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Nitric-Oxide-Induced Necrosis and Apoptosis in PC12 Cells Mediated by Mitochondria

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Abstract: Nitric oxide (NO) can trigger either necrotic or apoptotic cell death. We have used PC12 cells to investigate the extent to which NO-induced cell death is mediated by mitochondria. Addition of NO donors, 1 m*M* *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) or 1 m*M* diethylenetriamine–NO adduct (NOC-18), to PC12 cells resulted in a steady-state level of 1–3 μM NO, rapid and almost complete inhibition of cellular respiration (within 1 min), and a rapid decrease in mitochondrial membrane potential within the cells. A 24-h incubation of PC12 cells with NO donors (SNAP or NOC-18) or specific inhibitors of mitochondrial respiration (myxothiazol, rotenone, or azide), in the absence of glucose, caused total ATP depletion and resulted in 80–100% necrosis. The presence of glucose almost completely prevented the decrease in ATP level and the increase in necrosis induced by the NO donors or mitochondrial inhibitors, suggesting that the NO-induced necrosis in the absence of glucose was due to the inhibition of mitochondrial respiration and subsequent ATP depletion. However, in the presence of glucose, NO donors and mitochondrial inhibitors induced apoptosis of PC12 cells as determined by nuclear morphology. The presence of apoptotic cells was prevented completely by benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (a nonspecific caspase inhibitor), indicating that apoptosis was mediated by caspase activation. Indeed, both NO donors and mitochondrial inhibitors in PC12 cells caused the activation of caspase-3- and caspase-3-processing-like proteases. Caspase-1 activity was not activated. Cyclosporin A (an inhibitor of the mitochondrial permeability transition pore) decreased the activity of caspase-3- and caspase-3-processing-like proteases after treatment with NO donors, but was not effective in the case of the mitochondrial inhibitors. The activation of caspases was accompanied by the release of cytochrome *c* from mitochondria into the cytosol, which was partially prevented by cyclosporin A in the case of NO donors. These results indicate that NO donors (SNAP or NOC-18) may trigger apoptosis in PC12 cells partially mediated by opening the mitochondrial permeability transition pores, release of cytochrome *c,* and subsequent caspase activation. NO-induced apoptosis is blocked completely in the absence of glucose, probably due to the lack of ATP. Our findings suggest that mitochondria may be involved in both types of cell death induced by NO donors: necrosis by respiratory inhibition and apoptosis by opening the permeability transition pore. Further, our results indicate that the mode of cell death (necrosis versus apoptosis) induced by either NO or mitochondrial inhibitors depends critically on the glycolytic capacity of the cell. **Key Words:** Nitric oxide—Mitochondria—Glucose—Necrosis—Caspase activation—Apoptosis.
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In the central nervous system, nitric oxide (NO) at physiological levels acts as an intercellular messenger; however, at higher concentrations it can be neurotoxic (Gross and Wolin, 1995; Dawson and Dawson, 1996). NO is implicated in many pathological processes, such as brain ischaemia, neurodegeneration (Bolaños et al., 1997), and inflammation (Jenner and Olanow, 1996; Peuchen et al., 1997). Many different mechanisms of NO-induced cell death have been proposed, including the following: DNA damage-induced p53 accumulation, leading to apoptosis; DNA damage-induced poly(ADP)-ribose polymerase activation, leading to NADH and ATP depletion and necrosis; glutamate release and excitotoxicity; and oxidative damage due to inhibition of catalase, depletion of glutathione, release of free iron, and production of peroxynitrite (Murphy, 1999).

Some of the cytotoxic effects of NO may be mediated by inhibition of mitochondrial respiration. NO can rapidly, potently, and reversibly inhibit mitochondrial respiration at respiratory complex IV (cytochrome oxidase), whereas peroxynitrite ($ONOO^-$, formed in the reaction between NO and superoxide) can inhibit mitochondrial

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Abbreviations used: amc, 7-amino-4-methylcoumarin; CsA, cyclosporin A; DEVD, L-aspartic-L-glutamic-L-valyl-L-aspartic acid; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; IETD, L-isoleucyl-L-glutamic-L-threonyl-L-aspartic acid; mPTP, mitochondrial permeability transition pore; NO, nitric oxide; NOC-18 or DETA-NONOate, diethylenetriamine–nitric oxide adduct; SNAP, *S*-nitroso-N-acetyl-DL-penicillamine; TPP^+, tetraphenylphosphonium cation; YVAD, L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

aconitase, complexes I, II, and V, as well as opening the mitochondrial permeability transition pore (mPTP) (Brown, 1999).

We have shown previously that NO can cause acute inhibition of mitochondrial respiration in neuronal nerve terminals (Brown and Cooper, 1994) and subsequent glutamate release (McNaught and Brown, 1998). We also found that astrocytes and macrophages activated with cytokines and endotoxin to express inducible NO synthase produce up to 1 μM NO and inhibit their own cellular respiration and that of coincubated cells via the NO inhibition of cytochrome oxidase (Brown, 1995, 1998; Brown et al., 1995).

NO can cause cell death by either necrosis or apoptosis. Necrosis is often characterized by swelling of the cell and cytoplasmic organelles (especially mitochondria), followed by rupture of the plasma membrane. Apoptosis is regarded as an active and progressive response to physiologic or pathologic stimuli. It is characterized by early condensation of nuclear chromatin, enzymatic cleavage of the DNA into oligonucleosomal fragments, loss of plasma membrane phospholipid asymmetry, and segmentation of the cells into membrane-bound "apoptotic bodies." Cells dying by apoptosis can be phagocytosed by macrophages without damaging nearby cells. In contrast, the rupture of the necrotic cells releasing lysosomal and other enzymes causes inflammation of the surrounding cells (Hirsch et al., 1997; Kruman and Mattson, 1999).

The signalling pathways leading to apoptosis involve the activation of cysteine proteases known as caspases. They play a crucial role in the regulation and execution of apoptotic cell death. Caspases are constitutively present in cells as nonactive zymogens and require proteolytic cleavage into the catalytic active heterodimer. Two different pathways of caspase activation have been described. The first pathway involves death receptors such as Fas or tumor necrosis factor (TNF) receptors, which lead to activation of caspase-8 and subsequently can activate downstream caspases, such as caspase-3, -6, or -7 (Nicholson and Thorneberry, 1997; Chul et al., 1999). In the second pathway, various proapoptotic signals provoke the release of apoptogenic proteins, cytochrome *c*, and apoptosis-inducing factor (AIF) from mitochondria into the cytoplasm (Li et al., 1997; Susin et al., 1999). The release of these intermembrane proteins may be mediated by the PTP (Mignotte and Vayssiere, 1998; Susin et al., 1999). It is well documented that when cytochrome *c* is present in the cytoplasm, it binds to cytosolic protein Apaf-1 and, in the presence of ATP, activates procaspase-9. Active caspase-9 then cleaves and activates procaspase-3. Thus, caspase-3 can be activated by two different mechanisms: directly by caspase-8 or indirectly by mitochondria-mediated mechanisms.

Although some mechanisms of NO-induced cytotoxicity have been proposed, the crucial pathways involved in apoptotic or necrotic cell death induced by NO are still unclear. In the present studies, using PC12 cells as a model system, we investigated the extent to which NO-induced cell death is mediated by mitochondrial events. We tested whether inhibition of mitochondrial respiration by NO donors [*S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) or diethylenetriamine–NO adduct (NOC-18 or DETA-NONOate)] or inhibitors of mitochondrial respiration (rotenone, myxothiazol, azide) caused apoptosis or necrosis in PC12 cells cultured in the presence or absence of glucose. In the case of apoptosis, the activity of caspase-1-, 3-, and 8-like proteases has been studied. Simultaneously, the concentration of cytochrome *c* in the cytoplasm has been investigated to test whether the activation of caspases is accompanied by the release of cytochrome *c* from mitochondria. The activity of caspases and the release of cytochrome *c* were also studied in the presence of cyclosporin A (CsA) to test whether opening of the mPTP is involved in the release of cytochrome *c* and the activation of caspases.

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources: SNAP and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) from Alexis; NOC-18 from RBI; Dulbecco's modified Eagle's medium (DMEM) from Gibco-BRL; L-aspartic-L-glutamic-L-valyl-L-aspartic acid 7-amino-4-methylcoumarin (DEVD-amc); L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid (YVAD)-amc and L-isoleucyl-L-glutamic-L-threonyl-L-aspartic acid (IETD)-amc from Alexis; and CsA from Calbiochem. Other reagents were obtained from Sigma.

Cell culture

The rat pheochromocytoma cell line PC12 was purchased from ATCC (American Type Culture Collection) and maintained in DMEM supplemented with heat-inactivated horse serum (10%, vol/vol), fetal bovine serum (5%, vol/vol), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, in a humidified atmosphere of 5% CO_2. Cells were passaged every 5 days. The cells were plated in 24-well plates, covered with collagen (Sigma) at the density of $1.5 \times 10^4/cm^2$. In all studies, the medium was changed to glucose-free medium 1 day before experiments, and then to fresh medium (with or without 20 m*M* glucose) just before the exposure to NO donors (1 m*M* SNAP or 1 m*M* NOC-18) or mitochondrial inhibitors (2 μM myxothiazol, 2 m*M* azide, or 2 μM rotenone) for various intervals of time (3, 6, 8, 12, 18, or 24 h). Glucose-free medium (supplemented with serum) was used to minimize the ATP supply from glycolysis. In control culture of PC12 cells, the absence of glucose for 48 h did not increase cell death by either necrosis or apoptosis. PC12 cells can grow normally without glucose (in the presence of serum) for up to 4 days (Shimizu et al., 1996).

Assessment of cell viability and morphology

After 3 or 24 h of incubation with NO donors or mitochondrial inhibitors, cell viability was estimated by trypan blue exclusion (0.4%, light microscope) and propidium iodide staining (10 μM, fluorescence microscope). Propidium iodide and trypan blue stained only the cells with disrupted plasma membrane integrity, so these cells were considered as necrotic. The nuclear morphology of the cell was assessed using DNA dye Hoechst 33342 (1 μM, fluorescence microscope). The cells with chromatin condensation and nuclear fragmentation were

considered as apoptotic, and those with blue round nuclei as viable cells. Necrotic or apoptotic cells were counted in three microscopic fields with a minimum of 100 cells per field, in each well (three wells per treatment) and expressed as percentage of necrotic or apoptotic cells over the total number of cells. Each exposure was repeated at least three times.

Cell death was also studied after exposure of PC12 cells to the degraded solutions of NO donors. SNAP (1 m*M;* old solution, 72 h kept at room temperature) did not cause any death of PC12 cells after 24 h of exposure. However, 1 m*M* NOC-18 (old solution, 72 h kept at room temperature) caused a low level of necrosis of PC12 cells (5–10%) in the presence and absence of glucose when compared with the control culture.

Measurement of NO generation and oxygen consumption

Measurement of NO levels released by NO donors (1 m*M* SNAP or 1 m*M* NOC-18) and oxygen consumption in the presence of PC12 cells was performed using a Clark-type NO electrode (World Precision Instruments) inserted through the top of a Clark-type oxygen electrode, permitting simultaneous measurement of NO and oxygen levels. The PC12 cells cultured in DMEM at standard conditions (see Cell culture) were scraped, centrifuged (800 *g*, 5 min), and then resuspended in Krebs-HEPES buffer. Experiments were performed in 1 ml of Krebs-HEPES buffer consisting of 1.5 m*M* $CaCl_2$, 5.6 m*M* glucose, 10 m*M* HEPES, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.1 m*M* $MgSO_4$, 118 m*M* NaCl, pH 7.4, at 37°C, in the presence of PC12 cells ($\sim 1.5 \times 10^6$ cells), stirred continuously at a constant speed. The rate of oxygen consumption by PC12 cell was calculated before and after the addition of NO donors and expressed in nanomoles of oxygen per minute per number of cells. NO release from 1 m*M* SNAP or 1 m*M* NOC-18 was measured in the presence and absence of cells. The electrode was calibrated by adding aliquots of 1 μM NO-saturated water into the incubation chamber containing 1 ml of Krebs-HEPES buffer at 37°C, before and after the experiment. NO-saturated water was prepared by purging a gas-tight glass vial filled with distilled deionized water with nitrogen for ~15 min and then bubbling pure NO gas through the deoxygenated vial for a further 15 min or until saturation. The concentration of NO in NO-saturated water was taken as 2.0 m*M* at 20°C.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential of PC12 cells, in the presence and absence of NO donors (1 m*M* SNAP and 1 m*M* NOC-18) or mitochondrial inhibitor (2 μM myxothiazol), was measured in an oxygen-electrode chamber (the same as described above) with a tetraphenylphosphonium cation (TPP^+) electrode inserted from the top into the medium. As a membrane potential probe, TPP^+ was used because the lipophilic cation distributes across the cell and mitochondrial membranes according to the electric potentials. The TPP^+ uptake into the cells was measured at 37°C in a final volume of 1 ml of Krebs-HEPES buffer, pH 7.4 (Borutaite et al., 1995).

Measurement of intracellular ATP

ATP was determined luminometrically (Jade luminometer, Labtech International) using an ATP bioluminescence assay kit (Boehringer Mannheim). In brief, after the exposure of PC12 cells to NO donors or myxothiazol for 24 h, the cells were harvested by scraping and then centrifuged (800 *g*, 5 min). The pellet was resuspended in dilution buffer and lysed, and then the aliquots of cellular extract were assayed for ATP content using the ATP dependency of the light-emitting luciferase-catalyzed oxidation of luciferin. ATP concentrations were expressed as nanomoles per 10^6 cells.

Determination of lactate accumulation

After the exposure of PC12 cells to NO donors or mitochondrial inhibitor, in the presence or absence of glucose for 24 h (see Cell culture), aliquots of deproteinized medium were assayed for lactate using a L-lactic acid kit (Boehringer Mannheim) according to the protocol. The amount of lactate was measured by monitoring the oxidation of L-lactic acid by NAD in the presence of L-lactate dehydrogenase to pyruvate. The equilibrium of the reaction was displaced in favour of pyruvate and NADH formation by glutamate-pyruvate transaminase in the presence of glutamate. NADH formation was monitored at 340 nm and was proportional to lactate concentration.

Assay for caspase activity

After various time intervals of incubation with NO donors or mitochondrial inhibitors (in the presence or absence of glucose) with or without CsA, the cells were washed with Krebs-HEPES solution, pH 7.4, and then resuspended in lysis buffer (100 m*M* HEPES, pH 7.4, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1 m*M* EDTA, 10 m*M* dithiothreitol (DTT), 1 m*M* phenylmethylsulphonyl fluoride (PMSF), 10 mg/ml pepstatin, 10 mg/ml leupeptin) and left on ice for 40 min. Then cells were repeatedly passed through a syringe (25-gauge needle) until most of the cell membrane was disrupted, and centrifuged at 10,000 *g* for 10 min. Activity of caspases was measured using fluorogenic substrate peptides; DEVD-amc for caspase-3-, YVAD-amc for caspase-1-, and IETD-amc for caspase-8-like proteases (caspase-3-processing proteases). Cytosolic protein (50 μg) was incubated with 100 μM substrate peptide in 50 μl of incubation buffer (50 m*M* HEPES, 10% sucrose, 1 m*M* $MgCl_2$, 1 m*M* DTT, pH 7.4) at 37°C for 60 min. The release of amc was measured using a Perkin–Elmer fluorimeter (excitation at 380 nm, emission at 460 nm). Amc at 10 n*M* was used as standard. Caspase activity is expressed as nanomoles of amc per minute per milligram of total cytosolic protein [method of Lowry et al. (1951), Sigma protein assay kit].

Western blot

The cytosolic fractions that were prepared for measurement of caspase activity were also used for the detection of cytochrome *c*. The same amount of protein from control and treated PC12 cells with NO donors or mitochondrial inhibitors was electrophoresed on sodium dodecyl sulphate–polyacrylamide gel (SDS-PAGE) (12% separation gel and 5% stacking gel). Proteins were electrotransferred to blotting nitrocellulose membrane and then incubated with primary monoclonal antibodies against cytochrome *c* (7H8.2C12 mouse IgG, PharMingen, 1 μg/ml) at room temperature for 1 h. Finally, proteins were visualized using a peroxidase-conjugated antibody to mouse Ig and a chemiluminescence detection system. The results were quantified densitometrically by using a Leica Q500 image analyzer as described by Hargreaves et al. (1994).

RESULTS

NO donors inhibit oxygen consumption, decrease mitochondrial membrane potential, decrease cellular ATP, and increase lactate production

To determine the extent to which NO donors affect oxygen consumption and mitochondrial membrane po-

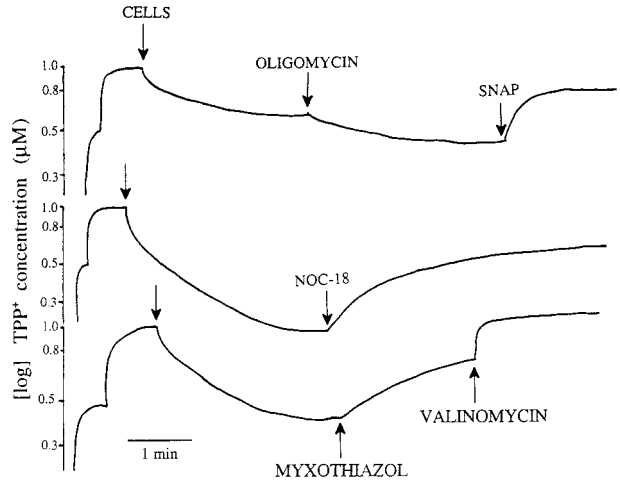

FIG. 1. The effect of NO donors (1 m*M* SNAP or 1 m*M* NOC-18) and mitochondrial inhibitor (2 μM myxothiazol) on the mitochondrial membrane potential ($\Delta\Psi_m$) of PC12 cells was assessed using a TPP^+ electrode. Addition of SNAP or NOC-18 caused a significant fall in $\Delta\Psi_m$ as recorded by a TPP^+ efflux from the cells. Similar results were obtained after addition of the respiratory inhibitor, myxothiazol. Oligomycin (0.6 μg/ml; an inhibitor of ATP synthesis) caused an increase in TPP^+ uptake and did not prevent SNAP-induced efflux of TPP^+. Valinomycin (0.5 μg/ml; a potassium ionophore) was used to set the mitochondrial membrane potential to zero and thus find the minimum level of TPP^+ uptake.

tential of PC12 cells, we measured these with an oxygen electrode and TPP^+ electrode in 1 ml of Krebs-HEPES buffer (pH 7.4 at 37°C) before and after addition of NO donors. Before the addition of NOC-18, the oxygen consumption of PC12 cells was between 5.3 and 5.6 nmol of O/min/10^6 cells, but within minutes of adding NOC-18, it was between 0.00 and 0.31 ± 0.03 nmol of O/min/10^6 cells, so NOC-18 inhibited mitochondrial respiration between 94 and 100%. Similar results were obtained with 1 m*M* SNAP, but were difficult to quantify because SNAP itself induces some oxygen consumption. The time course of the inhibition of respiration was parallel to the NO release from the NO donors (1 m*M* SNAP or 1 m*M* NOC-18). In the case of 1 m*M* SNAP, the release of NO was variable, but in most cases the steady-state level of NO measured by electrode was 1.2–2.9 μM in Krebs-HEPES buffer (1.0–1.5 μM in DMEM) after 10–15 min. Because the release of NO from SNAP was very rapid, 90% of inhibition of mitochondrial respiration was already observed within <1 min. In the case of NOC-18, the release of NO was less variable and the steady-state level between 1.5 and 2.5 μM in Krebs-HEPES buffer (0.9–1.4 μM in DMEM) was observed after 15–25 min. In the first minute, the inhibition of PC12 mitochondrial respiration was between 50 and 70% and complete inhibition between 15 and 20 min.

Addition of 2 μM myxothiazol or 2 μM rotenone caused complete inhibition of oxygen consumption of the cells.

The addition of either NO donors (1 m*M* SNAP or 1 m*M* NOC-18) or respiratory inhibitor (myxothiazol) decreased TPP^+ uptake into the cells (Fig. 1), as we could expect after inhibition of mitochondrial respiration and a decrease in mitochondrial membrane potential. Oligomycin increased the TPP^+ uptake presumably due to an increase in mitochondrial membrane potential resulting from inhibition of the ATP synthase, and NO donors still decreased the TPP^+ uptake in the presence of oligomycin, suggesting that the NO-induced decrease in TPP^+ uptake was due to the inhibition of respiration, rather than any effect on ATP consumption.

As NO-induced mitochondrial inhibition might result in energy depletion, we studied whether NO donors or a mitochondrial inhibitor decreased the ATP level in PC12 cells. Indeed, exposure of PC12 cells to NO donors (1 m*M* SNAP or 1 m*M* NOC-18) as well as a mitochondrial inhibitor (2 μM myxothiazol) resulted in almost total depletion of ATP when the cells were cultured in the medium deprived of glucose (Fig. 2). However, the same exposure of PC12 cells to NO donors or myxothiazol, but in the presence of glucose, resulted in relatively small decreases in ATP levels (Fig. 2).

The high level of ATP in the presence of glucose (20 m*M*) was accompanied by a high concentration of lactate in the medium (control culture, 3.1 ± 0.16 m*M*), and NO donors and myxothiazol increased the lactate level (1 m*M* SNAP, 5.83 ± 0.84 m*M;* 1 m*M* NOC-18, 5.34 ± 0.15 m*M;* myxothiazol, 7.15 ± 0.67 m*M*), consistent with an activation of glycolysis after mitochondrial inhibition. In the absence of glucose, the level of lactate in the medium was between 0.91 and 0.98 m*M* after expo-

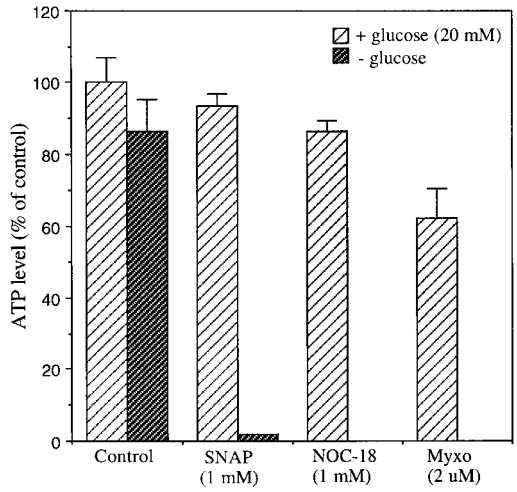

FIG. 2. The effect of NO donors (1 m*M* SNAP or 1 m*M* NOC-18) and mitochondrial inhibitor [2 μM myxothiazol (Myxo)] on ATP concentration in PC12 cells. In the absence of glucose, SNAP and NOC-18, as well as myxothiazol, caused complete depletion of ATP. However, in the presence of glucose, the levels of ATP decreased relatively little in the presence of NO donors or myxothiazol. The data are expressed as % of the control (100%, 1.78 ± 0.12 nmol of ATP/10^6 cells) from two independent experiments (means ± SD, three independent measurements from each experiment).

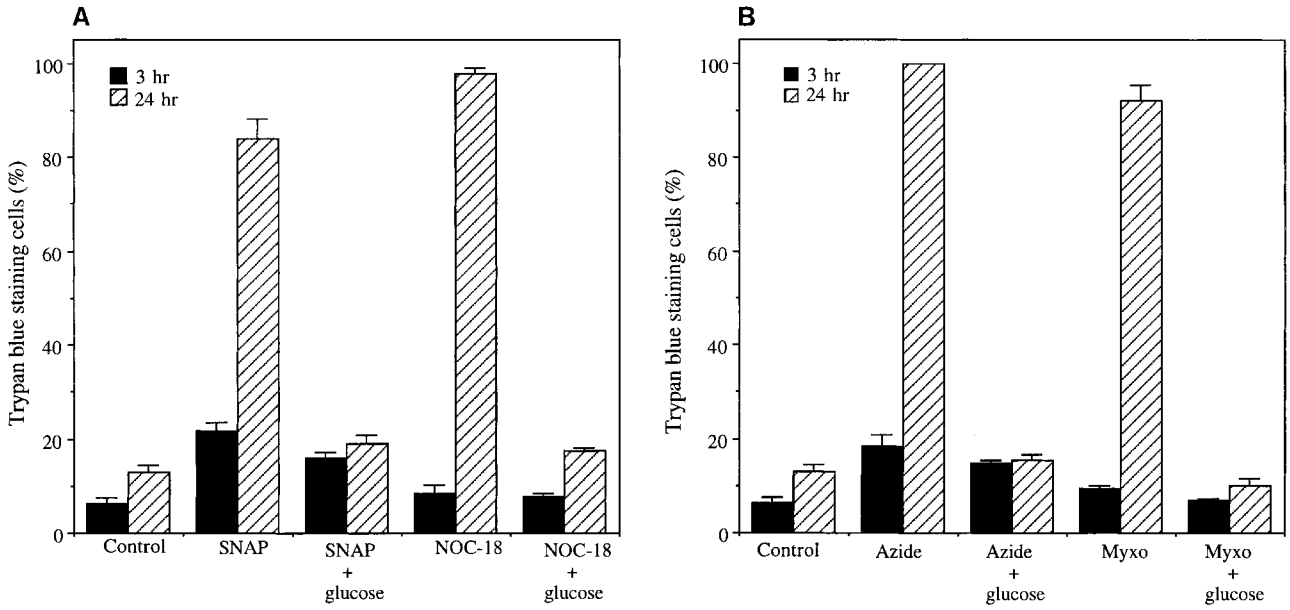

FIG. 3. The induction of necrosis in PC12 cells by (**A**) NO donors (1 m*M* SNAP or 1 m*M* NOC-18) and (**B**) mitochondrial inhibitors [2 μM myxothiazol (Myxo) and 2 m*M* azide] for 3 or 24 h in the presence or absence of glucose. Note the significant decrease in the number of necrotic cells in the presence of glucose after 24 h of incubation. Percentages of trypan blue-positive cells were quantified (see Materials and Methods), and values represent means ± SD of determinations made in four separate cultures.

sure to NO donors or myxothiazol and 0.5 m*M* in the control culture.

NO donors and respiratory inhibitors cause necrosis in the absence of glucose, but not in its presence

To test whether the mitochondrial inhibition induced by NO could cause cell death, PC12 cells were exposed for 24 h to nitrosylating (1 m*M* SNAP) and non-nitrosylating (1 m*M* NOC-18) NO donors (Fig. 3A) or to inhibitors of the respiratory chain (2 m*M* azide or 2 μM myxothiazol) (Fig. 3B) in DMEM deprived of glucose (see Materials and Methods). Both NO donors and respiratory inhibitors caused 80–100% necrosis as characterized by intact chromatin (Hoechst 33342 staining) and loss of plasma membrane integrity (trypan blue and propidium iodide staining) (Fig. 4B). PC12 cells were exposed, in the presence of glucose, for 24 h to NO donors (1 m*M* SNAP or 1 m*M* NOC-18) or mitochondrial inhibitors (2 m*M* azide and 2 μM myxothiazol) to determine whether the presence of glucose would protect PC12 cells against necrosis. Indeed, the presence of glucose reduced the number of necrotic cells to a level similar to the control in the case of both NO donors (Fig. 3A and 4C) and the mitochondrial inhibitors (Fig. 3B). This suggests that the necrosis induced by NO donors in the absence of glucose was due entirely to respiratory inhibition and subsequent ATP depletion.

NO donors and respiratory inhibitors cause apoptosis in the presence of glucose, but not in its absence

However, in the presence of glucose, both NO donors and mitochondrial inhibitors induced apoptosis of the cells as estimated by nuclear morphology. In these cells, chromatin condensation and nuclear fragmentation were observed (Fig. 4C), whereas the control culture had round blue nuclei of viable cells (Fig. 4A, Hoechst 33342 staining). The number of apoptotic cells was much higher in the culture treated with NO donors (Fig. 5A) than with the mitochondrial inhibitors (Fig. 5B). The 3-h incubation with either NO donors or mitochondrial inhibitors did not cause significant cell death in any experiment. In the absence of glucose, apoptosis was not observed with either NO donors or respiratory inhibitors.

NO donors and respiratory inhibitors induce apoptosis via caspase activation

To address whether or not caspases contribute to mitochondrial inhibitor- and NO donor-induced apoptosis, PC12 cells were incubated with NO donors (SNAP or NOC-18) or mitochondrial inhibitors (azide, myxothiazol, or rotenone) in the presence or absence of z-VAD-fmk, a cell-permeable nonspecific caspase inhibitor. The presence of apoptotic cells was completely prevented by z-VAD-fmk in a concentration-dependent manner (Fig. 5A and B), indicating that apoptosis was mediated by caspases. We therefore studied the time course of the activation of caspase-1-, -3-, and -8-like proteases (caspase-3-processing proteases). Cytosolic fractions from PC12 cells exposed to NO donors and mitochondrial inhibitors for various times (3, 8, 12, 18, or 24 h) were tested for the cleaving activity of three fluorogenic substrates (DEVD-, YVAD-, and IEDT-amc) (see Materials and Methods). DEVD-amc cleaving activity was clearly increased after incubation with NO donors (Fig. 6A) or mitochondrial inhibitors (Fig. 6C) only in the

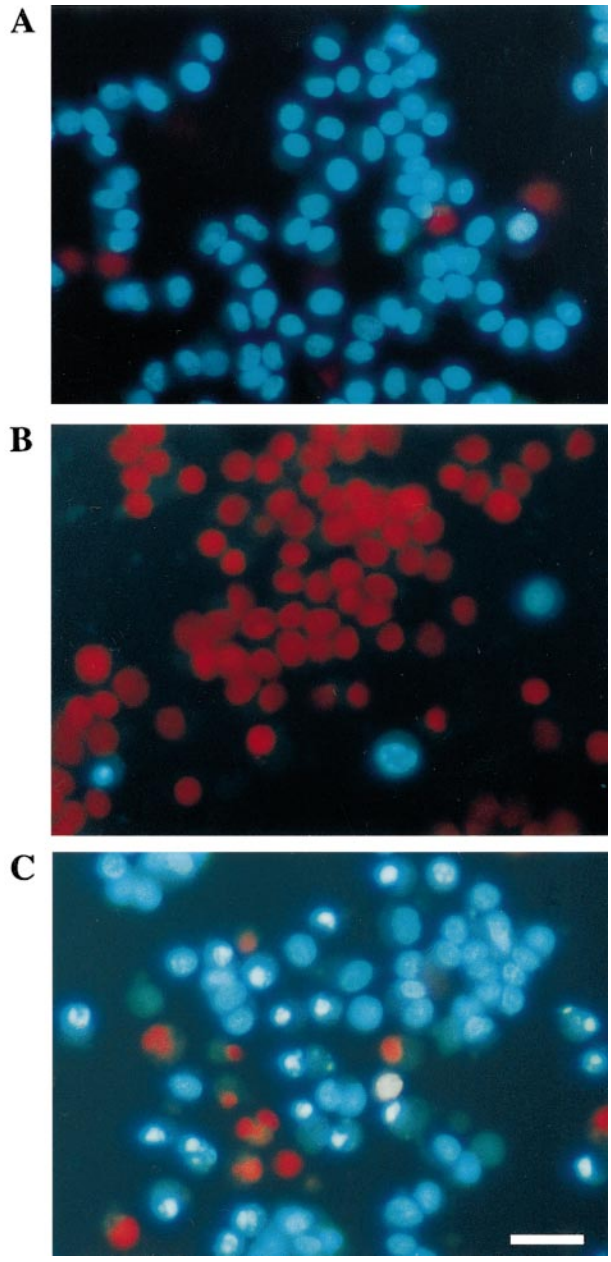

FIG. 4. Morphological analysis of nuclear chromatin in PC12 cells stained with the DNA-binding fluorochrome Hoechst 33342 and propidium iodide using a fluorescence microscope. In control culture (no glucose for the last 48 h) (**A**), viable cells show blue round nuclei (Hoechst 33342 staining). Necrotic cells were observed very rarely (propidium iodide, red colour of nuclei, present only in the cells with disrupted plasma membrane integrity). Apoptotic cells were hardly observed in control culture. In the absence of glucose (**B**), NOC18 induced necrosis in PC12 cells after 24 h of incubation as assessed by propidium iodide, staining. Apoptotic cells were not observed. In the presence of glucose and NOC-18 (**C**), the number of necrotic cells was greatly decreased (propidium iodide-positive cells), but apoptotic cells were present as characterized by chromatin condensation and nuclear fragmentation of Hoechst 33342-stained cells. Bar = 50 μm.

presence of glucose. Maximal DEVD-cleaving activity was detected at 24 h following NOC-18 or SNAP (Fig. 6A) and azide treatment (Fig. 6C). Myxothiazol-induced DEVD cleaving was the highest after 8 h of incubation (Fig. 6C). After exposure of PC12 cells to NO donors (Fig. 6B) or mitochondrial inhibitors (Fig. 6D), IETD-cleaving activity was increasing with time (only in the presence of glucose) to reach the highest values after 24 h of treatment almost in all cases. YVED-amc was not cleaved under any of the experimental conditions examined here (data not shown).

NO-induced caspase activation is partially prevented by CsA

To determine whether the mPTP is involved in NO-induced apoptosis, we have studied the activity of caspase-3- and caspase-3-processing proteases in PC12 cells after 8 h of exposure to NO donors or mitochondrial inhibitors in the absence or presence of 3 μM CsA (inhibitor of mPTP). CsA prevented the activity of caspase-3- (Fig. 7A) and caspase-3-processing proteases (Fig. 7B) in the case of the treatment with SNAP, and only partially after treatment with NOC-18, and was not effective in the case of myxothiazol and azide. CsA was always more effective in the inhibition of IEDT- (Fig. 7B) than DEVD-cleavage activity (Fig. 7A).

Caspase activation is accompanied by cytochrome *c* release into the cytosol

The same cytosolic fractions that were prepared for measurement of caspase activity were used for western blot analysis to test whether the caspase activity was accompanied by the release of cytochrome *c*. Cytochrome *c* content in the cytosol fraction was increased significantly after 8 h of incubation (only in the presence of glucose) with SNAP and NOC-18, but not with mitochondrial inhibitors (myxothiazol and azide) (Fig. 8). Furthermore, simultaneous treatment of PC12 cells with NO donors (or mitochondrial inhibitors) and 3 μM CsA decreased the content of cytochrome *c* in the cytosol fractions after exposure to SNAP, partially after exposure to NOC-18, and was not effective in the case of mitochondrial inhibitors (myxothiazol and azide) (Fig. 8).

DISCUSSION

We have investigated the causes of NO-induced cell death and the extent to which this involves NO inhibition of mitochondrial respiration. The two different NO donors caused strong and rapid inhibition of cellular oxygen consumption and depolarization of the mitochondrial membrane potential. The levels of NO released should have caused strong inhibition of cytochrome oxidase (Brown and Cooper, 1994; Brown, 1995), and this is probably the reason for the inhibition of oxygen consumption and depolarization of the mitochondria. Long-term (hours) incubation of cells with NO donors can cause inhibition of mitochondrial respiration at other sites (complexes I, II, and V and aconitase) (Lizasoain

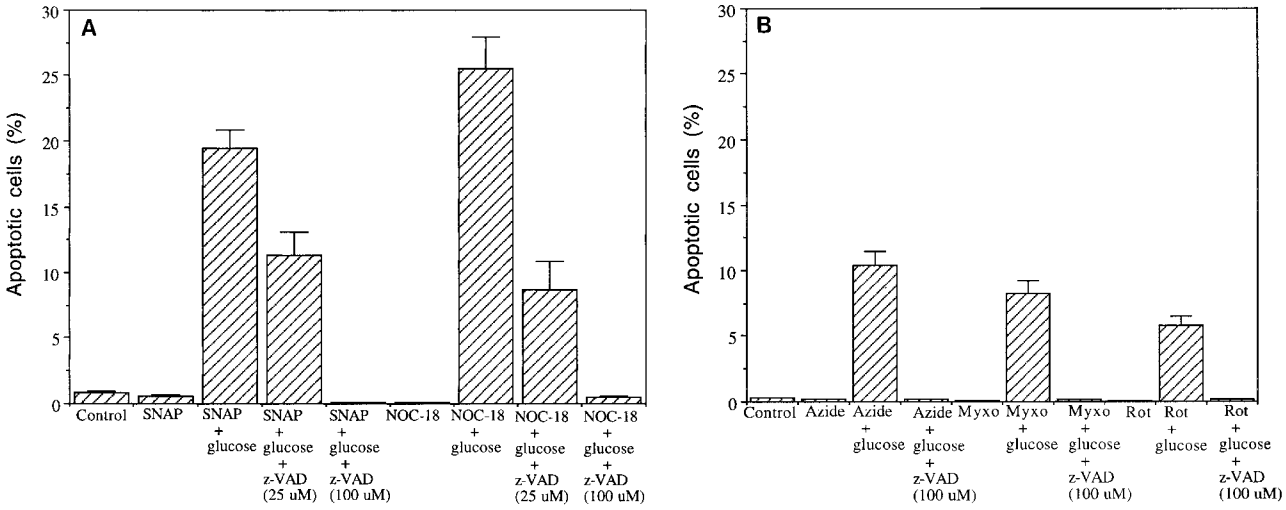

FIG. 5. The induction of apoptosis in PC12 cells by (**A**) NO donors (1 m*M* SNAP or 1 m*M* NOC-18) and (**B**) mitochondrial inhibitors [2 μM myxothiazol (Myxo), 2 m*M* azide, or 2 μM rotenone (Rot)] after 24 h of exposure in the presence of glucose. The presence of apoptotic cells was completely prevented by z-VAD-fmk (z-VAD; 100 μM). The cells were stained with DNA dye Hoechst 33342, and nuclei with chromatin condensation and nuclear fragmentation were counted (see Materials and Methods). Values represent the means ± SD of four or more separate cultures.

et al., 1996; Clementi et al., 1998), but we did not investigate whether this occurred in these cells. Rather we were interested in the extent to which the NO-induced inhibition of respiration causes subsequent cell death, and under what conditions.

In the absence of glucose, the NO donors caused total depletion of ATP resulting in 80–100% necrosis after 24 h. Specific respiratory chain inhibitors caused a similar level of necrotic cell death. As the NO donors inhibited cellular respiration, and specific respiratory inhibitors caused cell death over a similar time course to the NO donors, it follows that NO donors must cause cell death by respiratory inhibition, unless the NO does something additional to preempt this form of cell death. However, the fact that glucose prevents both NO- and respiratory inhibitor-induced ATP depletion and necrosis indicates that the NO-induced necrosis is due entirely to the ATP depletion following respiratory inhibition. This is an important conclusion because a variety of other mechanisms of NO-induced necrosis have been suggested. However, our conclusion might apply only to conditions of glucose deprivation, and may not generalize to other cell types. In the presence of glucose, 1 m*M* SNAP and 1 m*M* NOC-18 producing high levels of NO for a sustained period caused very little necrosis of PC12 cells (particularly if the necrosis secondary to apoptosis is subtracted off). So if NO can cause necrosis by other means, these PC12 cells are resistant to these mechanisms, at least under the conditions used here.

It might be argued with some justification that NO-induced cell death in the absence of glucose is of little physiological or pathological relevance. However, it should be noted that PC12 cells, as with most cell lines, have a high glycolytic capacity, so that inhibition of mitochondrial production has relatively little impact on their ATP turnover (Lin et al., 1993). Whereas most primary cells in the body rely more heavily on mitochondrial ATP production, particularly neurons (Erecińska and Wilson, 1992; Rolfe and Brown, 1997), many die necrotically in response to mitochondrial inhibitors or anoxia (Shimizu et al., 1996). Thus, whether NO-induced inhibition of mitochondrial respiration will cause necrotic cell death depends in part on the glycolytic capacity of the cell. In some conditions, NO (or more likely its derivative peroxynitrite) inhibits glycolysis at glyceraldehyde-3-phosphate dehydrogenase (Yasuda et al., 1998; Mohr et al., 1999), and if both glycolysis and respiration are substantially inhibited, a cell will inevitably die by necrosis.

Although NO donors did not cause much necrosis in the presence of glucose, they induced a substantial level of apoptosis. Specific respiratory chain inhibitors also induced apoptosis in the presence of glucose, but the level of apoptosis at 24 h was between a half and a third of that induced by NO donors. This suggests that the mechanism by which NO donors induced apoptosis is not solely via respiratory inhibition. NO must be doing something in addition to respiratory inhibition to cause the level of apoptosis that we observed. However, this should not obscure the possibility that NO-induced respiratory inhibition causes a significant proportion of the apoptosis or is a contributory cause of all of it.

In the absence of glucose, where total ATP depletion was found, apoptosis and caspase activation were not observed with either NO donors or respiratory inhibitors. This lack of apoptosis was not due to the high level of necrosis, because even expressed as a fraction of the remaining viable cells, the level of apoptosis was much lower than that in the presence of glucose. The probable cause for this inhibition of apoptosis in the absence of

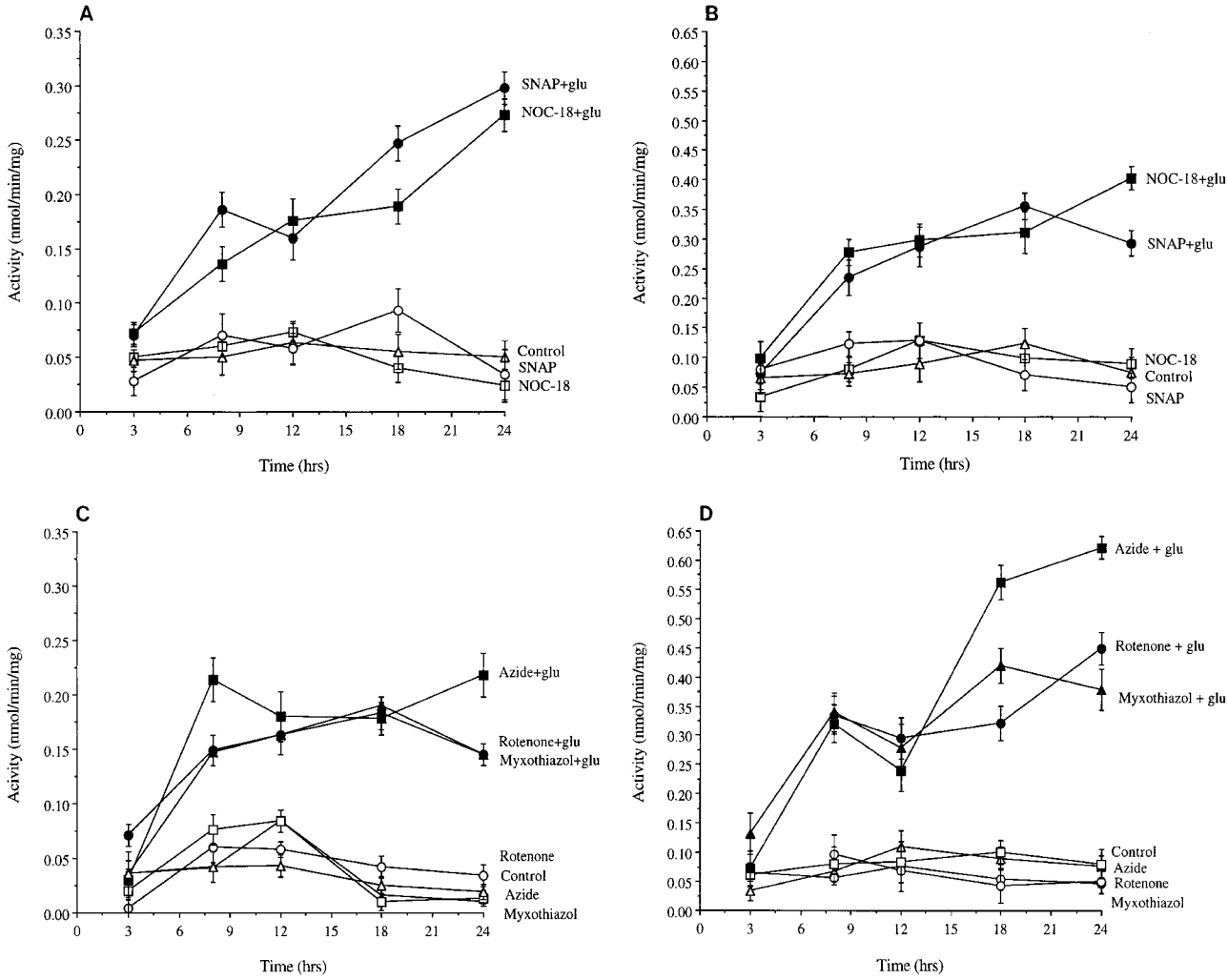

FIG. 6. Time course of the activation of (**A**) DEVD- and (**B**) IETD-cleaving activity in the cytosolic fraction of PC12 cells incubated with 1 m*M* SNAP (●, ○) or 1 m*M* NOC-18 (■, □) in the presence or absence of glucose (glu; filled and empty symbols, respectively). (**C**) DEVD- and (**D**) IETD-cleaving activity after exposure to the inhibitors of mitochondrial respiration [2 μM myxothiazol (▲, △), 2 m*M* azide (■, □), or 2 μM rotenone (●, ○) in the presence or absence of glucose (glu; filled and empty symbols, respectively)] for different intervals of time. Note that the DEVD- or IETD-cleaving activity was observed only in the presence of glucose and was clearly increased within 6–8 h of incubation and continued to increase to reach maximal activity after 24 h of exposure. Values represent the means ± SD of three or more experiments and are expressed as nmol/min/mg of protein as described in Materials and Methods.

glucose is the well known ATP requirement for several steps in the apoptotic pathway (Richter et al., 1996; Eguchi et al., 1997). Thus, the glycolytic capacity of the cell determines not only whether necrosis will occur in response to NO and respiratory inhibition, but also whether apoptosis will occur, such that if the glycolytic capacity is sufficient, as in the case of PC12 cells, apoptosis will occur, whereas if it is insufficient, necrosis will occur.

The apoptosis induced by NO donors and respiratory inhibitors was mediated by caspases, as demonstrated by the findings that caspase activities were activated prior to nuclear apoptosis and a nonspecific caspase inhibitor (z-VAD-fmk) completely prevented apoptosis. NO donors and respiratory inhibitors induced a caspase-3-like protease activity (results with DEVD-amc) and a caspase-3-processing protease activity (results with IETD-amc) to a similar extent and with a similar time course. Neither DEVD-amc nor IETD-amc is entirely specific for particular caspases (Bossy-Wetzel and Green, 1999) and thus the results with DEVD-amc and IETD-amc are probably best interpreted simply as increases in caspase activity. The absence of caspase activation in the absence of glucose (severe ATP depletion) indicates that hypoglycemic block on apoptosis is prior to caspase activation, as has been observed by others (Richter et al., 1996; Leist et al., 1999).

Cytochrome *c* was released into the cytoplasm by NO donors and respiratory inhibitors to the level that correlated with the level of nuclear apoptosis. Cytochrome *c* release is a central event in mitochondrial triggered apoptosis leading to caspase activation (Kroemer et al.,

1997; Li et al., 1997; Suzuki et al., 1999). However, cytochrome *c* release can also be caused by the nonmitochondrial pathway of apoptosis induction (such as Fas-induced apoptosis) (Krippner et al., 1996; Adachi et al., 1997). CsA partially blocked NO donor and respiratory inhibitor-induced caspase activation and cytochrome *c* release. This blockage was almost total in the case of SNAP, partial for NOC-18, and in the case of respiratory inhibitors, only significant for IETD-amc activity. CsA blocks the mPTP. Thus, these findings suggest that cytochrome *c* release and caspase activation were mediated by opening of the mitochondrial pore at least in the case of SNAP. SNAP has been shown to activate directly the pore in isolated mitochondria (Hortelano et al., 1997), whereas NOC-18 and other nonnitrosylating NO donors are less able to open the pore (V. Borutaite and G. C. Brown, unpublished observations). Thus, the NO donors probably caused caspase activation, partly by opening the pore at least in the case of SNAP.

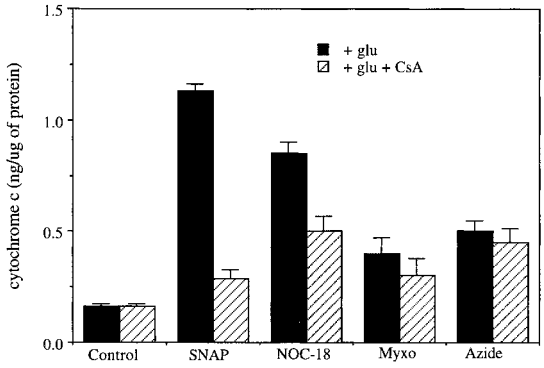

FIG. 8. Effect of CsA (3 μM) on the concentration of cytochrome *c* in the cytosol fractions prepared from PC12 cells incubated for 8 h in the presence of glucose (glu) with NO donors (1 m*M* SNAP or 1 m*M* NOC-18) or mitochondrial inhibitors [2 μM myxothiazol (Myxo) or 2 m*M* azide]. The amount of cytochrome *c* present in cytosolic fractions was studied by western blot and assessed by densitometry. The data present the means ± SEM of integrated optical density of three or more independent blots.

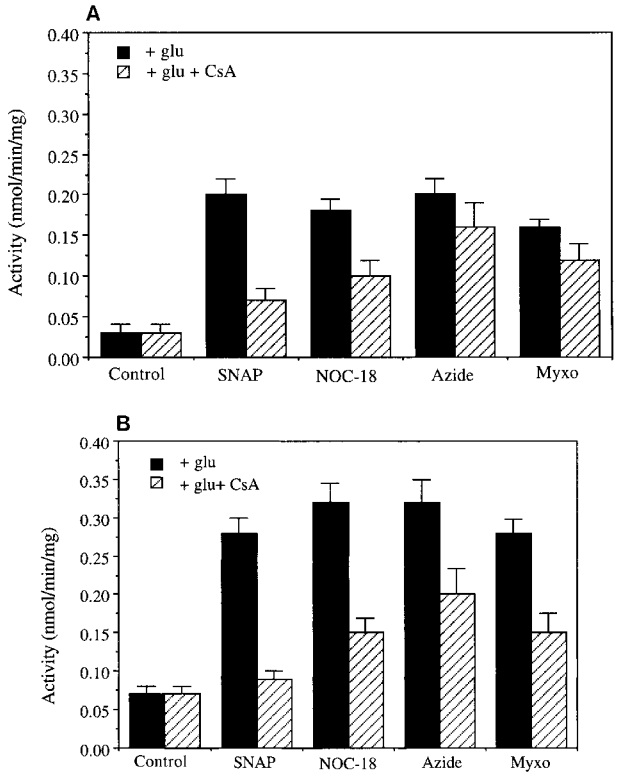

FIG. 7. Effect of CsA (3 μM) on the activity of (**A**) caspase-3- and (**B**) caspase-3-processing proteases after 8 h of incubation of PC12 cells in the presence of glucose (glu) with NO donors (1 m*M* SNAP or 1 m*M* NOC-18) or mitochondrial inhibitors [2 m*M* azide or 2 μM myxothiazol (Myxo)]. Note that CsA prevented the activity of caspase-3- and caspase-3-processing proteases in the case of SNAP, only partially in the case of NOC-18, and was not effective after treatment with mitochondrial inhibitors. Activity of caspases was measured using fluorogenic substrate peptides (see Materials and Methods). Values represent the means ± SEM of at least three experiments and are expressed as nmol/min/mg of protein.

In conclusion, NO donors caused necrosis in the absence of glucose due to inhibition of respiration (and subsequent total ATP depletion), but in the presence of glucose (to maintain ATP level via glycolysis) NO donors caused apoptosis, probably partially due to respiratory inhibition and/or opening of the mPTP, leading to cytochrome *c* release and caspase activation. Mitochondrial inhibitors themselves caused necrosis in the absence of glucose, and apoptosis in the presence of glucose; and apoptosis was due to caspase activation accompanied by cytochrome *c* release, but mitochondrial permeability transition was apparently not involved.

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