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Nitric Oxide and Peroxynitrite Induce Cellular Death in Bovine Chromaffin Cells: Evidence for a Mixed Necrotic and Apoptotic Mechanism With Caspases Activation

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Treatment of chromaffin cells with nitric oxide (NO) donors (SNP and SNAP) and peroxynitrite produces a time- and dose-dependent necrotic and apoptotic cell death. Necrotic cell death was characterized by both an increase in lactate dehydrogenase and ATP release and changes in nuclei and cell morphology (as seen with fluorescence microscopy analysis with propidium iodide and Hoechst 33342). Apoptotic cell death was characterized by nuclear fragmentation and presence of apoptotic cell bodies, by a decrease in DNA content, and by an increase in DNA fragmentation. Treatment of chromaffin cells with lipopolysaccharide (LPS) or cytokines (interferon-γ, tumor necrosis factor-α) resulted only in apoptotic cell death. Apoptotic effects of NO-inducing compounds were specifically reversed, depending on the stimuli, by the NO scavenger carboxy-PTIO (CPTio) or by the NOS inhibitors L-NMA and thiocitrulline. NO-induced apoptotic death in chromaffin cells was concomitant to a cell cycle arrest in G_0G_1 phase and a decrease in the number of chromaffin cells in the G_2M and S phases of cell cycle. All NO-producing compounds were able to induce activation of caspase 3 and cytochrome c release, and specific inhibitors of caspase 3 and 9, such as Ac-DEVD-CHO (CPP32) and Ac-Z-LEHD-FMK, respectively, prevented NO-induced apoptosis in chromaffin cells. These results suggest that chromaffin cells could be good models for investigating the molecular basis of degeneration in diseases showing death of catecholaminergic neurons, phenomenon in which NO plays an important role.

Key words: nitric oxide; nitric oxide synthase; cellular death; chromaffin cells; glutamate; peroxynitrite; apoptosis; necrosis; adrenal medulla

Nitric oxide (NO) is a messenger molecule involved in several processes, including smooth muscle relaxation, neurotransmission, tumor cells, and bacterial killing (Moncada et al., 1991; Nathan, 1992; Garthwaite and Boulton, 1995). NO exerts its physiological effects by regulating the guanylate cyclase activity and possibly by mild, partially reversible covalent protein modifications, such as S-nitrosylation (Stamler, 1994; Martínez-Ruiz and Lamas, 2004). However, induction of a high NO-output system in response to cytokines or a massive production of NO following accumulation of the excitatory neurotransmitter glutamate (Dawson et al., 1991; Lipton et al., 1993) can result in cell killing. Neurons (Stamler et al., 1992), pancreatic β-cells (Bergman et al., 1992), or macrophages (Albina et al., 1993) seem to be particularly sensitive to NO toxicity.

Necrosis and apoptosis are distinct mechanisms of cell death, with very different characteristics. Necrosis is caused by toxic or traumatic events, with passive cell swelling, injury to cytoplasmic organelles including mitochondria, and quick collapse of internal homeostasis. Necrosis leads to membrane lysis, release of cellular contents, and resulting inflammation (Kerr and Harmon, 1991; Schwartz et al., 1993). In contrast, apoptosis is an active process of neuronal cell destruction with specific defining morphologic and molecular features, such as membrane blebbing, chromatin condensation, and DNA fragmentation (Kerr et al., 1972; Bursch et al., 1992). Although in some systems NO reacts with other radicals and causes necrotic cell death, in others progressive

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intra- or extracellular generation of NO may cause apoptosis (Albina et al., 1993; Messmer et al., 1995; Ankarcrona et al., 1994).

Enzymatically produced NO can undergo a large variety of nonenzymatic reactions, e.g., with cellular thiols, metals, or superoxide (O_2^-). Products arising from these secondary reactions, particularly peroxynitrite ($ONOO^-$), formed from NO· and O_2^-, have been implicated in cortical neuronal and PC12 cell apoptosis (Bonfoco et al., 1995; Estévez et al., 1995).

Oxidative stress that results in generation of free radicals has been implicated in a final common pathway for neurotoxicity in a wide variety of acute and chronic neurological diseases (for review see Murphy, 1999). In these disorders, excessive stimulation of excitatory amino acid receptors may trigger the production of free radicals. Particularly, neurotoxicity associated with overstimulation of N-methyl-D-aspartate (NMDA) receptors is thought to be mediated by excessive Ca^{2+} influx, resulting in a series of neurotoxic events (Lipton and Rosemberg, 1994). One of these events is the activation of nitric oxide synthase (NOS) and the resulting production of NO (Dawson et al., 1991). Another neurotoxic event is the stimulation of phospholipase A_2 or mitochondrial Ca^{2+} overloading, leading to generation of superoxide anion (O_2^-). NO· can react with O_2^- to form peroxynitrite ($ONOO^-$), resulting in a dose-dependent neuronal damage (Lipton et al., 1993).

Adrenal chromaffin cells are neurosecretory cells derived from the neural crest. In rats, these cells proliferate extensively before birth and, in a milder way, also throughout life (Malvaldi et al., 1968; Tischler et al., 1989). Adult rat chromaffin cells proliferation is regulated, in vivo, by neurally derived signals and, in vitro, by stimulation with peptidic growth factors or by activation of adenylate cyclase or protein kinase C that mimics the effects of neurotransmitters in adrenal medullary nerve endings (Tischler et al., 1994). Therefore, these cells could be used as a model for development of the nervous system.

In bovine chromaffin cells, the presence of a constitutively expressed neuronal NOS (nNOS) has been demonstrated by both biochemical and immunocytochemical methods (Oset-Gasque et al., 1994, 1998; Schwarz et al., 1998; Vicente et al., 2002). In addition, the presence of NOS fibers closely associated with those choline acetyltransferase (ChAT)-positive fibers innervating rat chromaffin cells has also been shown (Holgert et al., 1995; Tanaka and Chiba, 1996). In these cells, the L-arginine/NO/cGMP pathway has an important inhibitory role in both basal and acetylcholine (ACh)-stimulated catecholamine (CA) secretion (Schwarz et al., 1998; Vicente et al., 2002).

Tumoral chromaffin PC12 cell lines have been widely studied as models of necrotic and apoptotic cell death induced by different stimuli (such as serum and trophic factors deprivation or NO challenge; Chung et al., 1999; Bal-Price and Brown, 2000). However, there is little evidence in the literature supporting the possibility that chromaffin cells could undergo physiological apoptotic death (Jordan et al., 2000, 2002), and it is not known whether NO, apart from its biological actions, could have a neurotoxic effect in these cells. Therefore, we studied the effects of NO on necrotic and apoptotic cell death in chromaffin cells. This study was extended to $ONOO^-$, to determine the possible participation of this toxic anion on NO-induced cell death.

Our results demonstrate that treatment of chromaffin cells with NO donors and/or cytokines, the last inducing NO formation by NOS activation, produced mainly an apoptotic cell death, whereas treatment with $ONOO^-$ mediated both necrosis and apoptosis, depending on the dose and time of stimulation. Therefore, we assessed that the intensity of original insult could be related to the chromaffin cell death pathway. These results seem to demonstrate that chromaffin cells could constitute a good model for studying the molecular mechanisms underlying catecholaminergic neuron degeneration.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium, fetal calf serum, HEPES, and RNAse A were purchased from Gibco BRL (Poole, United Kingdom); collagenase from *Clostridium histolyticum* (EC 3.4.4.19) was supplied by Boehringer Mannheim S.A. (Barcelona, Spain). The NOS inhibitor S-methyl-L-thiocitruline hydrochloride (thiocitrulline) was obtained from Tocris Cookson (Bristol, United Kingdom). Antibiotics, cytosine arabinoside, 8-fluoro-desoxiuridine (FDU), lipopolysaccharide (LPS), interferon (IFN)-γ, superoxide dismutase (SOD), Hoesch 33342, neutral red, propidium iodide, olygomicin, rotenone, and nerve growth factor were from Sigma (Madrid, Spain). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). The luciferin-luciferase (L-L) reagent was from BioOrbit (1243-118 ATP Biomass Kit) and the test Kinesis 50 (Kinesis 50, 470-0023) from Bio-Rad Laboratories (Richmond, CA); SITO-13 and hydroethidine were from Molecular Probes (Eugene, OR). Anticytochrome c monoclonal antibody and the caspase 3 fluorogenic substrate peptide Ac-DEVD-amc were supplied by BD PharMingen International (Becton Dickinson Co., Mountain View, CA). Peroxynitrite and carboxy-PTIO were purchased from Alexis Biochemicals (Lausanne, Switzerland), and 2,3-diaminonaphtalene (DAN), CPP32, and Z-LEHD-FMK were from Calbiochem-Novabiochem Co (La Jolla, CA). All other chemicals were reactive-grade products from Merck (Darmstadt, Germany).

Chromaffin Cell Culture and Drug Treatments

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Vicente et al. (2005). Cell viability was checked by trypan blue exclusion, and chromaffin cell purity was assessed by specific incorporation of neutral red to these cells. Both parameters were routinely higher than 90%.

Cells were suspended at a 0.5×10^6/ml density in Dulbecco's modified Eagle's medium (DMEM) containing 10%

fetal calf serum, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 40 mg/ml gentamicin), and cytostatics (10 mmol/liter fluorodeoxyuridine and 10 mmol/liter cytosine arabinoside), plated in 24 Costar cluster dishes, and used 3–7 days after plating. Three to seven days after plating, cells were washed three times with DMEM and exposed to NO donors or cytokines, by adding the different compounds to culture medium in concentrated form and mixing very gently. The cultures were then incubated as indicated for the figures. In the case of peroxynitrite treatment, to minimize reactions of this compound with bicarbonate and other components in culture medium, cells were incubated in 1 ml DMEM for 1–2 min before addition of a single bolus of this compound. Peroxynitrite was quickly added on one edge of the culture dish, and buffer was quickly swirled for 5 sec to distribute the peroxynitrite within the dish. Five minutes after peroxynitrite exposure, buffer was removed and replaced by 10% fetal calf serum (FCS) supplemented culture medium. What we used as a control treatment was decomposed peroxynitrite (Pd), obtained by adding peroxynitrite to the buffer before addition to the cells.

Quantitation of Cytotoxicity

Cytotoxicity was estimated by quantization of lactate dehydrogenase (LDH) and ATP content and efflux from cells into incubation medium after different times of treatment.

Measurement of LDH Activity. For these measurements, 3–7 days aged bovine chromaffin cells grown in 24-well culture dishes were stimulated with NO donors, peroxynitrite, or cytokines in DMEM medium at short (15 min) or long (24 hr) intervals. Then, medium was collected and cells were lysed by scraping them in 250 μl of a buffer containing 0.5% (v/v) Triton X-100 in a 0.1 M potassium phosphate buffer, pH 7.4. The buffer was then removed after centrifugation at 10,000 rpm for 5 min, and LDH activity was determined in both the medium and the lysis buffer in the presence of 0.5 mM pyruvate and 0.15 mM NADH by the spectrophotometric method of Vassault (1983). Antagonists or blockers were added 5 min before agonists. [Percentage of LDH release = LDH in medium (LDH in medium + LDH in lysis buffer)].

Determination of ATP Release and Content. ATP release in response to different NO-generating compounds was determined by using the luciferin-luciferase (L-L) reagent (BioOrbit, 1243-118 ATP Biomass Kit) according to the manufacturer's instructions. Briefly, after stimulation of cells (0.5×10^6) with different NO-generating compounds or vehicle in 250–500 μl of a Krebs-HEPES solution (Locke medium) containing (mM) NaCl 140, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.5, ascorbic acid 0.5, and HEPES 10, pH 7.5, for variable times, supernatants were collected and cells frozen in 200 μl Locke's medium at –80°C. After freezing, 25 μl of supernatants or 25 μl of cell pellets diluted 1/10 in the Locke's medium were added to 25 μl of ATP Releasing Reagent and 50 μl of 0.1 mM Tris-acetic buffer, pH 7.75, and incubated for 5 min at 37°C in a BioOrbit 1251 Luminometer with continuous stirring. Then, 25 μl of ATP monitoring reagent (luciferin-luciferase) was added and the ATP content determined by the increase in luminescence. For calibrating the light signals, 10 pmol of ATP dissolved in Tris-acetic buffer was injected into each sample, and luminescence signal was determined. ATP cellular content was expressed as pmol ATP/10^6 cells and ATP release as percentage of ATP released over total ATP cellular content.

Measurement of Superoxide Production. Superoxide production was monitored with 5 μM dihydroethidine (HEt; Molecular Probes) as previously described (Jordan et al., 2000). Background was subtracted, and fluorescence was recorded at different times (4 hr and 24 hr) by using an excitation filter of 535 nm and an emission filter of 635 nm in a spectrofluorimeter (Bio-Tek FL 600). Fluorescence was recorded every 20 sec during a 7-min period. Linear regression of fluorescence data was calculated for each condition, and the slope (a) of the best fitting line ($y = ax$) was considered as an index of O_2^- production.

NO Measurements

For measuring amounts of NO released by different NO-generating compounds, nitrites (the final stable products of NO) were determined by using the spectrofluorimetric method of Misko et al. (1993). This method is based on the reaction of nitrite with 2,3-diaminonaphtalene (DAN) under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent derivative product. After cell (0.5×10^6) stimulation with tested compounds dissolved in Locke's medium during different times, 100 μl of supernatants were mixed with 10 μl of freshly prepared DAN (0.05 mg/ml in 0.62 M HCl). After a 10-min incubation period, in the dark at 37°C, the reaction was stopped with 5 ml of 2.8 N NaOH. Formation of the fluorescent 2,3-diaminonaphthotriazole was measured with a Perkin Elmer LS-50 fluorimeter. Excitation and emission wavelengths were set to 365 and 450 nm, respectively. The DAN reagent was protected from light. Nitrite standards (more than 98% purity; purchased from Sigma) were routinely freshly made, dissolved in double-deionized water, and kept on ice prior to use.

Analysis of DNA Fragmentation

Internucleosomal DNA fragmentation was assessed by agarose gel electrophoresis analysis of fragmented DNA by a modified version of the method of Lyons et al. (1992). The dishes ($1–2 \times 10^6$ cells) were washed twice with ice-cold PBS, and lysis was accomplished with 1 ml of 20 mM EDTA, 0.5% Triton X-100, 5 mM Tris-HCl, pH 8.0. Dishes were gently shaken for 15 min at 4°C. After that, intact nuclei were pelleted and eliminated by centrifugation at 500*g* for 10 min, and the supernatant was centrifuged at 25,000*g* at 4°C for 30 min. DNA from the supernatant was precipitated with ethanol 2.5 V at –80°C, pelleted by microcentrifugation at 4°C for 15 min, dried, and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer). Then, it was incubated with 0.1 mg/ml RNAse A during 30 min at 37°C and with 0.25 mg/ml proteinase K for 2–3 hr at 37°C to eliminate RNA and proteins. DNA was purified by phenol-chloroform extraction, precipitated with ethanol at –80°C, and resus-

pended in TE buffer. Then, DNA, stained with 0.5 μg/ml of ethidium bromide, was electrophoresed in a 1.5% agarose gel.

Confocal Microscopy Analysis

Morphology of chromatin from apoptotic and intact nuclei was detected by confocal microscopy analysis of chromaffin cells attached to plates, incubated for 15 min with SYTO 13 (3 μM) and neutral red (NR; 1%) in PBS. SYTO 13 is a permeable dye that selectively stains nucleic acids of all cells, emitting green fluorescence, and NR was used to stain selectively amide phenolic compounds (catecholamines), emitting red fluorescence. The use of both colorants together allowed us to determine whether there were necrotic or apoptotic processes taking place in the chromaffin cell population. Apoptotic chromaffin cells were distinguished for having green SYTO 13-stained nuclei inside red-stained cytoplasm. Thus, by using this method, we can distinguish between intact nuclei and necrotic/apoptotic nuclei. MRC 1000 confocal microscopy (Bio-Rad, Hempstead, United Kingdom) was used, and digital images were printed by using laser graphics.

Fluorescence Microscopy Analysis

Morphology of nuclei and cells was detected by fluorescence microscopy analysis of chromaffin cells attached to plates and incubated for 15 min with 0.005% propidium iodide (PI) and 1 μM Hoechst 33342 in PBS. Only necrotic cells and late apoptotic cells can be labelled by PI, because PI only enters cells that have lost their plasma membrane integrity. Hoechst 33342 specifically stains DNA, being able to enter all cells. Viable cells were identified by blue fluorescence resulting from Hoechst staining excited by UV light. Injured (necrotic and apoptotic) cells were identified by the penetration and intercalation of PI in DNA. Cells were visualized in a Nikon Diaphot (Japan) fluorescence microscope.

Flow Cytometric Analysis

Analysis of DNA content and cell cycle was performed in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). DNA was stained with PI by using the Bio-Rad reagent kit (Kinesis 50, 470-0023; Bio-Rad, Richmond, CA), following the manufacturer's protocol. Measurements were carried out using a Double Discriminator Module in order to discriminate doublets. Ten thousand cells were acquired per sample. After that, the percentage of cells with DNA content lower than 2C was calculated, as well as the percentage of cells in the G_0G_1, S, and G_2M phases of the cell cycle, by using Multicycle software (Phoenix Software, Mountain View, CA).

Fluorometric Analysis of Caspase 3 Activity

After 24 hr incubation with NO donors or cytokines, the culture medium was decanted, and the cells (0.5×10^6/well) were quickly washed twice with PBS wash buffer (140 mM NaCl, 2.7 mM KH_2PO_4, pH 7.5) and then resuspended in a lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaPPi, and 0.5% Triton X-100; 1 million/ml). Cell lysates were repeatedly passed through a syringe (25-gauge needle) until most of the cell membrane was disrupted and were centrifuged at 10,000*g* for 10 min at 4°C. Activity of caspase 3 was measured by using the fluorogenic substrate peptide DEVD-amc (66081; BD Biosciences PharMingen). Cytosolic protein (~50 μg) was incubated with 20 μM substrate peptide in a 150-μl final volume of incubation buffer [20 mM HEPES, 10% glycerol, 2 mM dithiothreitol (DTT), pH 7.5] at 37°C for 2 hr. The release of fluorogenic amc was measured by using a Perkin-Elmer fluorimeter (excitation at 380 nm, emission at 460 nm). A negative control (lysis buffer without cells) was used throughout.

Cytochrome c Determination

For analyzing cytochrome c release, chromaffin cells (5×10^6) were resuspended in 250 mM sucrose, 25 mM Tris/HCl, pH 6.8, 2.5 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, 50 μg/ml trypsin inhibitor, and 40 μg/ml aprotinin. Samples were centrifuged at 13,000*g* for 3 min at 4°C. Supernatants (containing the cytosolic fraction) and pellets (containing the mitochondrial fraction resuspended in 120 μl of a mitochondrial lysis buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, 300 mM KCl, 5% glycerol, 0.2% Triton X-100, 5 mM DTT, 0.2 mM phenylmethylsulfonylfluoride, 40 μg/ml leupeptin, 50 μg/ml trypsin inhibitor, and 40 μg/ml aprotinin) were denatured, separated by SDS-12% polyacrylamide gel electrophoresis (50 μg protein/condition), and transferred to Immobilon-P membranes, as described above. In all cases, immunoblots were blocked in TTBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dried milk and were incubated overnight with the primary antibody (diluted 1:1,000 in TTBS + 0.5% nonfat dried milk). After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (1:5,000 in TTBS + 0.5% nonfat dried milk) for 2 hr, and the blot was developed with the ECL system (Amersham, Buckinghamshire, United Kingdom).

Statistical Analysis

Data were expressed as mean ± SEM values of three or four independent experiments with different cell batches, each performed in duplicate or triplicate. Statistical comparisons were assessed by using one-way analysis of variance (ANOVA; Scheffe's F-test), followed in some instances by a two-way ANOVA. Differences were accepted as significant at $P < 0.05$.

RESULTS

NO-Producing Agents and Peroxynitrite Induce a Decrease in Chromaffin Cell Viability

When chromaffin cell cultures were treated with SNP, an NO donor; LPS; or cytokines (IFNγ and TNFα), no significant cell damage was observed at 15 min (Table I). However, 24 hr treatment of chromaffin cells with SNP or cytokines, alone or in combinations, produced a cell deterioration detected by an increase in LDH release ranging from 1.5 to 2 times basal levels for cytokines and SNP, respectively. The cytokine-induced increases in LDH release were specifically reverted by the NOS inhibitors L-NMA and thiocitrulline at a

TABLE I. Effect of SNP and Cytokines on LDH Release at Short and Long Incubation Times[†]

Conditions	% LDH release (ratio over control) A) 15 Min	B) 24 Hr
Control	1 ± 0.08	1 ± 0.05
SNP 1 mM	1.05 ± 0.02 ns	$2.53 \pm 0.20^{***000}$
LPS 10 ng/ml	1.13 ± 0.03 ns	$1.48 \pm 0.02^{***000}$
IFNγ 50 Ui/ml	1.18 ± 0.04 ns	$1.32 \pm 0.03^{***00}$
TNFα 10 nM	1.10 ± 0.02 ns	$1.25 \pm 0.01^{**00}$
LPS + IFNγ	1.17 ± 0.05 ns	$1.76 \pm 0.06^{***000}$
LPS + IFNγ + TNFα	1.20 ± 0.06 ns	$1.62 \pm 0.04^{***000}$
P	$6.53 \pm 0.65^{***}$	$3.42 \pm 0.40^{***000}$

[†]Chromaffin cells were stimulated for 15 min (A) or 24 hr (B) with SNP, P (peroxynitrite), or different cytokines alone or in combinations. Cells stimulated with P for 24 hr were washed after 15 min of incubation. Results, expressed as %LDH release ratio over control, are mean ± SEM of three experiments each performed in duplicate. (C 15 min = 2.71% LDH release over total cell content; C 24 hr = 5.60 ± 0.04%). Statistics compare the effect of different agents over control (ns, non significant; $^{**}P < 0.01$, $^{***}P < 0.001$) and the effect of time exposure for each condition ($^{00}P < 0.01$, $^{000}P < 0.001$; two-way ANOVA test).

1 mM concentration (Fig. 1A,B) and were directly correlated with NO amounts released by these compounds (Fig. 1A, inset). These results indicate that cytokine-induced cytotoxicity at 24 hr is due to NO formation. In the same way, dose-dependent increases in LDH release induced by SNP were specifically reversed by 1 mM CPTio (a NO scavenger) at all studied doses (Fig. 2A), suggesting that cellular death induced by this compound is due to NO. However, in the case of SNP, CPTio inhibition of increase in LDH release induced by SNP was dependent on SNP concentration. That is, whereas CPTio was able to block completely LDH release induced by low doses of SNP (1–10 μM), it was able to inhibit only about 70% of LDH release induced by high doses of SNP (0.5–1 mM). These results indicate that LDH release induced by high SNP doses could be due to peroxynitrite formation. This idea was confirmed by results obtained with SOD, a superoxide scavenger, which was able to reduce by 30–40% LDH release induced by high doses of SNP (Fig. 2A).

Peroxynitrite ($ONOO^-$) is generally considered the terminal neurotoxic species formed from NO· and O_2^-. Direct addition of 1 mM peroxynitrite, as a single bolus,

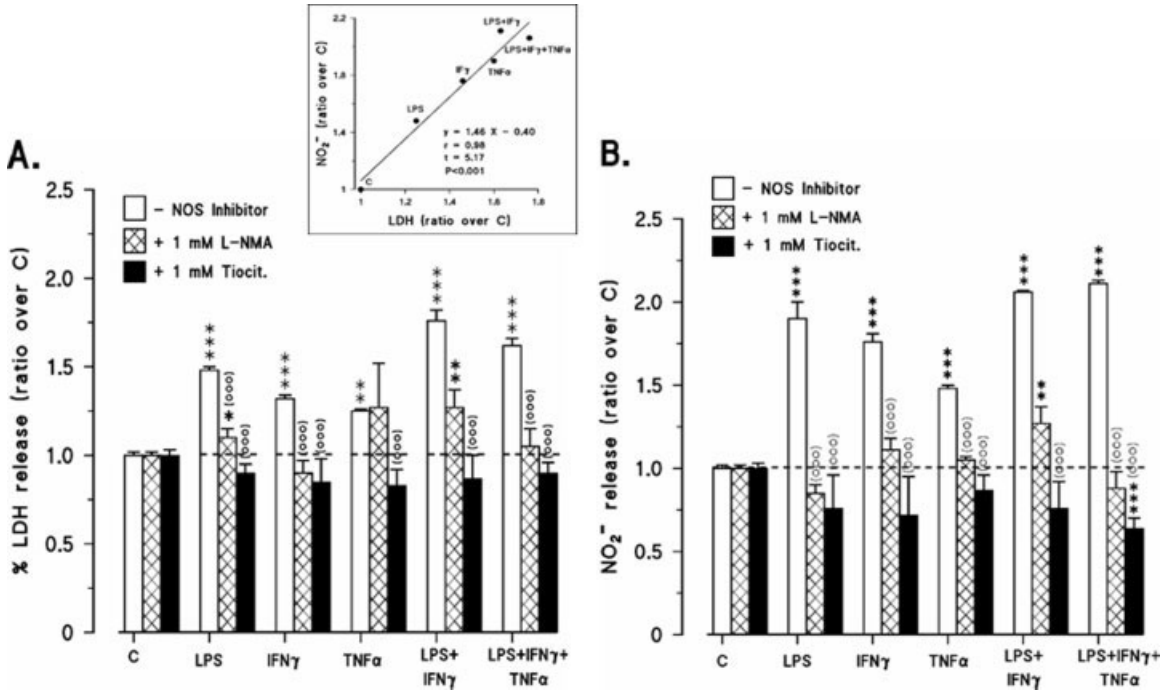

Fig. 1. Effect of LPS and cytokines on LDH release (**A**) and NO (nitrite) release (**B**) at long incubation times: reversion of effect by NOS inhibitors. Bovine chromaffin cells were treated during 24 hr with the indicated concentrations of cytokines, alone or in combination, and both LDH secretion and NO production were measured as described in Materials and Methods. Results were expressed in ratios of percentage LDH release or NO_2^- production over their control values. Data are mean ± SEM of three determinations performed in duplicated (n = 6). The statistical significances compare the effect of cytokines in the absence (open bars) and presence of 1 mM L-NMA (hatched bars) or 1 mM L-thiocitrulline (solid bars) over their own basal controls at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and the effect of NOS inhibitors on the increases in LDH release induced by each compound at $^{\circ\circ\circ}P < 0.001$ (two-way ANOVA). *Inset* shows linear correlation between LDH and NO (NO_2^-) induced by tested cytokines and their combinations. ($C_{\text{basal LDH secretion}} = 13.80\% \pm 0.50\%$ over total cellular content; $C_{\text{basal NO production (nitrite)}} = 0.58 \pm 0.04$ nmol/million cells); $LDH_{Tio} = 13.10\% \pm 0.40\%$; $NO_2^-{}_{Tio} = 0.52 \pm 0.02$ nmol/million cells; $LDH_{L\text{-}NMA} = 13.52\% \pm 0.51\%$; $NO_2^-{}_{L\text{-}NMA} = 0.48 \pm 0.06$ nmol/million cells.

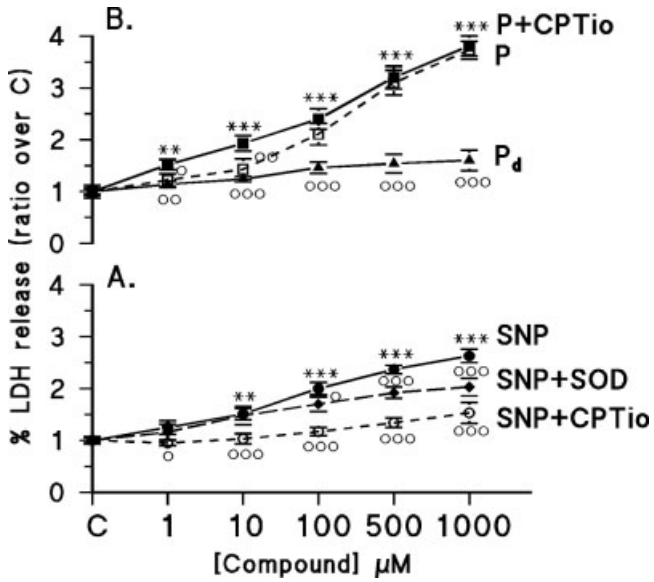

Fig. 2. Effect of SNP and peroxynitrite on LDH release at long incubation times: reversion by CPTio and SOD. Bovine chromaffin cells were treated for 24 hr with 1 mM SNP (**A**) or 1 mM P (peroxynitrite) or Pd (deactivated peroxynitrite; **B**) at the indicated concentrations, in the absence or presence of 100 μM CPTio or 50 UI/ml SOD, and LDH secretion was measured as described in Materials and Methods. Results, expressed in ratios of percentage LDH release over control values, are mean ± SEM of five determinations performed in duplicate (n = 6). Statistics compare the effect of SNP or P with the control value ($C_{basal\ LDH\ secretion}$ = 19.6% ± 0.04% over total cellular content; $^{**}P < 0.01$, $^{***}P < 0.001$) and the effect of CPTio and SOD on SNP and P-induced increase in LDH release ($^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$; two-way ANOVA).

to chromaffin cells produced a rapid time-dependent increase in LDH release of about six times the basal levels in 15 min (Table I). These results indicate the induction of an extensive necrotic cell damage characterized by acute cellular swelling and lysis, which could be observed rapidly after treatment. However, 5–10 min of incubation with P and subsequent washes, which eliminate detached cells, no longer showed that acute necrotic death. Thus, in these cells, after 24 hr of treatment with P, P-induced LDH release was lower than in cells incubated during acute, shorter times (these results being related to controls; Table I). P-induced LDH release at 24 hr was dose dependent and was greater than LDH release induced by SNP at the same doses. However, unlike SNP-induced LDH release, CPTio was able to reverse P-induced cellular death only at high doses of P (0.01–1 mM; Fig. 2B), indicating that this compound does not allow us to distinguish completely between the contributions of NO and P to NO donors-induced LDH release (at least at low levels of P formation), establishing the requirement of carrying out experiments with SOD (to trap O_2^- anions and therefore prevent P formation) when one want to study the contribution of NO and P to the NO donors effect. Nonetheless, P decomposition products (Pd) had no significant effect on cell viability at 24 hr (Fig. 2B), indicating the specificity of the P effect.

NO Induces an Increase in ATP Release but Not in ATP Depletion in Chromaffin Cells

ATP depletion has been associated with neurodegeneration. To determine whether NO-induced toxicity was parallel to a decrease in ATP levels, cultured cells were challenged to 1 mM SNP treatment in the presence or absence of 100 μM CPTio or 50 UI/ml SOD. SNP increased ATP release by about 10 times the control levels and diminished ATP cellular content by about 25% with respect to basal levels (Table II), these data suggesting the existence of necrotic cell death. However, insofar as total intracellular ATP content (obtained by adding intracellular ATP + released ATP) was not diminished with respect to control, the possibility of a concomitant apoptotic cell death was not excluded. To test whether the increase in ATP release by SNP could be due to NO release or to peroxynitrite formation, SNP-induced ATP release was measured in the presence of CPTio, an NO scavenger, or SOD, an enzyme that traps O_2^- anions, inhibiting P formation. These two compounds were able to reverse both SNP-induced effects, the increase in ATP release and the reduction in intracellular content (Table II), indicating that both NO and P species could be mediating the effects of SNP. To examine the possible NO donors' peroxynitrite formation, we monitored the O_2^- production induced by SNP and SNAP by a microfluorimetric assay as stated in Materials and Methods and compared these results with veratridine (and other products)-generated O_2^- production. Results in Table III show that chromaffin cells have a basal O_2^- production rate of 3.85 ± 0.21 a.f.u. min^{-1}, which does not vary significantly with time of incubation. By contrast, 50 μM veratridine (which prevents inactivation of voltage-dependent Na^+ channels), rotenone (which inhibits complex I in the mitochondrial respiratory chain), and oligomycin (an inhibitor to F_0F_1 ATP synthase) caused a time-dependent increase in the rate of O_2^- production, reaching values ranging from 2 to 4.5 times basal. O_2^- production by NO donors (SNP and SNAP) was also time-dependent, although smaller than with the above compounds, because the increase resulting from SNP and SNAP ranged between 1.2 and 1.9 times the basal values, indicating that, although NO· plus O_2^- formation of peroxynitrite participates in NO-donor-generated toxicity, this participation is small with respect to total cytotoxic effect of these compounds.

NO Donors, Cytokines, and Peroxynitrite Induce Morphological Chromaffin Cell Alterations Typical of Both Necrotic and Apoptotic Cellular Death

The microscopic observation of SNP- and cytokine-treated cells stained with Hoechst 33342 and PI indicates that a vast percentage of viable cells (round, blue nuclei in Fig. 3A′) displayed a homogeneous round

TABLE II. Effect of SNP on ATP Release and ATP Intracellular Content[†]

Conditions	ATP release (% over total cellular content)	Cell ATP (pmol/10^6cells)	Total ATP (pmol/10^6 cells)
Control	2.24 ± 0.12	605.41 ± 29.20	619.32 ± 30.14
SNP 1 mM	24.10 ± 0.29***	452.32 ± 18.42***	602.94 ± 10.45
CPTio 100 μM	2.34 ± 0.33	607.30 ± 18.64	621.92 ± 24.60
CPTio + SNP	11.65 ± 0.83***	557.73 ± 12.74**	630.41 ± 33.12
SOD 50 Ul/ml	2.04 ± 0.28	601.34 ± 27.28	614.09 ± 28.23
SOD + SNP	10.63 ± 0.90***	523.17 ± 31.49**	589.61 ± 35.94

[†]Chromaffin cells were treated for 24 hr with normal Locke (control), 100 μM CPTio, or 50 Ul/ml SOD in the absence and in the presence of 1 mM SNP. Then, medium was collected and cells lysed. After that, ATP release and content were measured as indicated in Materials and Methods. Results are mean ± SEM of three experiments each performed in duplicate. Statistical significances were calculated by one-way ANOVA test, tested conditions vs. the corresponding controls (**$P < 0.01$, ***$P < 0.001$).

TABLE III. Effect of NO-Producing Compounds on Superoxide Anion Production at Short and Long Incubation Times[†]

	A) 4 Hr		B) 24 Hr	
Conditions	Slope (y = ax)	Ratio	Slope (y = ax)	Ratio
Control	3.85 ± 0.21	1.00 ± 0.05	3.80 ± 0.65	1.00 ± 0.17
Veratridine 50 μM	9.82 ± 1.91***	2.55 ± 0.50	16.38 ± 2.23***	4.31 ± 0.59^{000}
SNP 1 mM	4.84 ± 0.53*	1.26 ± 0.14	7.10 ± 0.60***	1.87 ± 0.16^{000}
SNAP 1 mM	4.64 ± 0.47*	1.21 ± 0.12	6.51 ± 0.95***	1.71 ± 0.25^{000}
Rotenone 5 μM	7.46 ± 0.98***	1.94 ± 0.25	13.84 ± 0.32***	3.64 ± 0.08^{000}
Oligomycin 1 μg/μl	7.67 ± 1.39***	1.99 ± 0.36	12.07 ± 1.14***	3.18 ± 0.30^{000}

[†]Chromaffin cells (10^6) were incubated for 4 hr (A) or 24 hr (B) in the absence (control) or presence of indicated ROS-producing compounds in DMEM containing 10% FCS. Afterwards, 50 μM hydroethidine was added and the ethidium fluorescence production measured as indicated in Materials and Methods. The slopes of the lines (a) fitting the fluorescence intensity changes (an index of the rate of superoxide production) were individually calculated and averaged. Data are the mean slope values ± SEM of four different experiments each performed in triplicate. Statistics compare the effect of every different agent vs. control at 4 hr or 24 hr of incubation at *$P < 0.05$, ***$P < 0.001$ or the differences between the same condition comparing 24 hr to 4 hr at $^{000}P < 0.001$ (two-way ANOVA test for multiple variables).

shape, with membrane blebs, whereas small numbers were notably sunken. Nuclei of nonviable cells (red nuclei in Fig. 3B′–D′) showed chromatin condensation and, in numerous cases, nuclear fragmentation and apoptotic cell bodies. These images contrast with the morphology of control cells (blue cells in Figure 3A′), which displayed a typical polygonal to round shape, with no nuclear staining. Semiquantitative analysis of nonviable cells assessed by PI staining showed the following percentages of cell death: 10.1% ± 2.2% of dyed cells in nontreated cells (control) and 54.0% ± 10.0, 29.2% ± 5.1, 30.8% ± 4.6%, and 32.8% ± 6% of dyed cells in 1 mM SNP-, 10 μM LPS-, 10 nM TNFα-, and 50 UI/ml IFNγ-treated cells, respectively.

Because the morphological changes in cells treated with NO-generating compounds were highly suggestive of apoptosis, we performed a study of morphology of the entire cell population by confocal microscopy after staining of cell cultures with SYTO 13 and neutral red, to determine whether apoptosis was produced only in the chromaffin cell population. SYTO 13 stains DNA of all cell populations with a blue-green color, whereas neutral red (NR), which is avidly accumulated by chromaffin cells (Winckler, 1974; Brooks et al., 1992), selectively stains cytoplasm of chromaffin cells. Treatment with 1 mM SNP (Fig. 4B,C) caused the presence of rounded cells with a tendency to be detached, as well as the presence of fragmented nuclei and apoptotic cell bodies (Fig. 4, inset). These changes were induced after 8 hr of treatment (Fig. 4B), reaching maximal levels after 24 hr (Fig. 4C), when a great number of detached dead cells and a number of apoptotic-like cells could be observed. These morphological changes were observed mainly in the chromaffin cell population with red-stained cytoplasm. Endothelial cells, which appear in a lower plane with a certain refringence, were not red stained and died by necrosis in the very first hours of treatment. Treatment with 1 mM P induced more important morphological changes than treatment with SNP, these changes appearing after 6 hr of treatment (Fig. 4D). Cells suffering apoptosis were mainly chromaffin cells, which were rounded and had a tendency to be detached from well dishes. Endothelial cells appear below chromaffin cells, blue stained, with cell nuclei bigger than those of chromaffin cells and with broken connections. Cell death induced by P was time dependent, being maximal at 24 hr (Fig. 4F). At this

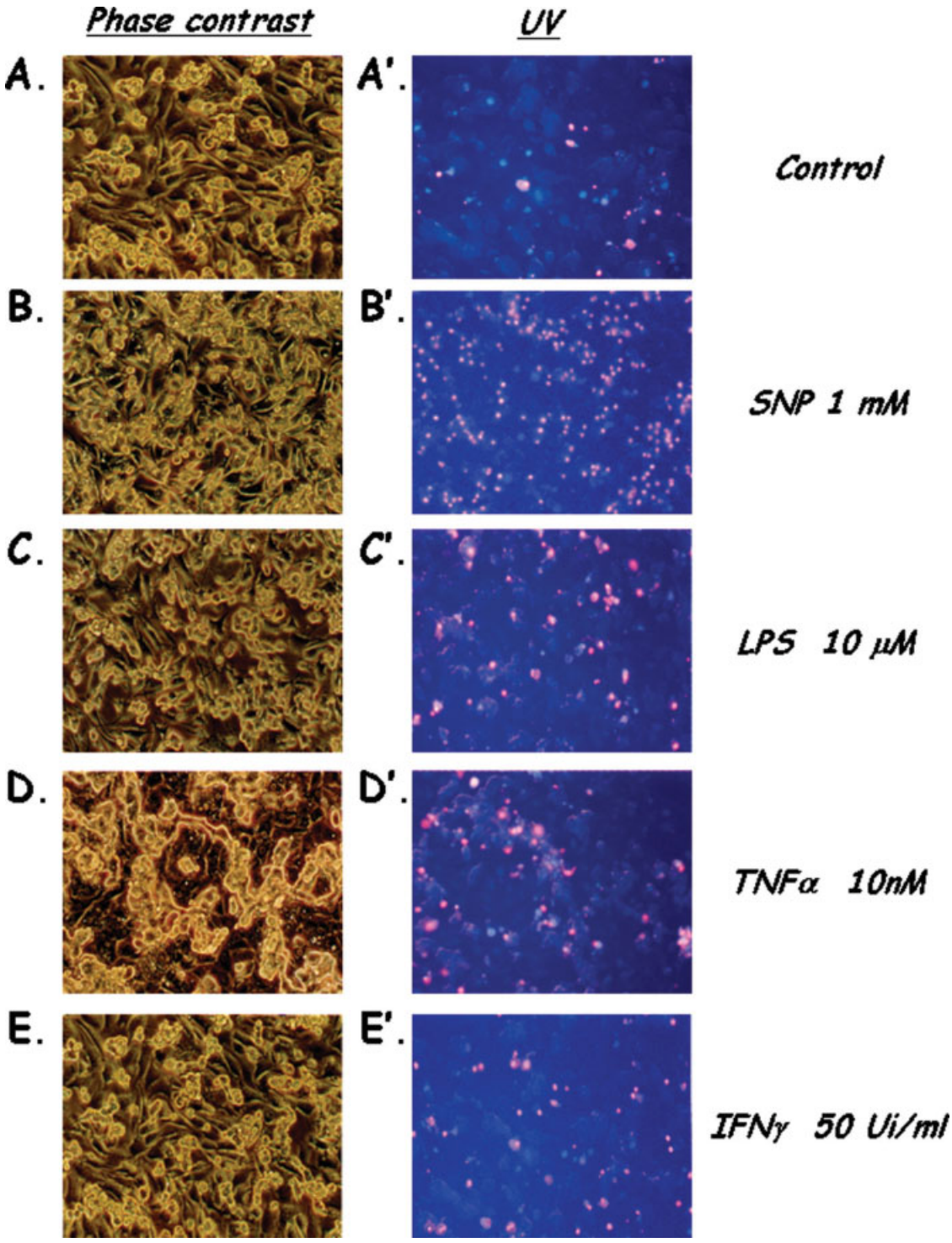

Fig. 3. Morphological analysis of nuclear chromatin in chromaffin cells stained with the DNA binding fluorochrome Hoechst 33342 and propidium iodide with a fluorescence microscope. In control cultures (**A**), viable cells show blue, round nuclei (Hoechst 33342 staining). Necrotic cells (propidium iodide, nuclei red) were present only in the cells with disrupted plasma membrane. Apoptotic cells were hardly observed in control cultures. In the presence of SNP (**B**) or cytokines (**C–E**), NO-induced necrosis in chromaffin cells after 24 hr of incubation was assessed by propidium iodide staining and apoptotic cells by the characteristic chromatin condensation and nuclear fragmentation of Hoechst 33342-stained cells and propidium iodide-stained cells.

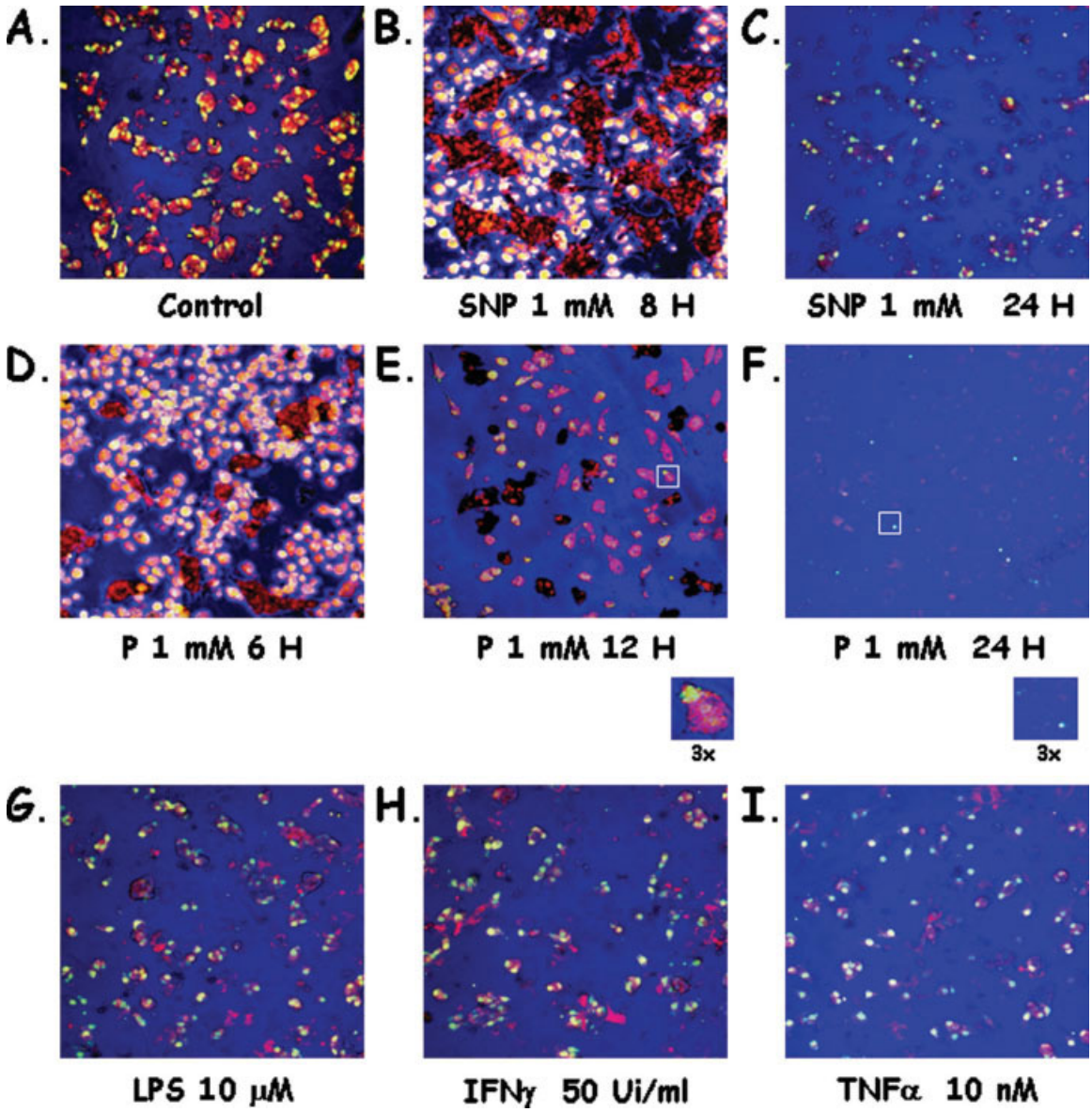

Fig. 4. Morphological analysis of nuclear chromatin in chromaffin cells stained with the DNA binding fluorochrome SYTO 13 and with the chromaffin cell cytosol fluorochrome neutral red via confocal microscopy. Chromaffin cells were treated for 24 hr with culture medium (control; **A**); 1 mM SNP for 8 and 24 hr (**B,C**); 1 mM P for 3, 12, and 24 hr (**D–F**); or LPS and cytokines for 24 hr at indicated doses (**G–I**). Cells were stained for 15 min with SYTO 13 and neutral red as described in Materials and Methods and then analyzed via confocal microscopy.

time, only a few apoptotic bodies and a high amount of cell debris appeared. In these cases, both chromaffin and endothelial cells displayed cellular death. However, analysis of morphological characteristics seems to show that, although chromaffin cells suffered mainly an apoptotic process, endothelial cells suffered mainly a necrotic death (Fig. 4F), although these experiments do not allow us to discount the possibility that some endothelial cells could suffer apoptosis. The fact that only a few cells appear after 24 hr of P treatment is due to prior washes for eliminating serum.

Finally, treatment of chromaffin cell cultures for 24 hr with LPS or cytokines only slightly modified chromaffin cell morphology, which shows numerous apoptotic bodies, the effects of TNFα being more powerful than those of the other two cytokines (Fig. 4G–I). These compounds do not seem to affect the contaminant endothelial cells. Taken together, these results indicate that both exogenously and endogenously generated P and NO induce characteristic morphological changes of cellular death in chromaffin cells, apoptotic features being predominant.

TABLE IV. Effect of SNP and Peroxynitrite on Apoptosis at Short and Long Incubation Times†

Conditions	% M1 (apoptosis)	
	A) 8 Hr	B) 24 Hr
Control	1.40 ± 0.14	2.86 ± 0.31^{000}
-FCS	1.90 ± 0.50 ns	$3.80 \pm 0.40^{\star 000}$
SNP 1 mM	1.10 ± 0.25 ns	$5.00 \pm 0.45^{\star\star 000}$
P 1 mM	$2.47 \pm 0.26^{\star}$	$16.80 \pm 2.03^{\star\star\star 000}$
Pd 1 mM	1.59 ± 0.05 ns	3.47 ± 0.66^{000}

†Chromaffin cells were stimulated for 8 hr (A) or 24 hr (B) with SNP, P (peroxynitnite), or Pd (deactivated peroxynitrite) in the presence of FCS. Results (mean ± SEM of three experiments, each performed in duplicate) were compared with apoptosis obtained in the absence of FCS (-FCS), and expressed as % M1 (apoptosis). Statistics compare the effect of different agents and control at 8 hr or 24 hr ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$) and the effect of time exposure for each condition ($^{000}P < 0.001$); (two-way ANOVA test).

NO Donors, Peroxynitrite, and Cytokines Increase the Number of Cells With Low DNA Content and Modify the Number of Cells in Different Phases of the Cell Cycle

For quantifying the NO- and P-induced apoptotic cell death in chromaffin cells, their DNA content was first analyzed by flow cytometry after staining with PI. SNP and P treatment induced a time-dependent increase in the percentage of cells with DNA content below 2C, which is not reproduced by Pd (Table IV). Peaks of 2 mM SNP-, SNAP-, and P-treated cells presenting DNA content below 2C after 24 hr are shown in Figure 5A. SNP, SNAP, and P at concentrations ranging from 10 μM to 2 mM induce a dose-dependent increase in apoptosis (Fig. 5B,C), the smaller effect corresponding to SNAP, followed by SNP (Fig. 5B) and then P (Fig. 5C; two, three, and seven times basal levels at 2 mM concentrations of SNAP, SNP, and P, respectively).

For studying the effect of these NO donors and P on the cell cycle, the flow cytometry analysis of cellular DNA stained with PI was also used and results compared with their effects on apoptosis. SNP and SNAP at concentrations ranging from 10 μM to 2 mM induce a small increase in the percentage of cells in G_0G_1 phase, which is accompanied by a parallel dose-dependent decrease in the percentage of cells in G_2M and S phases of cell cycle (Fig. 6A). By contrast, P induced statistically nonsignificant variations in G_0G_1 phase but induced an increase in the percentage of cells in both G_2M and S phases of cell cycle at concentrations ranging from 10 to 500 μM, followed by a decrease in the number of cells in both phases of the cell cycle (Fig. 6B). Changes induced by P were not mimicked by Pd which, as much the same as in apoptosis, did not produce any significant change in different phases of cell cycle.

With regard to the effects of LPS and cytokines on apoptosis and cell cycle, these compounds, alone or in combinations, induced, just like NO donors and P did, a time-dependent increase in the percentage of cells with a DNA content below 2C (Fig. 7A,B). This effect was inhibited by the NOS inhibitors L-NMA and thiocitrulline (Fig. 7B), compounds that do not produce apoptosis by themselves (Fig. 7B, inset). This increase in apoptosis was parallel to a decrease in the number of chromaffin cells in phase G_2M, as happens in the case of NO donors and P (Fig. 7C). However, in contrast to these compounds, cytokines did not induce any change in the number of cells in G_0G_1 phase and increased, not decreased, the number of cells in S phase (Figs. 7C).

For studying the specificity of changes induced in the cell cycle by NO-generating compounds and to confirm that these changes were specifically produced in chromaffin cells, we studied the ability of NGF (whose receptors are expressed only in chromaffin cells) to reverse these effects. We can see in Table V that changes in G_2M and S phases of the cell cycle induced by all these NO-generating compounds were reversed by NGF, which was able not only to protect chromaffin cells from NO-induced apoptosis but also to reverse the SNP and P induced decrease in percentage of cells in G_2M and S phases. NGF was also able to increase significantly the percentage of cells in G_2M phase decreased by cytokines and even to produce a significant increase in the number of cells in S phase not affected by cytokines and LPS (Table V). Insofar as NGF receptors are expressed only in chromaffin cells, these results seem to confirm that changes induced by NO on cell cycle are produced in chromaffin cells, ruling out the contribution of contaminant endothelial cells to the observed effect of NO on cell cycle.

NO Donors, Peroxynitrite, and Cytokines Induce DNA Fragmentation in Chromaffin Cells

For confirming that NO donors and P induce a process of apoptosis, some other biochemical methods were used, such as analysis of the presence of DNA fragments in the cytoplasmic fraction by agarose gel electrophoresis and measurement of caspase 3 activity. Study of the extranuclear DNA in cells untreated or treated with NO donors, P, or cytokines for 24 hr revealed an induction of DNA fragmentation by all these compounds, as shown in Figure 8. NO donors-, cytokines-, and P-treated cells presented different low-molecular-weight DNA fragments, resembling a ''ladder'' (not present in control cells), showing the cleavage of genomic DNA occurring in cells undergoing late apoptosis. The NO- and P-induced DNA fragmentation was specific, in that KFeCN (the vehicle of SNP) and Pd cause a very small effect on DNA cleavage.

Caspase 3 and 9 Activation and Cytochrome c Release Mediates NO-, Cytokine-, and P-Induced Apoptosis

For addressing whether caspases contribute to NO donors-, cytokines-, and P-induced apoptosis, chromaffin cells were incubated with SNP, LPS, cytokines, or P

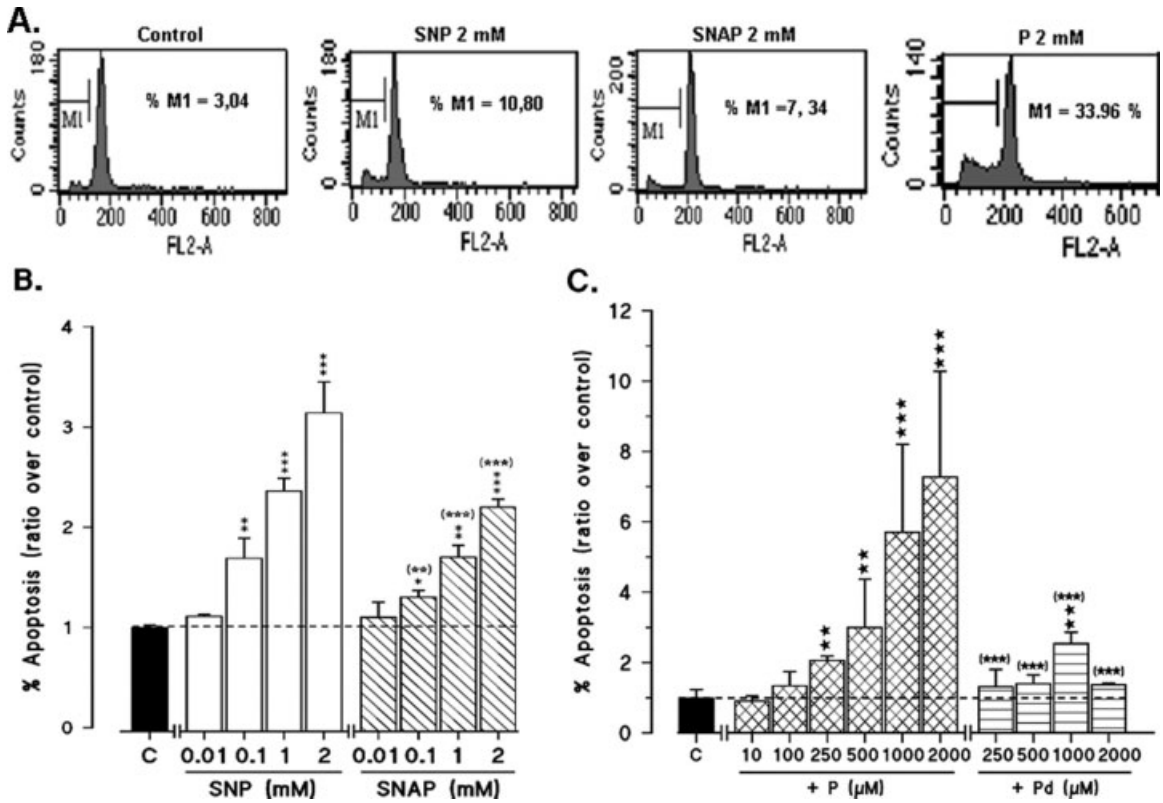

Fig. 5. NO donors and peroxynitrites induce a decrease in DNA content in chromaffin cells. **A:** DNA content histograms from flow cytometry measurements in bovine chromaffin cells untreated (control) or treated for 24 hr with 2 mM SNP, SNAP, or P carried out as described in Materials and Methods. M1 fractions represent the percentage of cells with DNA content below 2C (apoptotic cells). **B,C:** Dose-response curves showing apoptotic effect of NO donors (SNP and SNAP; B) or peroxynitrite (P and Pd; C). Percentage apoptosis was expressed as ratio over basal, and mean values ± SEM of three separate experiments each performed in duplicate are represented in each bar. Statistics compare the effect of SNP or P with the control values (vertical $^{**}P < 0.01$, $^{***}P < 0.001$) or in B- *differences between the same doses of SNP and SNAP or P and Pd (horizontal $^{\star}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$; two-way ANOVA).

for 24 hr in the presence or absence of Ac DEVD-CHO (CPP32), a cell-permeable compound that blocks caspase 3-like enzymes. DEVD-amc cleaving activity by caspase 3 was clearly increased after 24 hr of incubation with all these compounds (Fig. 9). The increase in caspase 3-like activity induced by all of these NO-generating compounds was almost completely reversed in the presence of 50 nM CPP32.

To determine whether caspase 3 and 9 were involved in NO-, cytokines-, and P-induced chromaffin cell apoptosis, we assayed the effect of CPP32 and Ac Z-LEHD-FMK (which block caspase 3- and caspase 9-like enzymes, respectively) on NO-induced apoptosis and inhibition of cell cycle. Both inhibitors, at 1 and 50 nM concentrations, produced a significant dose-dependent decrease in apoptosis induced by SNP, P, and cytokines such as IFNγ (Fig. 10A,B). Moreover, these compounds were also able to reverse the inhibition induced by all these NO-inducing compounds (especially the inhibition caused by SNP and cytokines) on the number of cells in G_2M phase, but they only slightly reversed the decrease in the percentage of cells in S phase in the case of P.

After viewing these results, we decided to study whether caspase activation induced by NO could be a consequence of cytochrome c release by mitochondria. After incubation of cells with NO-generating compounds, mitochondria were separated from cytosol, and cytochrome c release and content were analyzed by Western blot as described in Materials and Methods. As shown in Figure 11, cytochrome c release was significantly increased in chromaffin cells treated with SNP, P, LPS, and cytokines at 24 hr, the maximal effects being induced by SNP, P, and IFNγ. In contrast, levels of β-actin (control) remained unchanged (not shown).

DISCUSSION

NO is a diffusible messenger involved in several pathophysiological processes, including immune-mediated cytotoxicity and neural cell killing. NO or its redox products, such as P, can cause DNA damage and activate

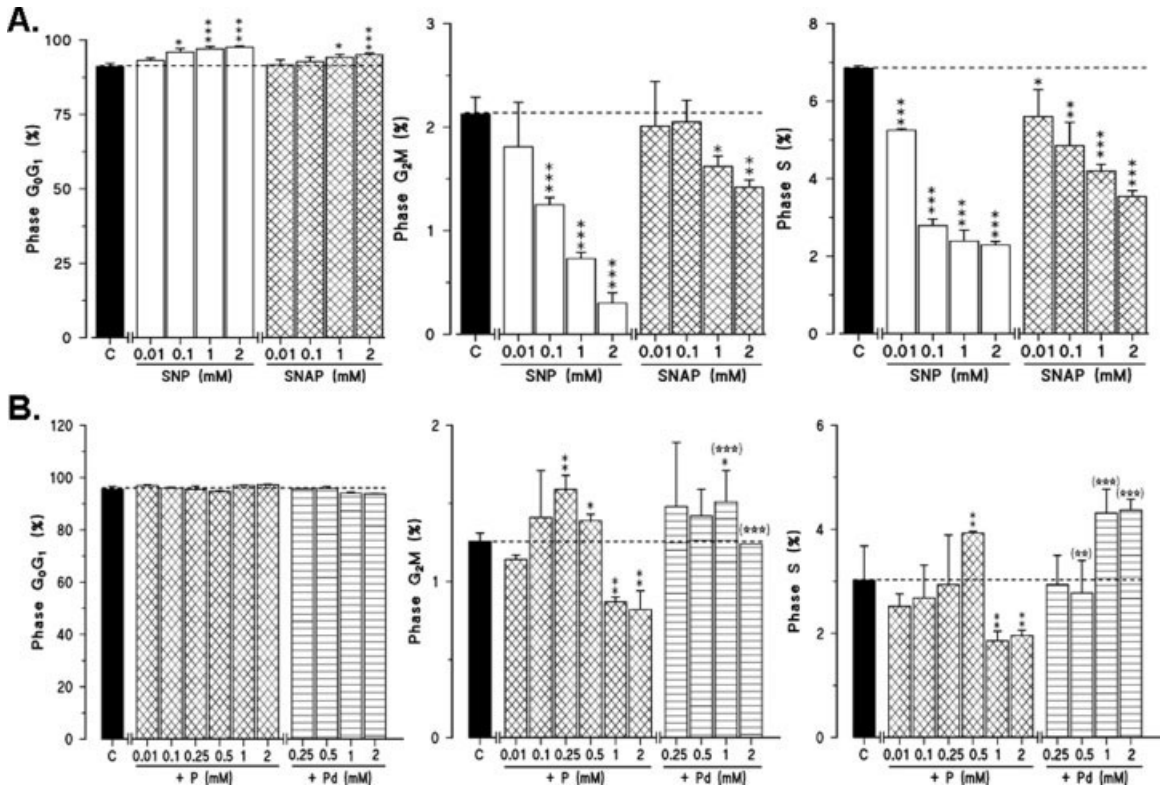

Fig. 6. NO donors and peroxynitrite induce a decrease in the number of chromaffin cells in G_2M and S phases of the cell cycle. Dose-response curves showing apoptotic effect of NO donors (SNP and SNAP; **A**) and peroxynitrite (P and Pd; **B**) on percentage of cells in different phases of the cell cycle. Results represents mean ± SEM of three determinations each performed in duplicated (n = 6). Statistics compare the effect of SNP or P with the control values ($^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$) or the difference between the same doses of P and Pd ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$; two-way ANOVA).

subsequent lethal reactions, including energy depletion and cell necrosis. NO induces apoptosis in several systems, regardless of its origin: endogenous production in response to cytokines or generation by chemical breakdown of donor molecules (Albina et al., 1993; Ankarcrona et al., 1994; Bal-Price and Brown, 2000; Figueroa et al., 2005). In this study, we demonstrate that in chromaffin cells NO and its oxidized derivate, P, are able to induce cellular death by a mixed necrotic and apoptotic mechanism, depending on time of stimulation and compound concentration.

Fluorescence and confocal microscopy analysis of morphological alterations in the presence of this compound showed three cell populations: completely blue-stained (live cells); broken cells with whole, red-stained nuclei (necrotic cells); and cells with blue-stained cytoplasm and red, fragmented nuclei (apoptotic bodies) typical of cells undergoing apoptosis. Specific staining of chromaffin cell cytoplasm with neutral red (Winckler, 1974; Brooks et al., 1992) helped us to see that, after 8 hr of treatment with NO donors, morphological apoptotic changes are produced mainly in chromaffin cells. Although P produces a mixed necrotic plus apoptotic cell death, apoptosis appearing earlier (from 3 hr incubations) and necrosis in a time-dependent way (being maximal at 24 hr of incubation), bacterial toxins and cytokines induce chromaffin cells to release NO, leading to cell death, mainly through an apoptotic mechanism. There are numerous examples in the literature demonstrating that NO and oxidized compounds are able to induce cell death by both necrotic and apoptotic mechanisms, depending on time of exposure and drug concentration. It has been postulated that mild stimuli produce mainly apoptosis, whereas strong stimuli produce necrosis over the long term and apoptosis over the short term (Bonfoco et al., 1995). For determining the quantity of NO stimuli required for inducing cell death, two important factors have to be taken into account: 1) the cellular content of antioxidant defenses and 2) the actual concentration of NO able to enter the cells. Chromaffin cells present high levels of GSH (about 5 nmol/5 $\times$ 10^6 cells; Jordan et al., 2002). We had previously seen that NO donors induced an increase in GSH levels, which could be related to the cells' attempt to protect themselves from the toxic NO stimulus. In contrast, P decreases GSH levels (unpublished results from our group). This result could be related to the strong death-inducing effect of this compound in chromaffin cells. In our

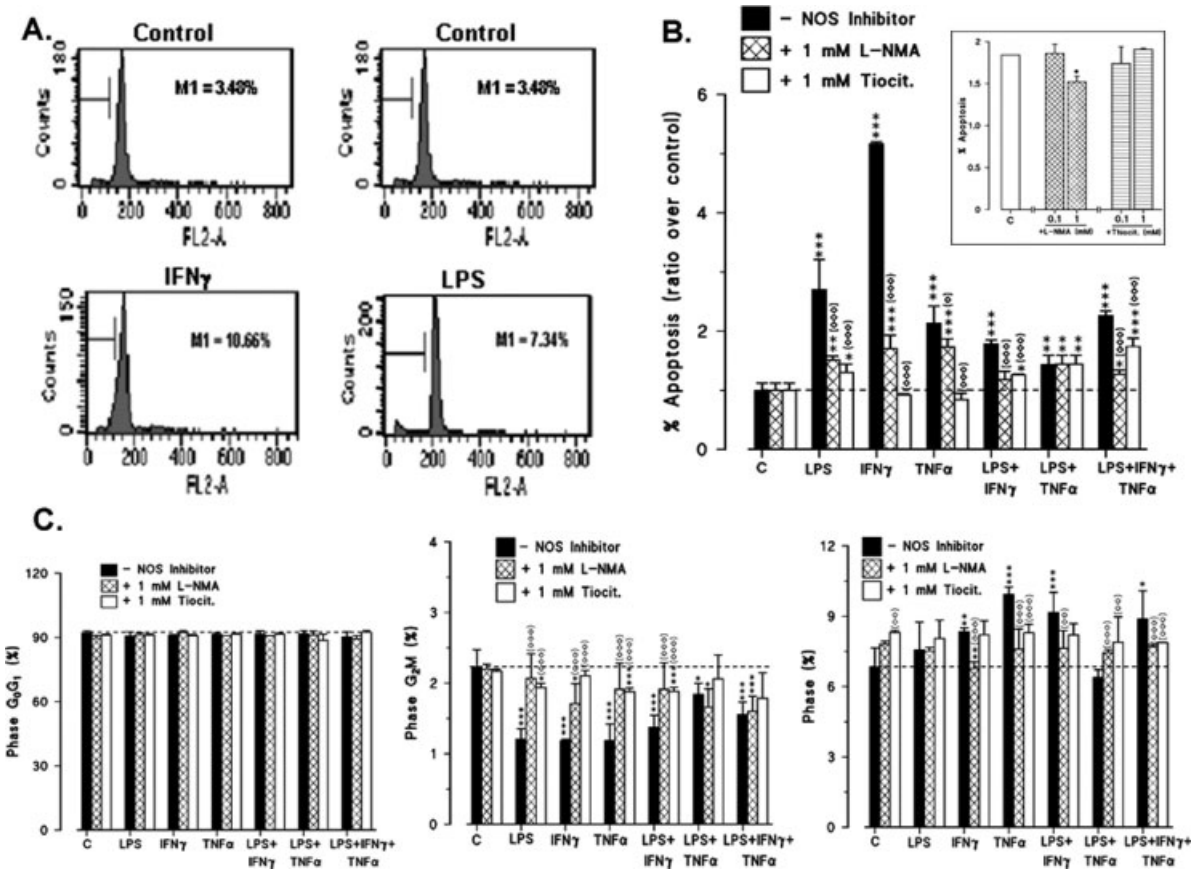

Fig. 7. Cytokines induce a decrease in DNA content specifically reversed by NOS inhibitors. Chromaffin cells were treated with 10 μM LPS, 50 UI/ml IFNγ, or 10 nM TNFα (alone or in specific combinations) for 24 hr, and DNA content and cell cycle were measured by flow cytometry. **A:** Representative records of DNA content histograms. M1 fractions represent the percentage of cells with DNA content below 2C (apoptotic cells). **B:** Quantification of apoptosis measures (mean ± SEM) from four experiments each performed by duplicate. Data in **inset** represent the basal effect of NOS inhibitors on apoptosis. **C:** Effect of cytokines (alone or in specific combinations) on different phases of the cell cycle in the absence or presence of NOS inhibitors L-NMA and S-methyl-L-thiocitrulline. Data are mean ± SEM from four experiments each performed in duplicate. Statistics compare the effect of cytokines with basal values at $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ and the effect of NOS inhibitors on the effect induced by each compound tested at $^{\diamond}P < 0.05$, $^{\diamond\diamond}P < 0.01$, $^{\diamond\diamond\diamond}P < 0.001$ (two-way ANOVA).

experiments, NO donors and P were used at high concentrations to ensure enough NO concentration inside the cells. It has been shown that PC12 cell stimulation with 1 mM P for 5 min produces submicromolar concentrations of this compound inside the cells during about 1 hr (Brunelli et al., 1995) and that millimolar concentrations of NO donors, such as SNP and SNAP, release small quantities of NO inside the cells for long periods of time (Bates et al., 1991), raising concentrations in the low micromolar range after cell stimulation (Ferrero et al., 1999). These NO levels are very similar to those produced by stimulation with cytokines, which stimulate NOS activity and expression in chromaffin cells or by activation of nNOS (2–4 μM) in conditions of brain ischemia (Malinski et al., 1993).

To analyze more deeply the type of NO-induced cell death in chromaffin cells, we studied the effect of these NO compounds on the release of LDH and ATP, nonspecific parameters that are altered in both necrotic and apoptotic cell death. At short incubation times (≤10 min), only P was able to increase LDH release, but, at long incubation times, all tested NO compounds increased the LDH release in a specific way, insofar as its effects were reversed by CPTio (an NO-trapping agent) in the case of NO donors and by NOS inhibitors in the case of cytokines. The effect of P, not reversed by CPTio at high concentrations of P, was partially reversed by NGF (90% at 40 ng/ml) and by glucose (50% at 50 μM concentration). These results seem to show the existence of a cell death with alterations in cell membrane permeability, which could be identified as a necrotic or late apoptotic cell death. This result was confirmed by measuring ATP release and ATP content. SNP was able both to increase ATP release and to decrease ATP intra-

TABLE V. Effect of NGF on Apoptosis and Changes in Cell Cycle Induced by NO-Generating Compounds in Chromaffin Cells[†]

Conditions	% M1	% G_0G_1	% G_2M	%S
NGF				
C	1.00 ± 0.12	1.00 ± 0.03	1.00 ± 0.20	1.00 ± 0.10
SNP 1 mM	1.97 ± 0.09***	1.03 ± 0.06	0.49 ± 0.09***	0.29 ± 0.02***
P 1 mM	6.47 ± 0.17***	0.87 ± 0.04*	0.33 ± 0.04***	0.34 ± 0.04***
Pd 1 mM	2.08 ± 0.20***	0.96 ± 0.02	0.45 ± 0.10***	1.11 ± 0.06
LPS	2.95 ± 0.56***	0.93 ± 0.05	0.61 ± 0.04**	1.02 ± 0.09
IFNγ	1.66 ± 0.10***	0.97 ± 0.04	0.77 ± 0.02*	1.13 ± 0.11
TNFα	2.41 ± 0.29***	0.96 ± 0.06	0.66 ± 0.08*	0.96 ± 0.05
+NGF				
C	0.97 ± 0.09	0.93 ± 0.06	1.52 ± 0.15**	1.70 ± 0.29***
SNP 1 mM	1.12 ± 0.06$^{\star 000}$	1.02 ± 0.03*	0.96 ± 0.10^{000}	0.69 ± 0.15$^{\star\star 000}$
P 1 mM	2.67 ± 0.15$^{\star\star\star 000}$	0.96 ± 0.03^{0}	1.16 ± 0.05^{000}	0.74 ± 0.02$^{\star\star 000}$
Pd 1 mM	1.25 ± 0.10$^{\star 000}$	1.01 ± 0.02	0.58 ± 0.10$^{\star\star 0}$	0.83 ± 0.10$^{\star 00}$
LPS	1.05 ± 0.05^{000}	0.95 ± 0.02	1.45 ± 0.10$^{\star\star 000}$	1.45 ± 0.19$^{\star\star\star 00}$
IFNγ	1.18 ± 0.10$^{\star 000}$	0.95 ± 0.01	1.72 ± 0.19$^{\star\star\star 000}$	1.33 ± 0.10$^{\star\star 0}$
TNFα	1.07 ± 0.04^{000}	0.94 ± 0.02	1.34 ± 0.10$^{\star\star 000}$	1.64 ± 0.24$^{\star\star\star 000}$

[†]Chromaffin cells were treated for 24 hr with indicated compounds in the absence or presence of 50 ng/ml NGF, and apoptosis and cells cycle were measured by flow cytometry as indicated in Materials and Methods. Results correspond to the mean ± SEM of three experiments each performed in duplicate. Statistical significances indicated by asterisks compare the effect of different compounds on apoptosis or cell cycle at $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ and that indicated by circles compare the effect of NGF on changes in apoptosis or cell cycle induced for each compound at $^{0}P < 0.05$, $^{00}P < 0.01$, $^{000}P < 0.001$ (two-way ANOVA test for multiple variables). Control basal values were % M1 = 3.17 ± 0.40; % G0G1 = 88 ± 3; % G_2M = 1.37 ± 0.3 and % S = 7.60 ± 0.8.

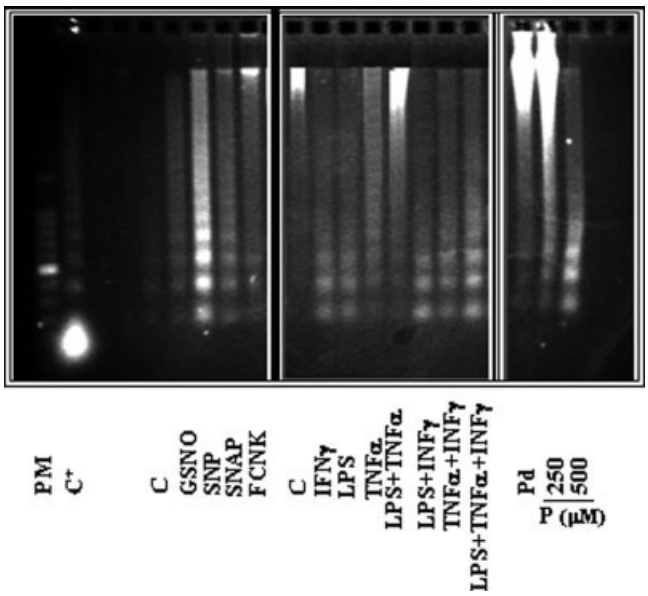

Fig. 8. Effects of NO donors, cytokines, and P on DNA fragmentation in bovine chromaffin cells. Representative agarose gels of DNA fragmentation. Chromaffin cell cultures were exposed to NO donors, cytokines, or P for 24 hr, and cytoplasmic DNA was extracted and analyzed as described in Materials and Methods. PM = molecular weight markers (100-bp ladder DNA); C^+ represents a positive control.

cellular content, effects that could be due to either an alteration in cell membrane permeability or a decrease in ATP synthesis. Given that, in SNP-treated cells, total ATP content (released ATP + intracellular ATP remaining content) is not significantly different from that in the control cells, it is likely that NO-induced ATP release could be due to an alteration in membrane permeability produced by a necrotic cell death or, as suggested by our recent results, that these effects are related to NO's ability to release cytosolic ATP by reversing the plasma membrane anion transporter (unpublished results). In this way, given that there is a 5:1 CAs:ATP ratio in chromaffin cells (Sillero et al., 1994) and that NO donors are not able to modify either CA secretion or its content (Vicente et al., 2005), the increase in ATP release must be cytosolic, not intragranular, and probably is related to its effect on specific anionic transporters. On the other hand, because the effect of NO donors on LDH and ATP release was reversed by both CPTio and SOD, it is plausible that the toxic effects of NO donors are due not only to NO but also to P. In fact, as shown in this paper, NO donors are able to increase the O_2^- production in chromaffin cells and, thus, to originate P.

Once determined that NO and its oxidative derivates are able to induce cell death in chromaffin cells, we tried to study NO-induced apoptosis in depth. Apoptosis was first assessed by flow cytometry, staining the cells with PI and measuring DNA content. All NO assayed compounds (cytokines and their combinations, NO donors, and P), were able to induce peaks of cells presenting a DNA content below 2C, which increase with dose and time of incubation with these NO com-

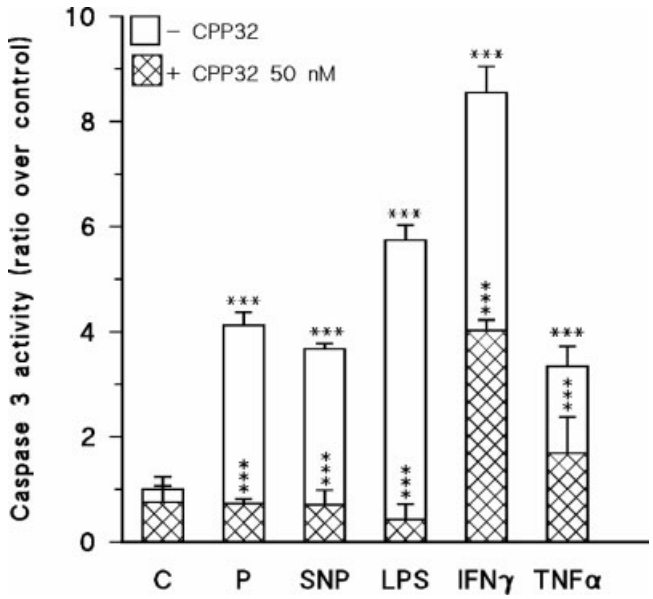

Fig. 9. NO induction of caspase 3 activity in bovine chromaffin cells and reversal of this effect by caspase 3-like activity specific inhibitor CPP32. Chromaffin cells were treated with NO donors (1 mM SNP, SNAP, and GSNO), 0.5 mM P, 10 μM LPS, 50 UI/ml INFγ, or 10 nM TNFα for 24 hr in the absence or presence of 50 nM CPP32. Caspase 3 activity was measured in cell lysates by using the fluorogenic substrate peptide DEVD-amc as indicated in Materials and Methods. Values represent the means ± SEM of at least three experiments each performed in duplicate and are expressed as a ratio over control (C = 7.20 ± 0.11 u.a.f./10^6 cells and CPP32 = 4.84 ± 0.1 u.a.f./10^6 cells). Statistical significances shown horizontally express the effect of different treatments on basal control (***$P < 0.001$), and statistical significances shown vertically represent the inhibition by CPP32 of caspase 3-like activity induced by the different treatments (***$P < 0.001$; multivariance analysis of ANOVA).

pounds. In the case of cytokines, the apoptotic effect was reversed in the presence of NOS inhibitors, indicating the participation of NO in the mechanism by which cytokines induce apoptosis. In the case of NO donors, the effect was dose-dependent and specifically reversed by CPTio and SOD, which means that, at least at high doses, P is also involved in the NO donor apoptotic effect. The effect of P was also specific, in that it was not mimicked by inactivated P. For confirming that these compounds induce an apoptotic process, we analyzed the presence of fragmented DNA in the cytoplasmic fraction by agarose gel electrophoresis, which reveals an induction of DNA fragmentation by all NO-assayed compounds. DNA from NO-treated cells presented different low-molecular-weight DNA fragments, resembling a ''ladder,'' that were not present in control cells, indicating the existence of apoptosis.

Activation of caspases is a final effector in the apoptotic cell death pathway. In chromaffin cells, NO donors were able to activate proteolytic caspases: caspase 9, the ''initiation'' caspase, and caspase 3, the ''effector'' caspase. Thus, on the one hand, all NO compounds tested were able to increase the activation of caspase 3, and, on the other hand, CPP32 and Ac Z-LEHD-FMK, specific inhibitors of caspase 3 and 9, respectively, were able to reverse NO-induced apoptosis. Because activated caspase 9 is involved in the activation of caspase 3, this being a key step in apoptosis, and cytochrome c release takes part, in turn, in caspase 9 activation, we studied the possible implication of cytochrome c release in NO-induced apoptosis. Results presented in this paper show that NO donors and cytokines were able to release cytochrome c from mitochondria to cytosol, showing the importance of mitochondrial involvement in the mechanism by which NO induces apoptotic death in chromaffin cells.

Results presented here demonstrate, for the first time, that NO is able to induce cell death in chromaffin cells by a mixed necrotic and apoptotic mechanism. There are very few studies described in the literature showing apoptotical death of chromaffin cells. Works by Jordan et al. (2000, 2002) show the effect of veratridine-induced apoptosis, mediated by superoxide anions, in chromaffin cells, and Ferrero and Torres (2001) show that YC-1, an NO-independent activator of soluble guanylyl cyclase that has a synergistic action with NO in stimulating cGMP synthesis, induces apoptosis in both endothelial and chromaffin adrenomedullary cells through a cGMP-independent mechanism. In our work, NO-induced apoptotic cell death in chromaffin cells is, at least in part, due to P formation and, possibly, carried out by S-nitrosylation or Tyr-nitration of specific proteins. The involvement of cGMP in this effect is very improbable, because, although in some experiments 8-Br cGMP was also able to induce apoptosis in a dose-dependent manner, neither ODQ, a specific inhibitor of soluble guanylate cyclase, nor KT5823, a specific PKG inhibitor, could reverse the effect of NO on apoptosis. Therefore, these results are in agreement with those of Ferrero and Torres (2001) on the apoptotic effect of YC-1 on chromaffin cells, which is independent of cGMP.

Our results on NO apoptotic effects in chromaffin cells are similar to those found by Desole et al. (1998), who showed apoptotic death induced by NO donors in PC12 cells, an effect in this case dependent of Fe mobilization by these cells, and are in agreement with those of Estévez et al. (1995) showing that these compounds induce cell death by two different mechanisms: necrosis, caused by P cell treatment with greater than 2 mM doses, and apoptosis, caused by treatment lasting for more than 4 hr. Because the cell cycle and apoptosis are two closely related events, we measured changes in the cell cycle parallel to measurements of apoptosis produced by NO compounds. In general, given the quiescent condition of chromaffin cells, changes in the cell cycle were very small, which is reflected by the high number of cells in the G_0G_1 phase. However, our results seem to indicate that apoptotic death induced by NO donors in chromaffin cells (increase in hypodiploid cell number) is

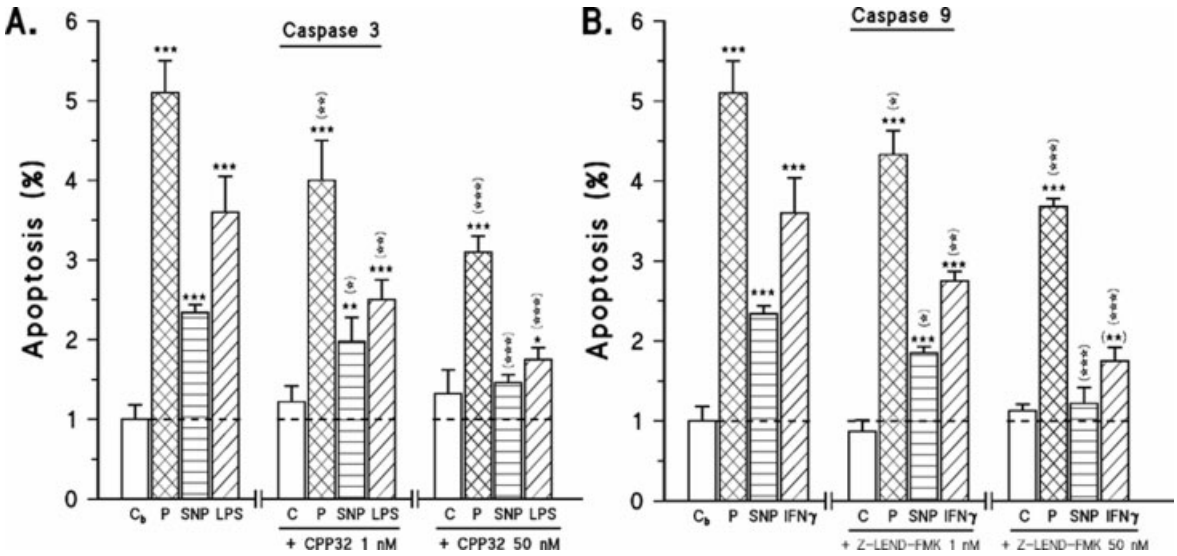

Fig. 10. Effect of caspase 3 (**A**) and caspase 9 (**B**) inhibition on apoptosis induced by different NO-increasing compounds. Chromaffin cells (10^6 cells/condition) were treated with the indicated compounds at concentrations indicated in Figure 8 for 24 hr in the absence or presence of CPP32 at 1 and 50 nM concentrations. Then, apoptosis was measured by flow cytometry as indicated in Materials and Methods. Values represent the means ± SEM of four experiments each performed in duplicate and are expressed as ratio over control. Statistical significances shown horizontally compare the effect of different treatments over the correspondent basal controls ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$), and statistical significances shown vertically represent the effect of CPP32 against the same treatment in the absence of this caspase 3 inhibitor ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; multivariance analysis of ANOVA).

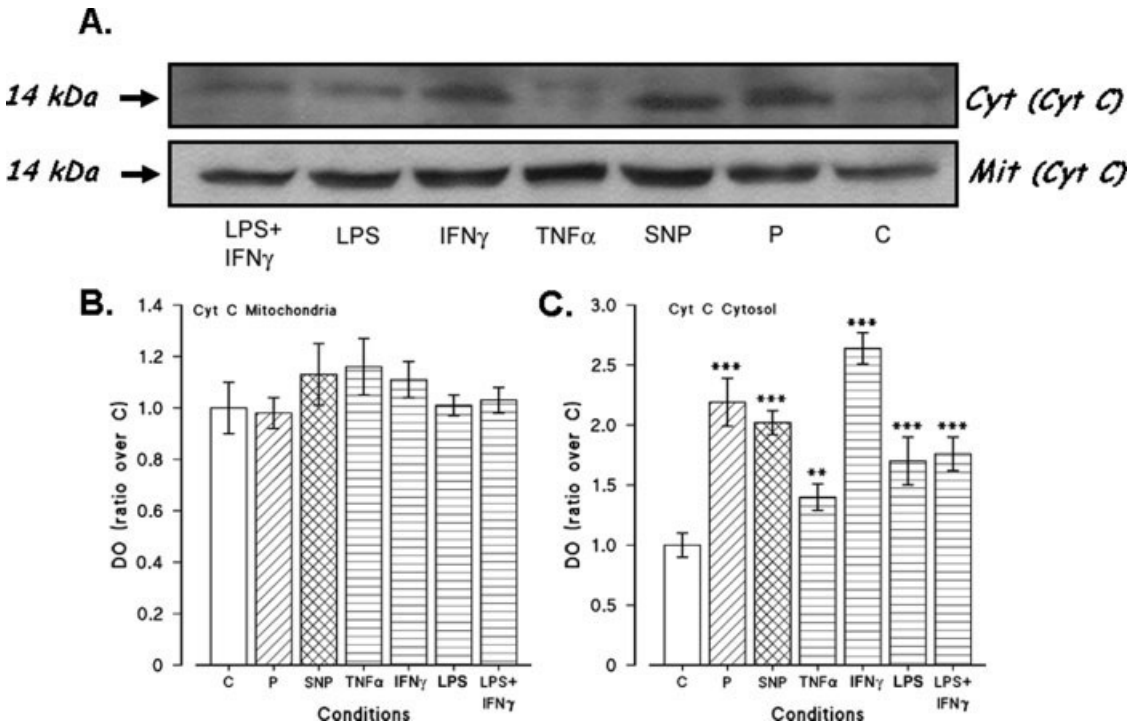

Fig. 11. Effect of NO-generating compounds on cytochrome c release into cytosol in chromaffin cells. Chromaffin cells (5×10^6 cells/condition) were treated for 24 hr with 0.5 mM P, 1 mM SNP, and 10 nM TNFα; 10 μM LPS; or 50 UI/ml IFNγ alone or at indicated combinations. Then, cells were lysed, and cytosolic (Cyt) and mitochondrial (Mit) fractions were separated and subjected to polyacrylamide gel electrophoresis and immunoblot analysis using an antibody specific for cytochrome c (Cyt C), as described in Materials and Methods. **A:** Representative gel of Western blot assay. **B,C:** Densitometric analysis of mitochondrial cytochrome c content (B) and cytocrome c released into cytosol (C). Data are means ± SEM from four different experiments. Statistical significances compare the effect of different treatments with the corresponding basal mitochondrial or cytosolic controls ($^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$; one-way ANOVA).

concomitant with a cell cycle arrest in G_0G_1 phase and with a decrease in the number of chromaffin cells in G_2M and S phases, although, in the case of P, only high concentrations were able to induce changes in G_2M phase and, in the case of cytokines, we observed only a decrease in the number of chromaffin cells in G_2M phase.

Although effects of NO and P on the cell cycle have not been deeply investigated, there are numerous examples in the literature showing the inhibitory effect of NO on cell proliferation, mediated in many cases by cGMP. Thus, in prostatic cells, the apoptotic effect of NO as an inhibitor of cell proliferation seems to be due to cell cycle arrest in G1 phase, preventing the cells from entering into S phase (Guh et al., 1998). In vascular smooth muscle cells, apoptosis induced by NO is concomitant with a decrease in DNA synthesis mediated by the isoform Iα of PKG (Chiche et al., 1998). These results are in agreement with our results, although the participation of cGMP/PKG in these effects is very improbable, insofar as only high doses of the PKG inhibitor KT-5823 (5 μM), levels at which other kinases could also be inhibited, were able to reverse these effects partially. In fact, as shown by Ferrero and Torres, YC-1 also inhibits proliferation of chromaffin cells by a cGMP-independent mechanism. The induction of cell proliferation in cells derived from neural crest (such as sympathetic neurons, enterocytes, or chromaffin cells) by local neurogenic signals has been demonstrated not only in developing cells (Sweetser et al., 1997) but also in adult cells (Tischler et al., 1991). Thus, although adult chromaffin cells have been considered for many years mainly as postmitotic cells and completely undifferentiated, it has been shown that they can proliferate through life, being able even to induce hyperplasias and neoplasias, both spontaneously by an aging effect and after exposure to drugs such as reserpine, which produces, in short-term treatments, a CA depletion accompanied by an increase in cell proliferation, or with tumor-inducing agents in long-term treatments (Tischler et al., 1988). Although the underlying mechanisms of these effects are still unclear, neurogenic signals coming from preganglionic sympathetic cells, dorsal ganglia of spinal medulla, or other sources (including nitrergic signals) could be responsible for them. Another explanation for our results is that chromaffin cell cultures contain a small proportion of endothelial cells and that changes in cell cycle could be ascribed to these cells. The fact that caspase inhibitors are not completely able to block the effect of NO on the cell cycle but do block the effect of NO on apoptosis shows that the NO effects on these cells could be involved. However, results presented in this paper demonstrate that NO-induced changes in the cell cycle can be reversed by NGF, whose receptors are expressed only in chromaffin cells. In fact, NGF is able to induce an increase in G_2M and S phases under basal conditions by mechanisms involving the activation of both MAPKs and PI3K/PKB. This conclusion is supported by results from Lopez-Collazo et al. (1997) showing that exposure of adrenal cells from vascular endothelium to NO donors, proinflammatory cytokines, or LPS was unable to induce apoptosis in these cells. Therefore, our results seem to demonstrate that the effects on the cell cycle are produced in chromaffin cells and support the idea that these cells, with neuronal and endocrine properties, could be an interesting, simple model for elucidating the nature and regulation of neurogenic and transduction signals involved in neural cell cycle regulation.

In summary, all these results suggest that, in chromaffin cells, NO produces: 1) cellular death by necrosis, evidenced by LDH and ATP release, and 2) apoptosis, demonstrated by flow cytometry and DNA fragmentation. On the other hand, the caspases pathway and cytochrome c release are implicated in apoptosis, insofar as 1) NO donors, as well as cytokines (IFNγ and TNFα), are able to activate caspase 3 and cytochrome c release and 2) inhibitors of these enzymes reverse the NO-mediated apoptosis. Thus, these cells could be a good model for studying cell death from neurodegenerative diseases and neuroprotection. Given that NO plays an important role in apoptosis, the model of cell death and apoptosis by NO studied here could be very useful for elucidating the mechanisms by which catecholaminergic neurons die and the mechanisms by which neurotrophins and other neuroprotective agents may induce neuroprotection in important neurodegenerative diseases.

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