells (Cano-Abad et al. 1998). Mechanisms responsible for 6-OHDA-induced toxicity remain unclear (for review see Blum et al. 2001). However, this toxicity might be mediated either by selective uptake through the catecholamine transporter followed by intracellular actions (Shimada et al. 1991; Usdin et al. 1991), or by extracellular oxidation (Blum et al. 2000). This is because, under physiological conditions, 6-OHDA is rapidly and non-enzymatically oxidized by molecular oxygen to form 1,4-para-quinone and its degradation products (Saner and Thoenen 1971), or reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide radical (O₂⁻) and hydroxyl radical (OH⁻). Cells continuously produce ROS, and their levels are regulated by a number of enzymes and physiological antioxidants. Nevertheless, an exacerbated ROS generation contributes to cellular dysfunction and cytotoxicity in a variety of pathological conditions (Jenner 1996; Owen et al. 1997; Butterfield and Kanski 2001). One of the downstream
Effects of ROS to trigger death pathways is the formation of a voltage-dependent high conductance multiproteic channel in the mitochondria, referred to as a mitochondrial permeability transitory pore (PTP). As a result of the PTP opening, mitochondria lose their membrane permeability control, releasing internal protein content to the cytoplasm (cytochrome c, apoptosis inducing factor, SMAC/Diablo, caspase family members, etc.). This release leads to the activation of different pathways that cause cell death (Susin et al. 1999). Caspases are crucial effectors of the cell death pathway activated by virtually all apoptosis-inducing stimuli within neurones (Jordan et al. 1997) and non-neuronal cells (for review see Creagh and Martin 2001). Among caspases, caspase-3 appears to play a pivotal role and has been found to be necessary for regulated cell death during brain development (Kuida et al. 1996).

In the present work, we explored 6-OHDA-induced toxicity mechanisms in bovine chromaffin cells. We found that 6-OHDA underwent an auto-oxidation pathway, generating ROS that were toxic to the cells. Accordingly, several antioxidants including catalase, vitamin E, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) and ascorbic acid provided protection to chromaffin cells against 6-OHDA-induced toxicity. However, 6-OHDA-induced toxicity did not produce either mitochondrial swelling or caspase activation.

Materials and methods

Cell culture
Bovine chromaffin cells were isolated as previously described (Jordan et al. 2000). Briefly, glands were washed with Ca²⁺-free Locke’s solution (Locke medium) containing (in mM/L) NaCl 154, KCl 5.6, MgCl₂ 1, HEPES 10, glucose 10, pH 7, to remove remaining erythrocytes. Glands were then incubated with Ca²⁺-free Locke’s medium containing 0.2% collagenase (Boehringer-Mannheim, Indianapolis, IN, USA) and 0.5% bovine serum albumin (Calbiochem, La Jolla, CA, USA) for 45 (3 × 15) min. Glands were then opened, the medulla separated from the cortex and incubated with collagenase for an additional 30 min. After filtering through a nylon mesh, chromaffin cells were separated from erythrocytes in a Percoll gradient. Cells were plated, at different densities (2 × 10⁶ cells/ml in culture flasks, or at 2 × 10⁵ cells/ml on poly-L-lysine-covered coverslips), in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (50 μg/mL). Cells were incubated at 37°C under an atmosphere of 5% CO₂.

Toxicity treatments and cell viability
For toxicity experiments, 6-OHDA was added to the cultures on day-3 in vitro, and cell viability was analysed 24 h later using the fluorescein diacetate/propidium iodide double-staining procedure (Jordan et al. 1997). Living and dead chromaffin cells were counted on adjacent fields of each coverslip for a total of 200–250 cells. The percentage of chromaffin cells surviving was determined on three to four coverslips for each condition and normalized to parallel controls. Each coverslip was treated as a single observation. Cell viability was determined from at least three separate experiments using different batches of 6-OHDA. Results are expressed as means ± SE, and significance was determined by Student’s t-test.

Chromatin and DNA studies
Chromatin was analysed by staining chromaffin cells with the dye Hoechst 33342 (Molecular Probes, Inc., Eugene, OR, USA; excitation/emission 350/460 nm). Cultures were rinsed three times with phosphate-buffered saline (PBS) and then incubated with 1 ng/mL Hoechst 33342 for 20 min at room temperature. After two rinses with PBS, cell staining was analysed using a fluorescent microscope.

For DNA fragmentation detection, 10 × 10⁵ untreated control cells, or cells treated with 6-OHDA (0.1 mM) or hydrogen peroxide (H₂O₂) (100 mM) for 24 h, were used. DNA was extracted by a modification of a previously described method (Jordan et al. 2000). Cells were rinsed twice in PBS and, after centrifugation, the pellet lysed in 1.25 mg/mL proteinase K (Roche Diagnostics GmbH, Mannheim, Germany), 50 mM Tris (pH 8), 10 mM EDTA and 0.5% sodium dodecyl sulphate overnight at 50°C. Samples were treated with 3 mg/mL boiled pancreatic Ribonuclease A (Roche Diagnostics GmbH) for 1 h at 50°C. DNA was then extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and precipitated overnight at −80°C with 0.1 m sodium acetate and 2 volumes of ethanol. After two rinses with 80% ethanol, DNA was incubated for 10 min with loading buffer, loaded onto a 2% agarose gel with 0.3 mg/mL ethidium bromide and run in TBE buffer (Tris-base 0.09 M boric acid and 2 mM EDTA, pH 8.0).

Fluorescence measurements of H₂O₂ production
Hydrogen peroxide generation was measured flurometrically as previously described (Votyakova and Reynolds 2001). Basically, 6-OHDA was added, at room temperature, to a standard incubation buffer that contained: 125 mM KCl, 2 mM K₂HPO₄, 5 mM MgCl₂, 10 mM HEPES (pH adjusted to pH 7.0 with KOH), 10 μM EGTA and scopeolitin (2 μM) in the presence of 1 U/mL horseradish peroxidase. Scopeolitin fluorescence was monitored at excitation/emission wavelengths of 365 nm (slit 3 nm)/460 nm (slit 5 nm) in a Perkin-Elmer fluorometer (luminiscence-spectrophotometer LS50B; Perkin-Elmer, Wellesley, MA, USA).

Formation of quinoidal products by 6-OHDA auto-oxidation
The formation of quinoidal products by 6-OHDA auto-oxidation was determined using a cell-free system. Briefly, 0.1 mM 6-OHDA was incubated at room temperature (22–25°C) for 15 min in culture medium with or without catalase, MnTBAP, vitamin E or ascorbic acid, and the formation of quinoidal products of 6-OHDA auto-oxidation was monitored by absorbance at 490 nm as previously described (Tiffany-Castiglioni et al. 1982) using a microplate reader (Bio-Rad, Hercules, CA, USA).

Assay of caspase enzymatic activity
Twelve and 24 h after treatment with 0.1 mM 6-OHDA, bovine chromaffin cells were collected in a buffer with the following composition (in mM/L): HEPES 25; EDTA 5; EGTA 1; MgCl₂ 5; dithiothreitol (DTT) 5; phenylmethylsulfonyl fluoride (PMSF) 1;....

10 μg/mL each of pepstatin and leupeptin; pH: 7.5. The cellular material was left for 20 min on ice and was then sonicated. The lysate was centrifuged for 20 min at 10 000 g and the supernatant fluid was quickly frozen in a methanol dry ice bath and stored at −80°C. Protein concentration of the lysates was quantified using the BCA Protein Assay (Pierce, Rockford, IL, USA). Lysates (30 μg protein) were incubated at 37°C in a buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, 0.1 M 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) and 10 mM DTN, with the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (DEVD-AFC) [15 μM in dimethylsulfoxide (DMSO), Calbiochem System Products, San Diego, CA, USA] (Jordan et al. 1997). Substrate cleavage emitted a fluorescent signal that was quantified in a Perkin-Elmer fluorometer (luminiscence-spectrophotometer LS50B) (excitation 400 nm, emission 505 nm). Enzymatic activity is expressed as arbitrary fluorescent units (AFU).

Mitochondria isolation
Mitochondria were isolated from bovine adrenal medulla as previously described (Campo et al. 1992). Briefly, tissue was manually homogenized by four strokes with a Teflon pestle in solution I (Sol I) containing 230 mM mannitol, 70 mM sucrose, 1 mM EGTA and 50 mM HEPES, pH 7.4, on ice. After centrifugation 106 g for 80 s, 4°C, the supernatant fluid was layered on solution II (460 mM mannitol, 14 mM sucrose, 1 mM EGTA and 10 mM HEPES, pH 7.4) and centrifuged at 800 g (3 min, 4°C). The top layer was then centrifuged at 2000 g (5 min, 4°C). The mitochondrial pellet was resuspended in 215 mM mannitol, 71 mM sucrose, 10 mM succinate and 10 mM HEPES, pH 7.4, and kept on ice until mitochondrial swelling determinations.

Mitochondrial swelling
Mitochondrial swelling was monitored as the change in light absorbance of the mitochondrial suspension at 540 nm as previously described (Kristal et al. 2000). Specifically, bovine adrenal medulla mitochondria were suspended in buffer containing 215 mM mannitol, 71 mM sucrose, 10 mM succinate and 10 mM HEPES, pH 7.4, to reach 0.5 mg protein/mL concentration, and aliquots (200 μL) of this mitochondrial suspension were then added to 96-well microplates. Changes in absorbance at 540 nm (A540nm) were followed using a microplate reader (Bio-Rad). Initial A540 Values were ≈ 0.8, and minor differences in loading were compensated by representing the data as the fraction of the initial absorbance value remaining at a given time. The lines shown are generated by linearly connecting the data from all time points.

Assay for mitochondrial NAD(P)H levels
The chemical stability of nicotinamide adenine dinucleotide coenzyme (NADH/NAD+) and its derivatives (NADPH/NADP+) was investigated using changes in the UV-visible absorption spectra of these compounds. NAD(P)H fluorescence in intact mitochondria (1 mg/mL at 25°C) was measured fluorimetrically using excitation and emission wavelengths set at 340 nm (slit 3 nm) and 460 nm (slit 5 nm), respectively, in a Perkin-Elmer LS50B luminiscence-spectrophotometer using a quartz cell with a 1.0 cm optical path, as previously described (Rover Junior et al. 1998). The experiments were started by measuring basal autofluorescence of 1.5 mL buffer (in mM: 230 mannitol, 5 HEPES and 70 sucrose, pH 7.4) during 60 s. Then, a freshly-prepared mitochondrial resuspension was added and allowed to reach stabilization for 120 s. After this time, different 6-OHDA concentrations were added. Under these conditions, an increase in autofluorescence signal indicates an increase in the reduced state of the pyridine nucleotide, NAD(P)H, and a decrease in autofluorescence signal indicates an increased oxidation to NAD(P)⁺.

Results
6-OHDA induces cell death in bovine chromaffin culture
Consistent with previous reports (Kirpekar et al. 1983; Cano-Abad et al. 1998), our data show that 6-OHDA produces cell death in bovine chromaffin cell cultures. We used the fluorescein diacetate/propidium iodide double-staining method to analyse the effects of 6-OHDA (1 μM to 100 mM) on chromaffin cell cultures (3 days in vitro). As shown in Fig. 1(a), 6-OHDA induced a marked reduction in cell viability, in a dose–response fashion, measured after 24 h of treatment, the IC50 being about 3 mM (Fig. 1a). Lower 6-OHDA concentrations, up to 10 μM, did not compromise cell viability, while higher concentrations (0.1–100 mM) resulted in gross morphological changes and were no longer selective for chromaffin cells, affecting all cell types present in the culture with the few cells remaining adhered to the coverslips being round in shape (data not shown). Under these conditions, we observed that cell plasma membrane integrity was altered, becoming permeable to propidium iodide. DNA staining using Hoechst 33342 clearly showed chromatin condensation and fragmentation (Fig. 2b). In the next set of experiments, we analysed DNA degradation in cultures treated with 0.1 mM 6-OHDA for 24 h and found a random degradation of DNA (Fig. 2c, lane 2).

6-OHDA-induced cell death is mediated by hydrogen peroxide
According to the well-known 6-OHDA auto-oxidation feature, due to the inherent instability of its catechol moiety, drug addition turned the culture medium a darkish violet colour. Hydrogen peroxide is a resulting product of 6-OHDA auto-oxidation (Saner and Thoenen 1971), and it has been proposed to play a role in the genesis of different neurodegenerative diseases (Behl et al. 1994). To determine whether H2O2 was involved in 6-OHDA-induced chromaffin cell death, we investigated, using the scopoletin oxidation method (Votyakova and Reynolds 2001), whether 6-OHDA auto-oxidation generated H2O2. As shown in Fig. 3(a), 6-OHDA induced H2O2 generation in a dose-dependent manner. Concentrations up to 10 μM did not result in significant changes in fluorescence, while higher concentrations (0.1–1 mM) caused marked H2O2 generation (Fig. 3a).
We have previously shown that ROS might mediate cell death in chromaffin cells (Jordan et al. 2000). To corroborate that H₂O₂ was toxic to chromaffin cell cultures, we added 100 mM H₂O₂ to the culture medium. After 24 h of treatment, cellular death increased markedly and DNA analysis rendered a similar pattern of cell death to that observed following 6-OHDA treatment (Fig. 2, lane 3).

Next, we explored whether H₂O₂ might mediate 6-OHDA-induced cellular death. The rationale for these experiments was that if 6-OHDA toxic effects were mediated by H₂O₂, antioxidant drugs should protect from 6-OHDA-induced death. As shown in Fig. 1(b), the addition of catalase (10 U/mL), which transforms H₂O₂ to H₂O, protected bovine chromaffin cell cultures against 6-OHDA (0.1 mM) and maintained until the end of the experiment. Cell viability was determined 24 h after 6-OHDA additions. Results are given as a percentage of the control and represent the mean ± SEM of 12 coverslips. *p < 0.05; **p < 0.001 versus vehicle conditions using Student’s t-test.

Effects of antioxidant drugs on quinone formation
Besides H₂O₂ production, 6-OHDA auto-oxidation results in quinone formation that might react with different proteins to produce inactive/destroyed quinoproteins (Tiffany-Castiglioni et al. 1982). The formation of quinones can easily be followed spectrophotometrically at 490 nm. In the next set of experiments, we explored whether quinone formation could be modified by the presence of scavenger drugs. In Fig. 3(b), 6-OHDA-induced quinone formation is shown in the presence or absence of antioxidant drugs. The degradation of superoxide dismutase activity, with the lipophilic antioxidant vitamin E (50 μM; 4 h pre-treatment), or even with the addition of ascorbic acid (0.01% final concentration).
0.1 mM 6-OHDA into quinones was fast but it was significantly blocked by 50 μM vitamin E, 10 nM MnTBAP and 0.01% ascorbic acid, while in the presence of 10 U/mL catalase quinone production was about 20% higher than control values (Fig. 3b).

6-OHDA depletes mitochondrial NAD(P)H levels
In the next set of experiments, we studied reduced NAD(P)H levels as an index of the redox mitochondrial state using an autofluorescence method (Maciel et al. 2001). When mitochondria (500 μg) were exposed to 6-OHDA (0.1–1 mM), a dose-dependent depletion in fluorescence was noticed, suggesting a decrease in mitochondrial NAD(P)H levels (Fig. 4a). In the next set of experiments, we exposed a commercial NAD(P)H tetrasodium salt (5 μM) to 6-OHDA (0.1–1 mM). As shown in Fig. 4(b), 6-OHDA (0.1–1 mM) directly decreased nucleotide auto-fluorescence. This is in good correlation with a displacement of the redox mitochondrial state toward a more oxidized state.

6-OHDA does not induce mitochondrial swelling
Mitochondria are key players in apoptosis triggering and can irreversibly lead the cell to death pathways by opening a mitochondrial permeability transition pore (Susin et al. 1999). As a result of PTP formation, water comes in and mitochondria swell. To determine whether mitochondria play a role in 6-OHDA-induced bovine chromaffin cell death, we studied the effect of 6-OHDA on mitochondrial swelling in isolated adrenal medulla mitochondria. In Fig. 5, the lack of effect of 0.1 mM 6-OHDA on mitochondrial swelling can be observed. The swelling ability of the mitochondrial preparations used in these experiments was tested by adding...
Ca\(^{2+}\) (75 \text{mM}) (Fig. 5). It is important to note that, under our experimental conditions, no significant mitochondrial swelling was observed in the presence of any of the 6-OHDA concentrations tested ranging from 0.01 to 10 mM.

**Caspase-like activity does not mediate 6-OHDA-induced death**

Caspases are a family of cysteine proteases with aspartyl protease activity that are key mediators in death pathways (Nicholson et al. 1995; Nicholson 1999). To explore the role of caspases in 6-OHDA-induced death, we treated chromaffin cell cultures with 0.1 mM 6-OHDA for 12 and 24 h, and DEVD-like caspase activity in cell lysates was determined by measuring DEVD-AFC hydrolysis. 6-OHDA failed to increase caspase activity (Fig. 6). In a parallel experiment, cells were treated with paraquat, an ROS generator that induces cell death in chromaffin cells (Jordan et al. 2000), and caspase activity determinations were performed 24 h later. As shown in Fig. 6, paraquat (1 mM) treatment markedly increased DEVD-like caspase activity.

**Discussion**

Consistent with previous data, exposure of bovine chromaffin cells in culture to 6-OHDA resulted in cell death. Our experiments suggest that this effect is mediated by an increased ROS production, mainly H\(_2\)O\(_2\), accompanied by NADPH depletion. Molecular targets for 6-OHDA toxicity are not well known and the precise mechanisms through which 6-OHDA induces cell death remain a matter of research. Herein, we present evidence which suggests that, in our model, the 6-OHDA death signal pathway does not seem to be mediated by mitochondrial swelling or by group II caspase members, but by H\(_2\)O\(_2\) generation.

Exposure of chromaffin cultures to 6-OHDA resulted in a marked reduction in cell viability, measured 24 h later, in a dose–response manner. Under our experimental conditions, we observed that cell plasma membrane integrity was altered to become permeable to propidium iodide, and we also found a non-ordered DNA degradation.

At physiological pH, 6-OHDA reacts with molecular oxygen to form quinones as well as ROS. Quinones react with nucleotic groups of macromolecules, leading to inactive/destroyed quinoproteins; these quinoproteins do not seem to contribute significantly to the observed cytotoxic effects of 6-OHDA in chromaffin cells because catalase, which increases quinone formation, blocked 6-OHDA toxicity. Under our experimental conditions, 6-OHDA quickly and non-enzymatically forms H\(_2\)O\(_2\). It is noteworthy that 6-OHDA concentrations which were non-toxic to chromaffin cultures did not increase H\(_2\)O\(_2\) production significantly, strongly suggesting a key role for H\(_2\)O\(_2\) in 6-OHDA-induced chromaffin cell death. Pre-treatment of the cells with catalase (Tiffany-Castiglioni et al. 1982; Cano-Abad et al. 1998) also prevented 6-OHDA-induced cytotoxicity. Moreover, H\(_2\)O\(_2\) addition to cultures produced a pattern of chromaffin cell death similar to 6-OHDA. Hydrogen peroxide can enter into the cells and react with trace metals to form the highly reactive OH\(^-\) (Koppenol 2001) that can oxidatively damage proteins, lipids and DNA (Beckman and Ames 1997). In agreement with previous reports (Blum et al. 2000), we observed protection against 6-OHDA-induced toxicity in chromaffin cells by treatment with different antioxidant drugs, such as vitamin E (Storch et al. 2000), the manganese porphyrin MnTBAP or ascorbic acid, suggesting that ROS generation plays a key role in 6-OHDA-induced chromaffin cell toxicity.

Following an increase in ROS production it is likely that chromaffin cell redox equilibrium would be displaced to a more oxidized state. Accordingly, we observed that

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@article{isni:00000001466119,
  title = {6-OHDA generates H\(_2\)O\(_2\) in bovine chromaffin cells},
  journal = {J. Neurochem.},
  volume = {84},
  pages = {1066–1073},
  year = {2003},
  author = {International Society for Neurochemistry}
}
6-OHDA decreased total mitochondrial reduced pyridine nucleotide levels in isolated adrenal medulla mitochondria. NAD(P)H, besides acting indirectly as an antioxidant in the reduction of GSSG to GSH, operates as an antioxidant in the matrix space both by scavenging toxic free radicals and repairing biomolecule-derived radicals (Kirsch and De Groot 2001).

Mitochondria have been linked to neurodegenerative diseases and can irreversibly commit the cell to death by opening a mitochondrial permeability transition pore (Sheehan et al. 1997; Green and Reed 1998; Brown et al. 2000; Kroemer and Reed 2000). Our results exclude the possibility that 6-OHDA might induce mitochondrial swelling because even high 6-OHDA concentrations (10 μM) did not produce mitochondrial swelling. The mitochondrial effects observed by other authors in the same models (Youn et al. 2002) are probably due to a secondary effect of 6-OHDA oxidation on cytoplasmic proteins and ulterior organelle dysfunction.

In addition, the data indicate that 6-OHDA does not induce caspase activation in chromaffin cell cultures. This lack of caspase activation might be due to 6-OHDA-induced ROS that might oxidize the enzyme and inhibit its activity (Borutaite and Brown 2001; Mannick et al. 2002). However, paraquat, a ROS-generating drug, induced a marked increase in caspase activity (Fig. 6). In agreement with our results, observations in Caenorhabditis elegans showed that 6-OHDA-triggered neural degeneration does not require activation of the CED-3/CED-4 death pathway (Nass et al. 2002), and in 6-OHDA-lesioned adult rats, caspase-3 was not activated (Crocker et al. 2001).

In summary, our results provide evidence that 6-OHDA induces, after auto-oxidation, toxic levels of H₂O₂ that caused chromaffin cell toxicity and death. Our results also suggest that mitochondrial swelling and caspase activation do not play a direct role in 6-OHDA-induced death in bovine chromaffin cells.

Acknowledgements

This work has been supported, in part, by grants 1FD97-0500 and SAF99-0060 from CICYT, BF12001-1565 from Ministerio de Ciencia y Tecnología, 01050 from Consejería de Sanidad, PA102-031 from Consejería de Ciencia y Tecnología JCCM and from Fundación Campollano to JC; BF12001-1058 and GC02-029 to MFG. We are grateful for the excellent technical work of Juana Rozalén. MFG is a fellow from JCCM.

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