Biochemical Pharmacology, Vol. 51, pp. 1389–1395, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00065-2

Cytotoxic Effects of an Oxidative Stress on Neuronal-Like Pheochromocytoma Cells (PC12)

*Florence Vimard, André Nouvelot and Dominique Duval**

UNIVERSITÉ DE CAEN,-CNRS URA - 1829, LABORATOIRE DE NEUROSCIENCES, BOULEVARD H. BECQUEREL, BP 5098, 14043 CAEN CÉDEX, FRANCE

ABSTRACT. Although the generation of oxygen derivatives during ischemia and reperfusion is generally held as a major event in the process leading to neuronal death, the biochemical mechanisms responsible for cell degeneration remain poorly understood. To better understand the toxicity induced by oxidative stress in neural tissue, we have tested the effect of an exogenous hydroperoxide, cumene hydroperoxide (CHP), on the metabolism and viability of PC12 cells. Addition of CHP in the culture medium leads to significant cell death that becomes perceptible at concentrations above 1 μM and reaches a maximum (80–90% toxicity) at 100 μM. A time-course study shows that Trypan blue uptake is preceded by a rapid phase of cell rounding and detachment from the substratum (within 30 min) followed by a progressive uptake of the dye (60–120 min). During this 2-hr period, we failed to observe any major signs of membrane lipoperoxidation (such as MDA production or fatty acid release). On the other hand, we observed that cell death is preceded by a striking decrease in cellular ATP content and in the retention of rhodamine 123 (within 15–30 min of treatment); thus, suggesting that the mitochondria may be the primary target of hydroperoxide action. BIOCHEM PHARMACOL 51;10:1389–1395, 1996.

KEY WORDS. cumene hydroperoxide; neuronal-like PC12 cells; ATP depletion; disruption of mitochondrial membrane potential; cell necrosis

The generation of oxygen derivatives is generally held as a major event in the process leading to neuronal death during ischemia and reperfusion but, also, in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis [1–4].

Many attempts have, thus, been made to use natural and synthetic antioxidant molecules and free radical scavengers as neuroprotective agents [5–6].

However, despite extensive investigations, the biochemical alterations produced by these oxygen derivatives as well as the mechanisms leading to neuronal death remain elusive. Several possibilities have been proposed including peroxidation of membrane fatty acids leading to alterations of permeability and ionic balance; early depletion of intracellular ATP and pyridine nucleotides responsible for disruption of the cytoskeleton; impaired DNA reparation and mitochondrial collapse; or even induction of early genes leading to apoptosis [7–10].

To better understand the steps involved in oxygen derivative-induced neuronal cell death, we have studied the effects of an exogenous hydroperoxide CHP† on PC 12 cell metabolism and viability. Indeed, pheochromocytoma cells (PC12) cells have been widely used as a "neuronal model" line to investigate the toxic effect of 6 hydroxydopamine, hydrogen peroxide, MPTP, and hypoxia [11–13], whereas, CHP has been described as a good inducer of lipoperoxidation and exerts cytotoxic effects at relatively low concentrations in various cellular models [14–17].

MATERIALS AND METHODS

Cell Culture

We used a subclone of PC12 cells, able to grow on plastic without polylysine or collagen coating, but still able to differentiate in the presence of NGF or basic FGF. The cells were grown routinely in Dulbecco's Minimal Essential Medium supplemented with 10% heat-inactivated fetal calf serum, 5% inactivated horse serum (Biological Industrie, Israël), 1% of a solution containing 10.000 μ/mL penicillin G, 10 mg/mL streptomycin, and 25 μg/mL amphotericine B in 0.9% Nacl (Sigma, St. Louis, MO, U.S.A.) and 2 mmol/L L-glutamine (Sigma). The cells were subcultured twice a week by gentle scraping. For determination of the cytotoxic effect of CHP (Sigma), the cells were seeded at a concentration of 10,000 cells/cm^2 in 6-well plates (2 mL/well). Fourty-eight hours after seeding, the medium was renewed by fresh medium containing the desired drug concentration. After a 2-hr additional incubation, cell viability was determined using either the Trypan blue exclusion procedure or LDH release.

* Corresponding author.

† *Abbreviations:* MPTP, 1-méthyl-4-phényl-1,2,3-6-tetrahydropyridine; LDH, lactate dehydrogenase; CHP, cumene hydroperoxide; MDA, malondialdehyde; NGF, nerve growth factor; b FGF, basic fibroblast growth factor; HETE, hydroxyeicosatetraenoic acid.

Received 11 August 1995; accepted 11 January 1996.

Determination of Lactate Dehydrogenase Activity

The culture medium was centrifuged at 250 *g* for 10 min to remove any nonadherent cells. LDH activity was determined spectrophotometrically at 340 nm using the method described by Wroblewski and Ladue [18]. The basal activity present in the culture medium alone (in the absence of cells) was systematically subtracted from the value found in the supernatants.

Determination of Cellular DNA Content

The cells were lysed in a 0.1 mol/L phosphate buffer pH 7.4 containing 0.1% Triton × 100 and sonicated twice at 20 kHz for 15 sec. Aliquots (40–80 μL) of the cell lysates were then taken for the determination of DNA content using the fluorescent dye, Hoechst 33258, according to the method described by Labarca and Paigen [19] and using calf thymus DNA as a standard.

[^{14}C]-Arachidonate Incorporation

Twenty-four hours after seeding, each well received 37–74 kBq of [^{14}C]-Arachidonate acid. After a 24-hr incubation period, the medium was renewed by arachidonate-free medium with or without CHP. After an additional 2-hr incubation, the radioactivity released in the supernatant and the cell radioactivity were determined by liquid scintillation spectrometry. Aliquots of the supernatants were then extracted as previously described [20]. Following protein precipitation by acetone (2 vol.) the medium was extracted by 3 vol. of chloroform, brought to pH 3.5 with 70% citric acid. The chloroform extracts were then evaporated under a N_2 stream, dissolved in chloroform-methanol, and spotted onto silica gel plates (Watman LK_6DF). After development using the upper phase of the mixture ethylacetatet/isooctane/acetic acid/water (90:50:20:100 v/v) as solvent, the distribution of the radioactivity on the plate was analyzed by scraping off and counting the gel.

Determination of Intracellular ATP

The cells were gently scraped off, washed by 2 mL of phosphate buffer pH 7.4 and centrifuged at 15.000 × *g* for 2 min. The pellet was then resuspended in 3% perchloric acid and centrifuged again at 15,000 × *g* for 2 min.

The supernatant was neutralized using 0.5 M $NaHCO_3$ and centrifuged to remove any insoluble material. The adenine nucleotides were then analysed by liquid chromatography using a cyclohexyl 5 μm column (Analychem International provided by Varian, France) and a 0.1 M phosphate buffer pH 6.0 as the elution buffer. ATP and ADP were identified by their retention times as compared to those of reference molecules.

Determination of Malonaldialdehyde Formation

MDA concentrations in the cell pellets and supernatants were determined according to the method of Beljean-Leymarie and Bruna [21]. Aliquots (1 mL) of the samples were mixed with 0.2 mL of 0.01M 2,4 hydrazinobenzothiazole (HBT) and 1 mL phosphate buffer pH 2.5. After 30 min incubation at 70°C, the derivation product (HBT-MDA) was extracted with hexane containing HBT-acetylacetone as an internal standard. The products were then analyzed by gas chromatography using a thermoionic specific detector.

Fluorimetric Analysis of Rhodamine 123 Retention

The changes in mitochondrial membrane potential ($\Delta\psi$) were evaluated by following the cellular retention of rhodamine 123 [22–24]. The cells were incubated for 30 min at 37°C with rhodamine 123 (5 μM), then washed and allowed to stand at 37°C for 45 min in a rhodamine-free medium. After medium renewal, the cells were then treated by 60 μM CHP for various periods of time. At the end of the treatment, the dye remaining trapped into the cells was determined by fluorimetric analysis following ultrasonic disruption. The excitation wavelength was 490 nm and the emission wavelength was 515 nm. The fluorescence intensity was expressed as fluorescence arbitrary units per μg of DNA.

Determination of Membrane Fatty Acids

The cell pellets extracted with chloroform-methanol (2:1 v/v). After preparation of fatty acid methyl esters according to Hagenfeldt [25], the various fatty acids were analyzed by gas chromatography using a varian GC 3300 equipment and a flame ionization detector.

Protein Determination

The amounts of cellular proteins were determined using the colorimetric method of Lowry and coworkers [26] and bovine serum albumin as standard.

RESULTS

Cytotoxic Action of Cumene Hydroperoxide

We, first, examined the effect of increasing concentrations of CHP (1–100 μM) on PC12 viability, after 120 min of treatment. As shown in Fig. 1, increasing doses of CHP lead to a dose-dependent increase in LDH release that becomes perceptible at a concentration of 5 μM and reaches a maximum (3- to 4-fold increase as compared to control) at 100 μM. LDH release appears associated with cell rounding and detachment from the surface of the culture flasks; we have, thus, determined, using the Trypan blue dye exclusion procedure, both the total number of cells recovered in the supernatant after 120 min of treatment with CHP (5–100 μM) and the proportion of these floating cells that incorporate the dye. It should be stressed that, even in the presence of high concentrations of CHP (100 μM), a significant percentage of the cell population (which may vary from 10–40% according to the experiment) remains attached to the surface of the culture flasks.

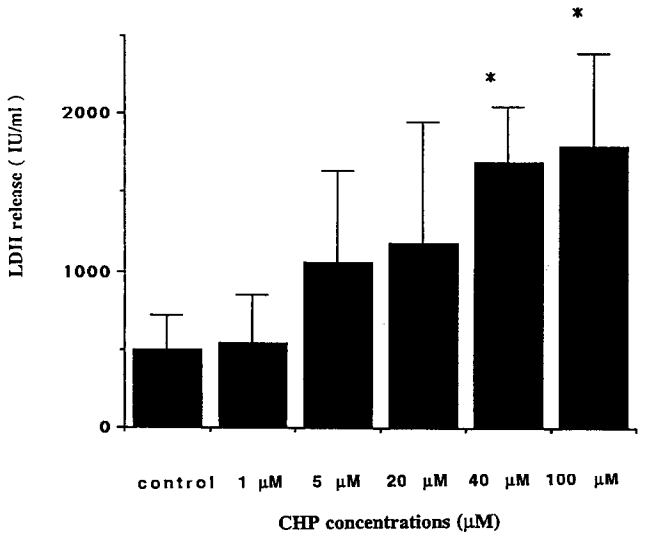

FIG. 1. Effect of increasing concentrations of CHP on cell viability. Fourty-eight hr after seeding, the cells were treated with various concentrations of CHP (1–100 µM). After 120-min incubation, the amount of LDH released into the culture supernatants was taken as an index of cell death. Each value represents the mean (±SD) of 3 different experiments. *$P < 0.05$ as compared to control, using student's *t*-test.

Figure 2 illustrates that increasing doses of CHP lead to a progressive detachment of the cells that becomes significant at concentrations higher than 5 µM and reaches a maximum at 100 µM. It appears, however, that the number of floating cells is always higher than the number of cells that take up the dye; thus, suggesting that cell detachment is not equal to cell death. To examine further this phenomenon, we have compared the kinetics of cell detachment and Trypan blue dye uptake during a 2-hr treatment by 60 µM CHP.

The results presented in Fig. 3 clearly show that cell detachment is not concomitant but precedes cell death, because we observed, after 15 or 30 min of treatment, that the vast majority of the detached cells are still refringent and, thus, still alive according to this criterion. Progressively, however, the proportion of dead cells among the floating population increases to reach almost 80% after 120 min of treatment.

To confirm that LDH release or Trypan blue dye uptake effectively correspond to cell death, we have tested the ability of treated cells to divide and grow following a 120-min treatment with 60 µM CHP. The floating cells were washed and plated again in the absence of CHP. After 24 hr or 48 hr of incubation, we observed that more than 95% of the plated cells do not attach to the surface of the culture flasks and, thus, fail to divide (data not shown).

Similarly, we observed that the cells remaining attached to the surface of the culture flasks after treatment by CHP (when fed with new culture medium without CHP), are also almost unable to divide and detached themselves within 24–48 hr of culture.

This finding suggests that the population that remains attached during CHP treatment is, indeed, sensitive to the

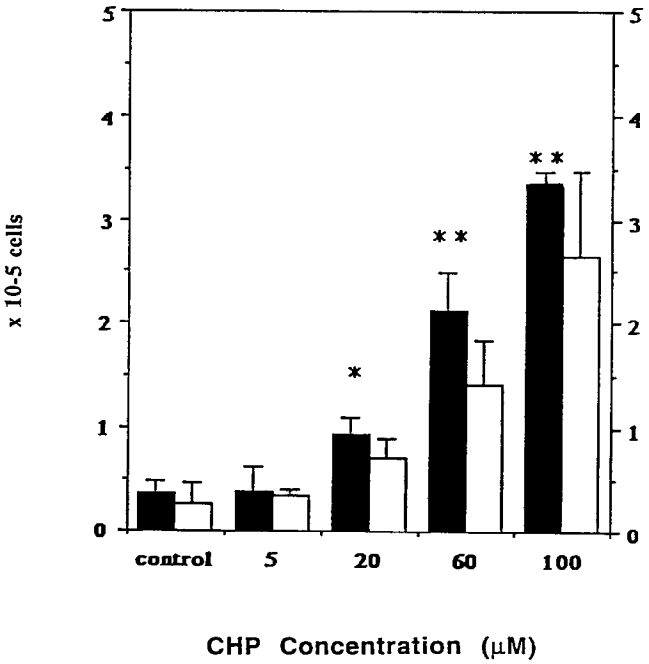

FIG. 2. Effect of increasing CHP concentrations on cell detachment and viability. The cells were incubated for 2 hr in the presence of increasing concentrations of CHP (5–100 µM). At the end of the incubation, the total number of floating cells (■) and of dead cells (□) were determined using the Trypan blue exclusion procedure. Each value represents the mean (±SD) of 3 different experiments. *$P < 0.05$ vs control; **$P < 0.01$ vs control.

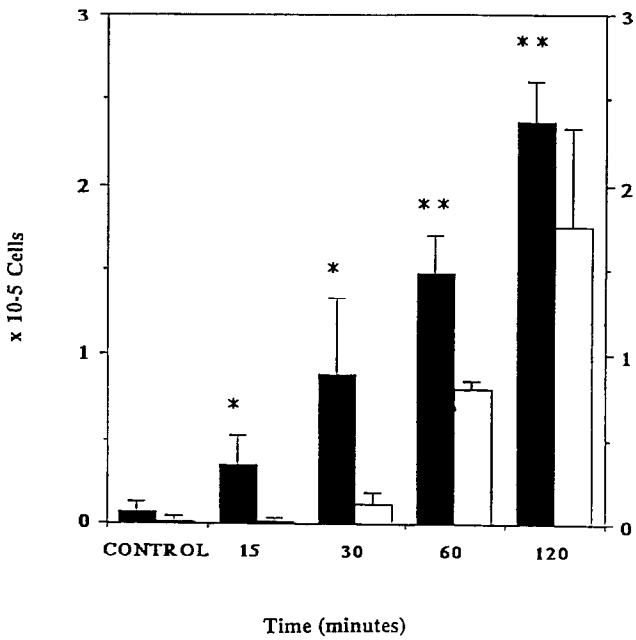

FIG. 3. Time-course of the effect of CHP (60 µM) on cell detachment and viability. After various periods of incubation (15–120 min) in the presence of CHP, the total amounts of floating cells (■) and of dead cells (□) were measured using the Trypan blue exclusion procedure. Each value represents the mean (±SD) of duplicate determinations in 3 different experiments. *$P < 0.05$ vs control; **$P < 0.01$ vs control.

oxidative stress but with a slower time course. Indeed, 24 hr of treatment with CHP (60 μM) leads to almost complete cell detachment and death (83% ± 15%, results not shown).

Is CHP-induced Cell Death Associated with Significant Signs of Lipoperoxidation?

To determine whether or not the cytotoxic action of CHP on PC12 cells might be the consequence of lipid peroxidation, we determined several parameters indicative of such a membrane attack in CHP-treated cells. Malonaldialdehyde is one of the major endproducts of the peroxidation of polyunsaturated fatty acids and is, thus, considered to be a good index of peroxidative mechanisms [27, 28]. Using a gas chromatography method selective of MDA formation, we only observed a slight increase (36%) in MDA liberation from 0.188 ± 0.06 μmoles/mL to 0.256 ± 0.09 μmoles/mL in CHP-treated samples ($P < 0.05$, $n = 4$) or even intracellular accumulation (not shown).

Loss of membrane phospholipids has often been considered as an index of membrane fatty acid peroxidation and degradation and we have, thus, measured by gas chromatography analysis the phospholipid-bound fatty acids in control and CHP-treated cells (60 μM, 2 hr). The fatty acid composition of control cell phospholipids is given in Fig. 4. This composition is characterized by a relatively high percentage of unsaturated fatty acids (~60%). We also noted the presence of polyunsaturated long-chain fatty acids, such as 20:4 (n-6), but also 22:5 and 22:6 (n-3). Nonetheless, we failed to detect any decrease in fatty acid content or composition following 2-hr treatment with 60 μM CHP, (i.e. in a situation where almost 80% of the cells are dead according to the Trypan blue exclusion procedure).

Because arachidonic acid is one of the major targets of polyunsaturated fatty acid peroxidation and may also be transformed into bioactive derivatives [29, 30], we have determined the effect of CHP on arachidonate release and metabolism. The cells were prelabeled with 0.5 μCi of [^{14}C] arachidonate for 24 hr, (i.e. conditions that give a steady-state incorporation level representing approximately 60–70% of the added radioactivity). We also observed that more than 90% of the incorporated radioactivity was associated with phospholipids (data not shown). The cells were then washed carefully and given fresh medium without radioactivity. After 120-min equilibration, we measured the kinetics of arachidonate release after treatment with 60 μM CHP. As shown in Fig. 5, this treatment, which induces the death of more than 70% of the cell population, is not associated with any significant liberation of membrane arachidonate but, rather, by a slight decrease during the first 60 min of treatment. Thin layer chromatography analysis of the radioactivity released in the medium after treatment with 60 μM CHP, fails to reveal any radioactivity metabolites of arachidonate with the exception of minor traces of HETE, but the bulk of released radioactivity corresponds to unmetabolized arachidonate (not shown).

It, thus, appears that CHP-induced cell death is not associated with a significant activation of phospholipase A_2 activity during the 120 min of CHP treatment.

Effect of CHP Treatment on Mitochondrial Activities

Because activities of mitochondria have been suggested, in various models of oxidative stress, to represent the major target of prooxidant molecules [31–33], we have determined the action of CHP on both cellular ATP content and rhodamine 123 uptake. Indeed, rhodamine 123 is known to be selectively taken up by mitochondria and to

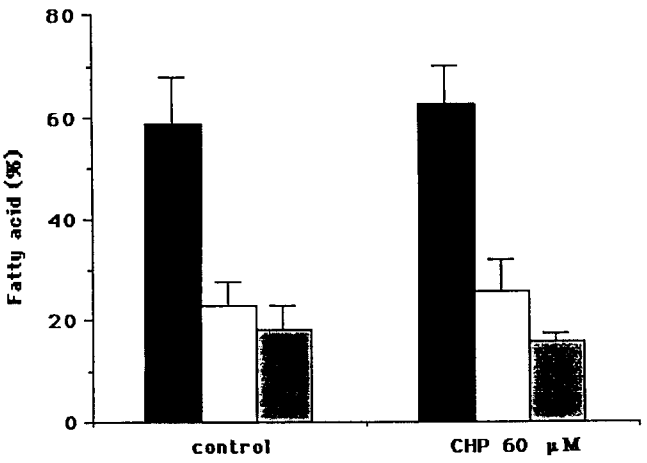

FIG. 4. Effect of CHP treatment (60 μM, 2 hr) on the relative composition of membrane fatty acids. Each value expressed as a percentage of the sum of membrane fatty acids, represents the mean (±SD) of 3 distinct experiments. (■) saturated fatty acids; (□) monounsaturated fatty acids; (□) polyunsaturated fatty acids.

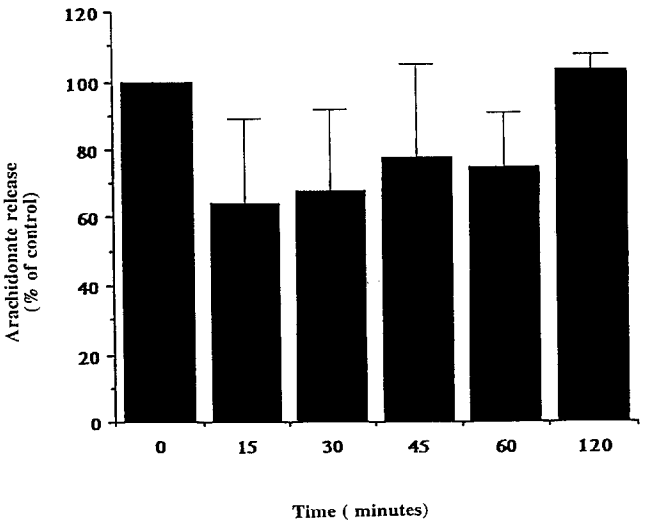

FIG. 5. Time-course of arachidonate release following CHP treatment. The cells were prelabelled for 24 hr with [^{14}C]-arachidonate, then washed and treated with 60 μM CHP for various periods of time. The amounts of radioactivity in the culture medium were then determined by liquid scintillation counting and expressed as a percentage of that released by untreated controls. Each value represent the mean (±SD) of duplicate determinations in 3 separate experiments.

reflect the maintenance of the mitochondrial potential $\Delta\psi$ [22–24].

As shown in Fig. 6, treatment with 60 μM CHP leads to a progressive decrease in intracellular ATP content, which reaches only 25–30% of control values after 30 min of treatment (i.e. at a time where most of the cells, while detached, remain nonpermeant to Trypan blue). Early kinetics carried out during the first 15 min of treatment indicate that the cellular ATP content remains almost constant during the first 10 min of treatment and markedly decreases thereafter (not shown). The decrease in intracellular ATP appears, thus, to slightly precede cell detachment (compare with Fig. 3).

We have measured in parallel the effect of CHP treatment on rhodamine 123 uptake and retention. For technical reasons, these measurements were only carried out on the cells remaining attached to the culture flasks during treatment. To take into account the progressive detachment of some cells during the experiment, the amount of rhodamine 123 remaining trapped by the attached, living cells, was expressed as fluorescence unit/ng of DNA.

As shown in Fig. 7, treatment by a cytotoxic dose of CHP (60 μM) leads to a significant drop of rhodamine 123 retention, which decreases to almost 30% of control value after 30 min of treatment. When analyzing the effect of increasing concentrations of CHP on rhodamine 123 retention, we observed that low doses (1–5 μM) do not reduce dye retention (but, rather, slightly stimulate retention), whereas this reduction is only seen in the presence of cytotoxic concentrations (60–100 μM) (Fig. 8).

These results, thus, indicate that cell detachment and death in the presence of CHP is associated with early alterations of mitochondrial functions such as ATP production and transmembrane potential maintenance.

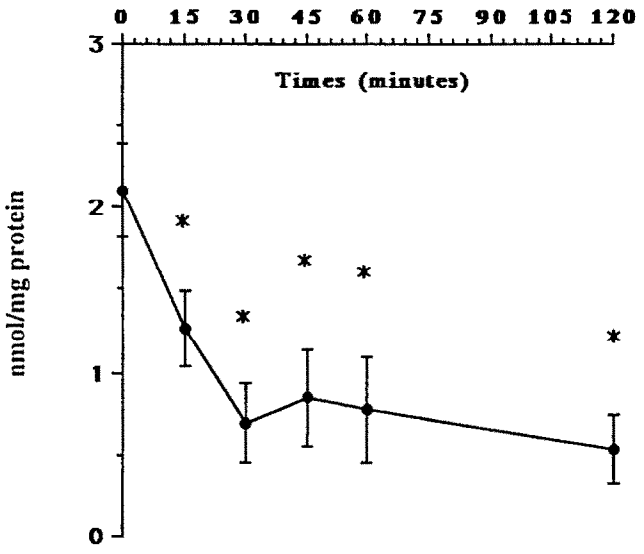

FIG. 6. Effect of CHP on the intracellular ATP content. The cells were incubated for various times with 60 μM CHP. At the end of the incubations, the intracellular ATP content was measured by liquid chromatography and expressed as nmol/mg protein. Each value represents the mean (±SD) of 3 different experiments. *$P < 0.05$ vs control.

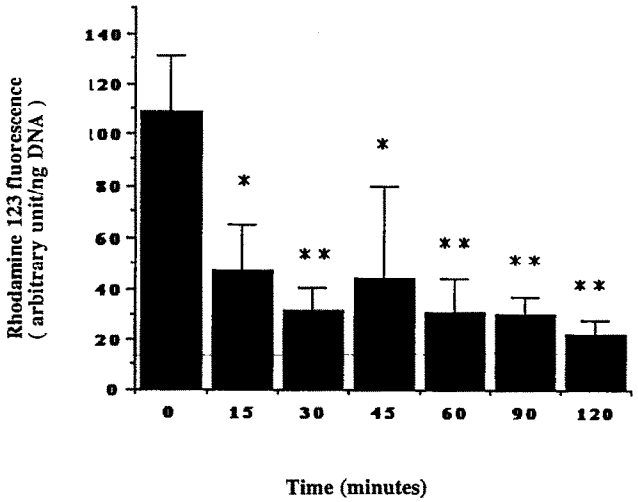

FIG. 7. Time-course of the effect of CHP on cellular rhodamine 123 retention. The cells were first loaded with 5 μM rhodamine 123 for 30 min at 37°C and then allowed to stand for 45 min in a dye-free medium. After various periods of treatment with 60 μM CHP, the amount of rhodamine 123 remaining trapped by the adherent cells was determined by fluorimetry and expressed per ng of cellular DNA. Each value represents the mean (±SD) of 3 different experiments. *$P < 0.05$ vs control; **$P < 0.01$ vs control.

DISCUSSION

It first appears from the present results that PC12 cells constitute an useful model to investigate the mechanisms of hydroperoxide-induced cell death. Indeed, the toxic effect of CHP, monitored by medium LDH accumulation or Try-

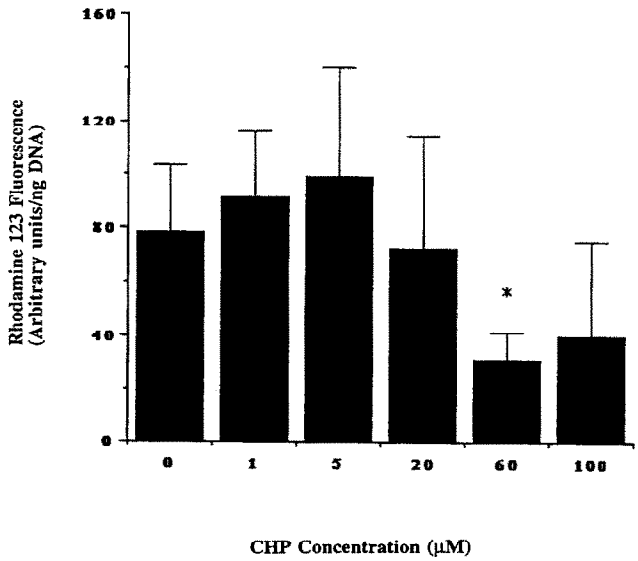

FIG. 8. Effect of increasing doses of CHP on cellular rhodamine 123 retention. After rhodamine 123 loading and rinsing (see the legend of Fig. 7), the cells were treated for 120 min in the presence of increasing concentrations of CHP. We then measure the intensity of rhodamine 123 fluorescence trapped by the adherent cells. Each value represents the mean (±SD) of 3 different experiments. *$P < 0.05$ vs control.

pan blue dye exclusion, is still noticeable at concentrations as low as 1–5 μM and reaches a maximum at 60–100 μM. In addition, this effect is very rapid in onset because the loss of cell viability is achieved within 90–120 min and does not markedly increase during the following 24 hr. Usually, a small proportion of the cells, ranging between 5–20% of the entire population, remains viable throughout the treatment but they are unable to recover and divide when fed a new, CHP-free, culture medium. Time-course experiments indicate that the loss of viability that occurs within 30–60 min of CHP treatment is clearly preceded by cell rounding and detachment from the bottom of the culture vial.

The purpose of our study was to investigate the role of lipoperoxidation during CHP-induced cell lysis. However, when using two current methods to detect lipoperoxidative membrane attack, we were unable to demonstrate such damage. MDA formation is generally accepted as a good reflection of membrane unsaturated fatty acid oxidative degradation. We have, thus, determined, using a specific gas chromatographic method [21], the effect of CHP on medium MDA release or cellular accumulation, but we only demonstrated a modest effect of CHP on MDA release. Other workers have also measured the loss of membrane phospholipid fatty acids as an index of oxidative stress [34, 35]. Results in Fig. 4 show that, after 120 min of CHP treatment (i.e. at a time where more than 70% of the cells were considered dead), no signs of membrane fatty acid loss could be observed. Similarly, treatment of cells prelabeled with [^{14}C] arachidonate by 60 μM CHP did not induce any significant release of free radioactive arachidonate or arachidonate derivatives within 60–120 min of treatment. We even observed a small, but not significant, inhibition of fatty acid release following CHP treatment. These results are in good agreement with those of Shimura *et al.* [16] who demonstrated that cumene hydroperoxide effectively induces lipid peroxidation in rat splenocytes but only at relatively high concentrations (200–600 μM).

Another question raised by the present findings is whether or not the toxic action of the CHP might be the result of an apoptotic event [10, 36, 37]. Although we have not yet carried out detailed studies of the morphological changes induced by CHP in PC12 cells, several elements indicate that its toxic action is likely due to necrosis. Cell death, which is achieved very rapidly (within 60–120 min of CHP treatment), could not be blocked by either actinomycin D or cycloheximide at concentrations able to block glutamate-induced cell death in the same PC12 population [38]. Determination of cellular and medium DNA contents after 120-min CHP treatment reveal only a very small loss of cell DNA content (less than 5%), without any release of DNA fragments in the supernatants (not shown). Furthermore, agarose gel electrophoresis did not reveal the "ladder" generally observed during apoptotic events [39] (not shown).

In contrast, when following mitochondrial activity either by measuring the intracellular ATP content or the retention of the fluorescent dye rhodamine 123, which is considered to reflect the mitochondrial transmembrane potential [22–24], we observed an early effect of CHP on both parameters. These effects are visible within 15 min of treatment, clearly precede Trypan blue dye uptake, and are almost concomitant with changes in cell shape and detachment from the surface of the culture flasks. Several groups have similarly noticed that oxidative stress is associated with an early and significant decrease in cellular ATP content [40, 41], whereas others described a drop in mitochondrial transmembrane potential associated with Ca^{++} ions leakage from these organelles [32, 33, 42]. At the present time, it remains unclear as to which is the primary event produced by an oxidative shock in PC12 cells and what the relationship is between these metabolic alterations and the morphologic changes observed following CHP treatment. Bershadsky *et al.* [43] have postulated that the cellular actin network might be a primary target of oxidative stress, whereas Jurkowitz-Alexander *et al.* described ATP depletion associated with membrane blebbing and independent of calcium in a glial cell line [44]. Although current evidence points toward a primary effect of cumene hydroperoxide on PC12 mitochondrial activity, additional experiments are now in progress to delineate more precisely the relationship between the various alterations observed during CHP treatment and to understand the role of calcium ions and of the cytoskeleton in cell lysis.

PC12 cells were kindly provided by D Barritault and J. Courty (Université Paris XII, Créteil, France). The authors acknowledge the help of Dr. A. Young and O. Mostéghanemi in the preparation of this manuscript. This work was, in part, supported by a grant from "The Fondation pour la Recherche Médicale" Paris.

References

1. Coyle JT and Puttfarcken P, Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262:** 689–695, 1993.
2. De Erausquin GA, Costa E and Hanbauer I, Calcium homeostasis, Free radical formation, and trophic factor dependance mechanisms in Parkinson's disease. *Pharmacol Rev.* **46:** 467–482, 1994.
3. Fahn S and Cohen G, The oxidant stress hypothesis in Parkinson's disease. *Ann Neurol.* **32:** 804–812, 1992.
4. Benzi G and Moretti A, Age and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Rad Biol Med.* **19:** 77–101, 1995.
5. Traystman RJ, Kirsch JR and Koehler RC, Oxygen radical mechanisms of brain injury following ischemia and reperfusion. *J. Applied Physiol* **71:** 1185–1195, 1991.
6. Colton CA, Pagan F, Snell J, Corton JS, Cummins A and Gilbert DL, Protection from oxidation enhances the survival of cultured mesencephalic neurons. *Exptl Neurol* **132:** 54–61, 1995.
7. Richter C and Kass GEN, Oxidative stress in mitochondria: its relationship to cellular Ca^{2+} homeostasis, cell death, proliferation and differentiation. *Chem Biol Interact* **77:** 1–23, 1991.
8. Bhatnagar A, Biochemical mechanism of irreversible cell injury caused by free radical-initiated reactions. *Mol Cell Biochem* **137:** 9–16, 1994.
9. Hinshaw DB, Sklar LA, Bohl B, Schraufstatter IU, Hyslop PA, Rossi MW, Spragg RG and Cochrane CG, Cytoskeletal

and morphologic impact of cellular oxidant injury. *Amer. J. Pathol* **123:** 454–464, 1986.
10. Silas-Santiago I, Greenlund LJS, Johnson EM and Snider WD, Molecular genetics of neuronal survival. *Curr Opin Neurobiol.* **5:** 42–49, 1995.
11. Jackson GR, Werrbach-Perez K, Ezell EL, Post JFM and Perez-Polo JR, Nerve growth factor effects on pyridine nucleotides after oxidant injury of rat pheochromocytoma cells. *Brain Res* **592:** 239–248, 1992.
12. Hinshaw DB, Miller MT, Omann GM, Beals TF and Hyslop PA, A cellular model of oxidant-mediated neuronal injury. *Brain Res* **615:** 13–26, 1993.
13. Cobuzzi RJ, Neafsey EJ and Collins MA, Differential cytotoxicities of N-methyl-β-carbolinium analogues of MPP^+ in PC12 cells: insight into potential neurotoxicants in Parkinson's disease. *J. Neurochem.* **62:** 1503–1510, 1994.
14. Vroegop SM, Decker DE and Buxser SE, Localization of damage induced by reactive oxygen species in cultured cells. *Free Rad Biol Med* **18:** 141–151, 1995.
15. Poot M, Verherk A, Koster JF, Esterbauer H and Jongkind J, Influence of cumene hydroperoxide and 4-hydroxynonenal on the glutathione metabolism during in vitro ageing of human skin fibroblasts. *Eur J Biochem* **162:** 287–291, 1987.
16. Shimura J, Shimura F and Hosoya N, Functional disability of rat splenocytes provoked to lipid peroxidation by cumene hydroperoxide. *Biochem Biophys Acta* **845:** 43–47, 1985.
17. Persoon-Rothert M, Egas-Kenniphaas JM, Van der Valk-Korshoorn EJM and Van der Larse A, Cumene hydroperoxide-induced changes in calcium homeostasis in cultured neonatal rat heart cells. *Cardiovasc. Res* **26:** 706–712, 1992.
18. Wroblewski F and Ladue JS, Lactic dehydrogenase activity in blood. *Proc Soc Exp. Biol. Med.* **90:** 210–213, 1955.
19. Labarca C and Paigen K, A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* **102:** 344–352, 1980.
20. Duval D, Effect of dexamethasone on arachidonate metabolism in isolated mouse thymocytes. *Prost Leuk Essential fatty acids* **37:** 149–156, 1989.
21. Beljean-Leymarie M, and Bruna E, Subpicogram determination of malondialdehyde by gas-liquid chromatography with phosphorus detector. Standardisation. *Anal. Biochem.* **173,** 174–184, 1988.
22. Johnson LV, Walsh ML, Bockus BJ, Chen LB, Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. *J Cell Biol* **88:** 526–535, 1981.
23. Lizard G, Chignol MC, Chardonnet Y and Schmitt D, Active cell membrane mechanisms involved in the exclusion of RH123 allow distinction between normal and tumoral cells. *Cell Biol Toxicol.* **10:** 399–406, 1994.
24. Johnson LV, Walsh ML, Chen LB, Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl Acad. Sci USA* **77:** 990–994, 1980.
25. Hagenfeldt L, A gas chromatographic method for the determination of fatty acids in plasma. *Clin. Chim. Acta* **13:** 266–268, 1966.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the folin phenol reagent. *J. Biol Chem* **193:** 265–275, 1951.
27. Vagnozzi R, Tavazzi B, Lazzarino G, Di Pierro D, Siragusa P, Giuffré R and Giardina B, Time dependence of plasma malonaldialdehyde, oxypurines, and nucleosides during incomplete cerebral ischemia in the rat. *Biochem Med. Metab. Biol.* **53:** 98–104, 1994.
28. Cini M, Fariello RG, Bianchetti A and Moretti A, Studies on lipid peroxidation in the rat brain. *Neurochem Res* **19:** 283–288, 1994.
29. Sumida C, Graber R and Nunez E, Role of fatty acids in signal transduction: Modulators and messengers. *Prost Leuk Essential fatty acids* **48:** 117–122, 1993.
30. Lands WEM, and Kulmacz RJ, The regulation of the biosynthesis of prostaglandins and leukotrienes. *Prog Lipid. Res* **25:** 105–109, 1986.
31. Takeuchi Y, Morii H, Tamura M, Hayaishi O and Watanabe Y, A possible mechanism of mitochondrial dysfunction during cerebral ischemia: inhibition of mitochondrial respiration activity by arachidonic acid. *Arch Biochem Biophys* **289:** 33–38, 1991.
32. Lötscher HR, Winterhalter KH, Carafoli E and Richter C, Hydroperoxide-induced loss of pyridine nucleotides and release of calcium from rat liver mitochondria. *J. Biol Chem* **255:** 9325–9330, 1980.
33. Van de Water B, Zoeteweij JP, de Bont HJGM, Mulder GJ and Nagelkerke JF. Role of mitochondrial Ca^{++} in the oxidative stress-induced dissipation of the mitochondrial membrane potential. Studies in isolated proximal tubular cells using the nephrotoxin 1,2 dichlorovinyl L. Cysteine. *J Biol Chem* **269:** 14546–14552, 1994.
34. Bromont C, Marie C and Bralet J, Increased lipid peroxidation in vulnerable brain regions after transient forebrain ischemia in rats. *Stroke* **20:** 918–924, 1989.
35. Freyss-Beguin M, Millanvoye Van Brussel E and Duval D, Effect of oxygen deprivation on metabolism of arachidonic acid by cultures of rat heart cells. *Amer J. Physiol* **257:** H444–H451, 1989.
36. Arends MJ, Morris RG and Wyllie AH, Apoptosis, the role of endonuclease. *Amer J Pathol* **136:** 593–608, 1990.
37. Mesner PW, Winters TR and Green SH, Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. *J. Cell Biol* **119:** 1669–1680, 1992.
38. Serghini R, Froissard P, Sola B and Duval D, Cycloheximide and actinomycin D block the toxic effect of glutamic acid on PC12 cells. *NeuroReport* **6,** 29–32, 1994.
39. Joseph R, Li W and Han E, Neuronal death, cytoplasmic calcium and internucleosomal DNA fragmentation: evidence for DNA fragments being released from cells. *Mol. Brain Res* **17:** 70–76, 1993.
40. Redegeld FAM, Moison RMW, Koster AS and Noordhoek J, Depletion of ATP but not of GSH affects viability of rat hepatocyte. *Eur J Pharmacol* **228:** 226–236, 1992.
41. Nieminen AL, Saylor AK, Herman B and Lemaster JJ, ATP depletion rather than mitochondrial depolarization mediates hepatocyte killing after metabolic inhibition. *Amer J Physiol* **267:** C67–C74, 1994.
42. Nishida T, Inoue T, Kanike W, Kawashima Y and Tagawa K, Involvement of Ca^{2++++} release and activation of phospholipase A_2 in mitochondrial dysfunction during anoxia. *J. Biochem* **106:** 533–538, 1989.
43. Bershadsky AD, Gelfand VI, Svitkina TM and Tint IS, Destruction of microfilament bundles in mouse embryo fibroblasts treated with inhibitors of energy metabolism. *Exptl Cell Res* **127:** 421–429, 1980.
44. Jurkowitz-Alexander MS, Altschuld RA, Hon, Johnson D, McDonald JS, Simmons TD and Horrocks LA, Cell swelling, blebbing and death are dependent on ATP depletion and independent of calcium during chemical hypoxia in a glial cell line (Roc-1). *J. Neurochem* **59:** 344–352, 1992.