Inhibition of Paraquat-Induced Autophagy Accelerates the Apoptotic Cell Death in Neuroblastoma SH-SY5Y Cells

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Autophagy is a degradative mechanism involved in the recycling and turnover of cytoplasmic constituents from eukaryotic cells. This phenomenon of autophagy has been observed in neurons from patients with Parkinson’s disease (PD), suggesting a functional role for autophagy in neuronal cell death. On the other hand, it has been demonstrated that exposure to pesticides can be a risk factor in the incidence of PD. In this sense, paraquat (PQ) (1,1′-dimethyl-4,4′-bipyridinium dichloride), a widely used herbicide that is structurally similar to the known dopaminergic neurotoxicant MPP⁺ (1-methyl-4-phenyl-pyridine), has been suggested as a potential etiologic factor for the development of PD. The current study shows, for the first time, that low concentrations of PQ induce several characteristics of autophagy in human neuroblastoma SH-SY5Y cells. In this way, PQ induced the accumulation of autophagic vacuoles (AVs) in the cytoplasm and the recruitment of a LC3-GFP fusion protein to AVs. Furthermore, the cells treated with PQ showed an increase of the long-lived protein degradation which is blocked in the presence of the autophagy inhibitor 3-methyladenine and regulated by the mammalian target of rapamycin (mTOR) signaling. Finally, the cells succumbed to cell death with hallmarks of apoptosis such as phosphatidylserine exposure, caspase activation, and chromatin condensation. While caspase inhibition retarded cell death, autophagy inhibition accelerated the apoptotic cell death induced by PQ. Altogether, these findings show the relationship between autophagy and apoptotic cell death in human neuroblastoma cells treated with PQ.

Key Words: paraquat; vacuoles; apoptosis; Parkinson

Autophagy is an intracellular lysosome-mediated catabolic mechanism that is responsible for the bulk degradation and recycling of damaged or dysfunctional cytoplasmic components and intracellular organelles (Klionsky et al., 2000). Autophagy is a cellular response to both extracellular (nutrient deprivation or hypoxia) and intracellular (accumulation of damaged organelles and cytoplasmic components) stress conditions. Autophagy, being an evolutionarily ancient cellular response to intra- and extracellular noxious stimuli, may precede or coexist with apoptosis, and it may be induced by apoptotic stimuli (Xue et al., 1999). Thus, autophagy and apoptosis may be interconnected (Bursch et al., 2000). Cellular autophagy is a physiological degradative process involved, like apoptosis, in embryonic growth and development, cellular remodeling, and the biogenesis of some subcellular organelles (Filonova et al., 2000; Hariri et al., 2000; Sattler and Mayer, 2000). Autophagic cell death involves accumulation of autophagic vacuoles (AVs) in the cytoplasm of dying cells as well as mitochondria dilation and enlargement of the endoplasmic reticulum and the Golgi apparatus. Autophagic cell death has been described during the normal nervous system development (Schweichel et al., 1973) and could be a consequence of a pathological process such as those associated with neurodegenerative diseases (Petersen et al., 2001). Of note, autophagy is highly enhanced in brain amyloidosis (Graeber et al., 2002), Alzheimer disease (Stadelmann et al., 1999), Huntington’s disease (Landles et al., 2004), and Parkinson’s disease (PD) (Anglade et al., 1997).

PD is a chronic progressive neurodegenerative disease, affecting at least 1% of the population over the age of 55. Genetic forms of the disease represent less than 5% of current cases, but the causes of the vast majority of sporadic cases of PD are still unknown. Accumulating evidence strongly points to environmental toxins as feasible triggers of neurodegeneration of nigrostriatal dopaminergic neurons, and the common use of pesticides in rural life has been correlated to parkinsonism in humans (Di Monte et al., 2000). In this sense, the herbicide 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat [PQ]), widely used as a cationic nonselective bipyridyl herbicide to control weeds and grasses in many agricultural areas, has emerged as a putative risk factor on the basis of its structural homology to 1-methyl-4-phenyl-pyridine (MPP⁺), the active metabolite of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine, a neurotoxicant that induces Parkinson’s-like features.
in rodents, nonhuman primates, and humans (Langston and Ballard, 1984; Tanner and Ben Shlomo, 1999).

Occupational PQ exposures have been associated with parkinsonism (Hertzman et al., 1990; Liou et al., 1997), although the mechanism of PQ toxicity is yet poorly understood. Several studies have suggested the involvement of reactive oxygen species (ROS) in its effect (Gonzalez-Polo et al., 2004; Mollace et al., 2003). Our group has demonstrated that free radicals play an active role in the PQ-induced apoptotic events that culminate with cell death (Gonzalez-Polo et al., 2004).

Here, we show for the first time that low concentrations of PQ induce several characteristics of autophagy in human neuroblastoma SH-SYSY cells, which are commonly selected as a cellular PD model (Lai et al., 1997; Lee et al., 2000; Shavali et al., 2004). Finally, the cells succumbed to cell death with hallmarks of apoptosis. Thus, we investigated the relationship between autophagy and apoptosis in neurons treated with PQ to propose a new and exciting strategy to the knowledge of the PD mechanism and its possible treatment.

MATERIALS AND METHODS

Cell line and culture condition. Human neuroblastoma SH-SYSY cells were grown in Dulbecco’s modified Eagle medium (Gibco BRL, Paisley, U.K.) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Hyclone, Brevieres, France). Cells were seeded at 5 × 10⁵ in a 75-cm² tissue culture flask (TPP, Trasadingen, Switzerland) and incubated at 37°C under a saturating humidity atmosphere of 5% CO₂/95% air.

Reagents and cell death induction. Confluent cells (~80%) in 75-cm² tissue culture flasks were trypsinized and seeded in tissue culture dishes at a concentration of 5 × 10⁴ cells/cm². Twenty-four hours later, the medium was aspirated and replaced with fresh medium alone or containing the indicated concentrations of PQ (Niso-Santano et al., 2006). Serum and amino acid starvation of cells were performed using serum-free Earle’s balanced salt solution medium (Sigma, St Louis, MO) (Boya et al., 2005). For caspase inhibition, a sublethal dose of N-benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD-fmk, 50μM, Bachem AG, Bubendorf, Switzerland) was added 1 h before PQ. The antioxidants N-acetylcysteine (NAC 1mM), vitamin E (100μM), glutathione (1mM), and the inhibitor of autophagy, 3-methyladenine (3-MA, 10mM), were added before the addition of PQ.

Flow cytometry. To determine apoptosis-associated changes by cytofluorometry, we used 3,3′-dihexyloxacarbocyanine iodide (DiOC₆(3), 40nM) for the mitochondrial transmembrane potential quantification, propidium iodide (PI, 1 μg/ml) for the determination of cell viability (Boya et al., 2003; Gonzalez-Polo et al., 2005a), and Annexin-V labeled with fluorescein isothiocyanate (all of them from Molecular Probes, Eugene, OR) for the assessment of phosphatidylserine exposure. The determination of superoxide anion generation, hydroethidine (HE, 10μM), was used. After different experimental conditions, cells were trypsinized and labeled with the fluorochromes at 37°C, followed by cytofluorometric analysis with a FACS Scan (Becton Dickinson, Franklin Lakes, NJ) (Gonzalez-Polo et al., 2005a,b).

Immunofluorescence and light microscopy. Cells were cultured on coverslips. After the experimental conditions, the cells were stained with CMFDA (5-chloromethylfluorescein diacetate, 1μM) (Molecular Probes) for 30 min at 37°C to visualize the vacuoles. Cells were fixed with paraformaldehyde (4% wt/vol) for CMFDA staining and immunofluorescence assays (Gonzalez-Polo et al., 2005a,2005b). Cells were stained for the detection of activated caspase-3 with a polyclonal antibody from Cell Signaling Technology, Inc. (Danvers, MA) developed with an anti-rabbit immunoglobulin Alexa fluor conjugate (Molecular Probes) and counterstained with Hoechst 33342, 2μM (Ho) (Sigma) before mounting. Fluorescence microscopy was analyzed with an Olympus IX51 equipped with a DC300F camera.

Western blot analysis. Following experimental treatments, the cells (cultured in 60 mm dishes) were rinsed twice with cold phosphate-buffered saline (PBS) and removed by scraping and then centrifuged at 900 × g for 5 min at 4°C. Cells were lysed in a buffer containing 50mM N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid pH 7.5, 100mM NaCl, 1% Triton X-100, 10% glycerol, 1mM ethyleneglycol-bis(aminohetero)-tetracetic acid, 5mM MgCl₂, 25mM NaF, and Protease Inhibitor Cocktail (Sigma). Cells were centrifuged at 13,000 × g for 5 min at 4°C. Supernatants were stored at −80°C until analysis by Western blot. Protein concentration was measured according to Bradford (1976) using bovine serum albumin (BSA) as standard. Equal amounts of proteins (20 μg) were loaded in a 12–15% sodium dodecyl sulfate gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes according to conventional partially modified methods (Fuentes et al., 2000). Briefly, proteins were transferred (250 mA for 60 min) to PVDF membranes using Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA). The procedure for immunodetection includes the transfer and blocking of the membrane (60 min at room temperature) with Tween–Tris-buffered saline (TTBS) (10mM Tris/HCl pH 7.5, 150mM NaCl, and 0.2% Tween-20) containing 10% nonfat-dried milk. Membranes were then incubated overnight at 4°C with primary antibodies, p-mTORser2448, and cleaved caspase-3 from Cell Signaling (Beverly, MA) and anti-LC3 from MBL Medical and Biological Laboratories Co., Ltd (Nagoya, Japan), all of them diluted 1:1000 in TTBS + 10% nonfat-dried milk or 5% BSA. After washing (for two 5-min periods with TTBS), membranes were incubated (60 min at room temperature) with peroxidase-conjugated secondary antibodies (1:5000 in TTBS with 10% nonfat-dried milk). After washing (for two 5-min periods and one 10-min period), the detection of bound antibodies was visualized by chemiluminescence using the ECL-plus reagent (GE Healthcare, Buckinghamshire, UK). Actin content was analyzed as a control by means of a rabbit polyclonal antibody (from Sigma).

Analysis of protein degradation. Human neuroblastoma SH-SYSY cells were incubated with 0.2 μCi/ml L-[1⁴C]valine (GE Healthcare) in complete medium (CM) for 24 h at 37°C. Unincorporated radioisotope was removed by washing the cells three times with PBS (pH 7.4). Cells were then incubated in Earle’s balanced salt solution buffer plus 0.1% BSA (nutrient-free [NF] medium) in the presence of 10mM cold valine, for 1 h (prechase period). After this time, the medium was replaced by the appropriate fresh medium (NF or CM) plus cold valine 10mM in the presence or absence of 10mM 3-MA and 10μM PQ for 4 h (chase period). Cells and radiolabeled proteins from the medium were precipitated in trichloroacetic acid at the final concentration of 10% (vol/vol). The precipitated proteins were separated from the soluble radioactivity by centrifugation at 600 × g for 20 min and then dissolved in 1 ml of 0.2 N NaOH. The rate of protein degradation was calculated by determining the ratio of acid-soluble radioactivity recovered from both cells and medium to the ratio of radioactivity in trichloroacetic acid-precipitated proteins obtained from both cells and medium (Pattingre et al., 2003).

Electron microscopy. Scraped cells were spun and immersed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.4) for 1 h at 4°C. Pellets were then rinsed in phosphate buffer, postfixed in 1% osmium tetroxide for 90 min, rinsed, dehydrated in a graded series of acetone, and embedded in Araldite resin. Ultrathin 90nm–thick sections were obtained using a Leica Reichert Ultracut ultramicrometer equipped with a glass knife. Sections were mounted on copper grids, stained with uranyl acetate and lead citrate, examined, and photographed with a Jeol JEM-100 CX II transmission electron microscope.

Transient transfections. SH-SYSY cells were transiently transfected with the Fugene procedure, with 1–2 μg DNA per 35-mm plate, according to the manufacturer’s protocol (Roche Diagnostics, GmbH, Mannheim, Germany) to label AVs with LC3-GFP plasmid (Kabeya et al., 2000).
Statistical analysis. Data are representative of at least three independent experiments each in triplicate determination. Statistical analysis of the data was performed using one-way ANOVA. Significance (*) was defined at $p < 0.05$.

RESULTS

PQ Induces Apoptotic Death in Neuroblastoma Cells

As previously described (Chun et al., 2001; Gonzalez-Polo et al., 2004; Niso-Santano et al., 2006), several cell lines, including human neuroblastoma SH-SY5Y cells, are sensitive to the PQ induced toxicity. We investigated, for the first time, the cell death type in neuroblastoma cells exposed to the bipiridinic pesticide PQ. As we show in Figure 1, the PQ treatment caused phosphatidylserine exposure on the outer leaflet of the plasma membrane, which can manifest before plasma membrane permeabilization. This phenomenon was increased in combination with PQ and starvation conditions. The cells treated with 10–25 μM of PQ exhibited a range of 25–50% loss of cell viability in CM and NF medium. In parallel

![FIG. 1. PQ-mediated sensitization of SH-SY5Y cells to starvation-induced cell death. The human neuroblastoma SH-SY5Y cells were cultured in the indicated conditions (CM, NF), in the absence (Co) or in the presence of the indicated doses of PQ for 24 h, followed by simultaneous staining of the phosphatidylserine exposure with Annexin-V fluorescein isothiocyanate (FITC) and viability with PI. Quantitative data (error bars are SE $n = 3$) are shown in (A), and representative FACS diagrams are shown in (B).]
experiments, we analyzed the involvement of caspases in the apoptotic death induced by 10\(\mu\)M of PQ (Fig. 2). The maximum level of caspase-3 activation was evident after 24 h of PQ incubation, shown by Western blot detection of a 17-kDa fragment (active form) and caspase-3 total, both in CM (Fig. 2A) and NF medium (Fig. 2B). Morphological analysis of cells subjected to the combined treatment of PQ 10\(\mu\)M and starvation indicate a typical apoptotic chromatin condensation (as detectable with the nuclear stain Hoechst 33342) associated with caspase-3 activation (Figs. 2C and 2D). Therefore, we wondered whether inhibition of caspases with the broad-spectrum caspase inhibitor Z-VAD-fmk would prevent cell death in this model. As shown in Figure 2E, Z-VAD-fmk retarded cell death of PQ-treated, SH-SY5Y cells, both in CM and NF medium.

**PQ Induces Reactive Oxygen Species Production in SH-SY5Y Neuroblastoma Cells**

The mechanism of action of PQ is as yet poorly understood. However, it is well known that PQ acts as a reactive oxygen species (ROS) producer (Bus *et al.*, 1984; Suntres 2002). These ROS interact with unsaturated lipids of membranes (lipid

![FIG. 2. Involvement of caspases in PQ-induced apoptosis in SH-SY5Y cells. Caspase-3 activation induced by PQ. (A–C). (A, B) Activation of caspase-3 was determined by Western blot as indicated in “Materials and Methods” section. Actin expression was determined as a loading control. Cells were incubated in CM (A) or NF medium (B) at the times indicated with 10\(\mu\)M of PQ. Blots are representative of at least three independent experiments. (C, D) SH-SY5Y cells cultured in poly-L-lysine–treated coverslips were incubated in the absence (Co) or in the presence of PQ (10\(\mu\)M) in the indicated medium for 24 h. Cells were fixed and immunostained for the detection of active caspase-3 (green fluorescence) and nuclear chromatin condensation (Hoechst 33342, blue fluorescence). Representative microphotographs are depicted in (C), and the frequency of the measured changes is analyzed in (D) (*\(p<0.05\) compared with control). (E) Effect of the caspase inhibitor Z-VAD-fmk on cell killing. Cells were cultured in the absence or presence of nutrients, PQ 10\(\mu\)M and/or Z-VAD-fmk 50\(\mu\)M for 24 h, and the frequency of cells with high Annexin-V fluorescein isothiocyanate (FITC) or PI incorporation was determined as in Figure 1A (*\(p<0.05\) compared with PQ treatment without Z-VAD-fmk).
peroxidation) and destroy organelles subsequently leading to cell death (Dodge, 1971). In this sense, several studies have suggested the involvement of ROS in the toxicity induced by PQ, including studies in vitro in several cell lines as in human lung epithelial cells (Cappelletti et al., 1998) or in cerebellar granule cells (Bus et al., 1984; Gonzalez-Polo et al., 2004), as well as studies in vivo (Mollace et al., 2003). In our study, we showed that exposure to low concentrations of PQ (10 μM) for 24 h caused an increase of intracellular levels of ROS in human neuroblastoma SH-SYSY cells (as measured by the oxidation of the nonfluorescent dye HE into its fluorescent product ethidium [Eth]) (Fig. 3). This production was enhanced when the cells were treated with a combination of nutrient depletion conditions and PQ. The ROS generation was abolished in the presence of several antioxidants as N-acetylcysteine as well as the ROS scavenger vitamin E (α-tocopherol) (data not shown).

The Treatment of SH-SYSY Neuroblastoma Cells with Low Concentrations of PQ Produces Characteristics of Autophagy

It has been described that autophagy is a cellular response to extracellular (nutrient deprivation, hypoxia) and intracellular stress conditions (accumulation of damaged organelles and cytoplasmic components). This phenomenon is characterized morphologically by the accumulation of AVs in the cytoplasm.

We have tested in our model that, in NF medium, that is, in the absence of serum and amino acids, as well as in CM, control cells did not develop any discernible vacuoles, while cells treated with PQ 10 μM exhibited an increase in cytoplasmic vacuolization visible as “holes” in CMFDA-stained cells of a time-dependent way (Figs. 4A and 4B). Moreover, these vacuoles induced in the presence of PQ showed, clearly, the characteristic double membranes of AVs as visible by transmission electron microscopy (Fig. 4C). Figures 4A and 4C show images in CM conditions. Microphotographs in NF conditions are equivalents (data not shown).

Similarly, after treatment of serum and amino acid starvation, 10 μM of PQ induced the recruitment of a LC3-GFP fusion protein to AV in discrete foci (Kabeya et al., 2000; Mizushima 2004) (Figs. 5A and 5B). In agreement with the microscopic observations, we detected by Western blotting the accumulation of the autophagosome marker LC3-II (Boya et al., 2005; Kabeya et al., 2000) both in CM (Fig. 5C) and under the conditions of starvation (data not shown) in SH-SYSY treated with PQ 10 μM. Furthermore, as compared to the prototypic autophagy inhibitor 3-MA, PQ (10 μM) induced the autophagy (as determined by quantifying the turnover of proteins in starved cells) as shown in Figure 6A. In this sense, exposure of human neuroblastoma cells with 10 μM of PQ produced a marked decrease in the phosphorylation (Ser2448) status of mTOR (Figs. 6B and 6C), protein involved in the autophagy signaling, with a maximal effect after 30 min of herbicide exposure in CM (Fig. 6B). In starved cells incubated with PQ, the activation of phosphorylated mTOR was invaluable to all the tested times (Fig. 6C).

The Inhibition of PQ-Induced Autophagy Accelerates the Apoptotic Cell Death in SH-SYSY Cells

We investigated the possible connection between the accumulation of autophagic phenomenon and the apoptotic cell death induced by PQ in SH-SYSY cells. The morphological analysis (Figure 7A) showed that the prototypic autophagy inhibitor 3-MA inhibited the PQ-induced autophagic vacuolization as well as protein degradation, already previously mentioned in Figure 6A. Moreover, cytofluorometric quantification revealed that a significant fraction of cells treated with 3-MA and/or PQ lost the capacity to retain the dye DiOC6(3) and hence dissipated the mitochondrial transmembrane potential (ΔΨm). Among this ΔΨm low population, a fraction of cells incorporated the vital dye PI and thus lost the barrier function of their plasma membrane (Fig. 7B). This event was more evident in combination of 3-MA and the herbicide PQ. In the same way, the caspase-3 activation, as measured by Western blot detection of 17-kDa fragment (Fig. 7C), was more increased with PQ and 3-MA together that each one separately. Therefore, the combination of these results clearly suggests that the inhibition of PQ-induced autophagy accelerates the apoptotic cell death in SH-SYSY cells.
DISCUSSION

Several studies have shown the possibility that environmental neurotoxicants such as pesticides may be related to the development of nongenetic PD (Cory-Slechta et al., 2005; Landrigan et al., 2005; Norris et al., 2004; Ritz et al., 2000; Sherer et al., 2001). PQ is one of the possible pesticides involved in PD genesis because of the similar chemical

FIG. 4. PQ induces accumulation of AVs. (A, B) SH-SY5Y cells were cultured in CM or NF in the presence of PQ 10μM the times indicated. The presence of cytoplasmic vacuoles was determined by staining with CFMDA as indicated in “Materials and Methods” section. Cytoplasmic vacuoles are visualized as holes. Representative microphotographs in CM are depicted in (A), and the percentage of vacuolated cell area is analyzed in (B) (error bars are SE n = 3). (C) Ultrastructure of PQ-treated SH-SY5Y cells. The cells were treated as in (A). Electron microscopy of representative cells are shown.
structure to the dopaminergic neurotoxicant MPP⁺ and the strong correlation between the incidence of the disease and the amount of PQ used (Ritz et al., 2000). In the present study we report, for the first time, that low concentrations of PQ induce molecular events compatible with autophagy in neuroblastoma SH-SY5Y cells. We also show that the inhibition of autophagy exacerbates PQ-induced apoptosis.

The human dopaminergic cell line SH-SY5Y, which has many qualities of nervous system neurons, was used as target neuronal cells. First, we confirmed that PQ induced neurotoxicity in SH-SY5Y cells in a dose-dependent manner both in CM and in starvation conditions (Fig. 1). This result was similar to the results reported by Gonzalez-Polo et al. (2004) and Yang et al. (2005). Our group found that PQ induced dose-dependent cell death and apoptosis in SH-SY5Y cells within a period of 24 h. PQ is a well-known oxidative-stress inducer (Gonzalez-Polo et al., 2004; Mollace et al., 2003), so most of the reports about it are focused on ROS production. In this sense, we show in our study that low concentrations of PQ (10μM) induce a robust ROS generation (Fig. 3), which is more evident in NF conditions. However, the major finding of this study is that low PQ concentrations produce characteristics of autophagy.

Autophagy was described for the first time by Schweichel et al. (1973). It is characterized by the appearance of numerous cytoplasmic AVs of lysosomal origin, followed by mitochondrial dilation and enlargement of the endoplasmic reticulum and the Golgi apparatus. Dysfunction of cellular degradation has been implicated in PD pathogenesis. Macropautophagy is the primary mechanism of degrading long-lived proteins and damaged organelles. The autophagic process begins with the remodeling of subcellular membranes into double-membrane vesicles that sequester cytoplasm and organelles in AVs. These vacuoles are fused with the lysosome to form the autophagolysosome. The content of the autophagolysosome are ultimately degraded by lysosomal degradative enzymes (Klionsky et al., 2000). The electron microscopy images in Figure 4 show that PQ induces the accumulation of double-membrane AVs in the cytoplasm. This event is accompanied by other autophagic characteristics as accumulation of the autophagosome marker LC3-II protein in cells treated with PQ 10μM the indicated times. Actin expression was determined as a loading control in the same conditions. Blots are representative of at least three independent experiments.

![FIG. 5. Effect of PQ on the subcellular localization and status of the AV marker LC3. (A, B) Redistribution of LC3-GFP. The cells were transfected with an LC3-GFP fusion construct as indicated in “Materials and Methods” section. Twenty-four hours after transient transfection, cells were incubated in the indicated medium, CM or NF, treated in the absence (Co) or presence of PQ 10μM for 15 h and fixed. Representative fluorescence microphotographs are shown in (A), and the frequency of cells exhibiting the accumulation of LC3-GFP in vacuoles was quantified (* p < 0.05 compared with control) in (B). (C) Immunoblot analyses of accumulating LC3-II protein in cells treated with PQ 10μM the indicated times. Actin expression was determined as a loading control in the same conditions. Blots are representative of at least three independent experiments.](image-url)
The pesticide induced, in the first place, the morphological appearance of autophagy (Figs. 4–6). But finally the cells succumbed in morphological and biochemical changes that are typical of apoptotic cell death, as phosphatidylserine exposure (Fig. 1), Δψₘ dissipation (Fig. 7), caspase-3 activation (Fig. 2), and chromatin condensation (Fig. 2). Interestingly, inhibition of caspases retarded cell death (Fig. 2), yet had no influence on autophagic vacuolization (data not shown). In this sense, there is some controversy in the bibliography regarding the precise role that autophagy might play in programed cell death. There are many reports describing situations in which autophagy activation accompanies apoptosis, but there are also cases in which autophagy enhancement leads to cell death in the absence of caspase activation (Bursch et al., 1996; Butler et al., 2000; Chi et al., 1999; Xiang et al., 1996). Moreover, autophagic cell death has been described in association with several neurodegenerative diseases, including PD (Anglade et al., 1997; Marino et al., 2004; Petersen et al., 2001). Increased levels of autophagy were observed in neuronal cell lines expressing mutant proteins associated with such diseases. Expression of PD-associated A53T, but not wild-type, α-synuclein in PC12 cells induced massive AV formation (Stefanis et al., 2001). In addition, dopaminergic neurons with AVs have been observed in the substantia nigra of PD patients, along with neurons displaying apoptotic features (Anglade et al., 1997). However, the functional consequence of increased autophagic activity for neurodegeneration is not clear but has been suggested that excessive autophagy may directly lead to neuronal cell death (Yuan et al., 2003).

In the literature, concentrations of high micromolar or millimolar range of PQ are normally used to study its neurotoxicity and apoptosis induction (Gonzalez-Polo et al., 2004; Kim et al., 2004; Peng et al., 2004). The evidence that PQ triggers apoptotic cell death has been demonstrated in other cellular models, as in human lung epithelial cells (Cappelletti et al., 1998) as well as in PC12 cells (Li et al., 1999). Our experiments show that PQ induces apoptosis in SH-SY5Y cells at low concentrations (10μM) at the same concentrations that...

FIG. 6. Study of the protein degradation and changes of mTOR phosphorylation in the PQ-mediated neurotoxicity. (A) The protein half-life was determined by pulse chasing with radioactive valine as indicated in “Materials and Methods” section. The cells were incubated in CM or NF and treated with PQ 10µM in the presence or absence of 3-MA 10mM for 6 h. *p < 0.05 compared with control. (B, C) mTOR dephosphorylation induced by PQ. The SH-SY5Y cells were incubated with 10µM of PQ the indicated times in CM (B) or in NF medium (C). Cell lysates were analyzed for mTOR activation by Western blotting using a phospho-specific mTOR (ser2448) antibody as indicated in “Materials and Methods” section. Actin expression was determined as a loading control in the same conditions. Blots are representative of at least three independent experiments.
produces autophagic events. These low concentrations are not normally used in the PQ toxicity study, and nobody in the present has described evidences that relate PQ-induced apoptosis and autophagy.

In our study, we have shown for the first time that PQ initially induces a morphological appearance of autophagy but it finally succumbed to a typical apoptotic cell death caspase dependent. Hereby, we demonstrated that, with the prototypic autophagy inhibitor 3-MA 10mM, not only the vacuoles formation and the protein degradation induced by PQ are inhibited but also the apoptotic cell death was accelerated and the caspase-3 activation increased. In this regard, recent studies (Boya et al., 2005; Gonzalez-Polo et al., 2005a) already have demonstrated that the inhibition of autophagy triggers apoptosis, so in this case autophagy would be considered as a defense mechanism and not as a type of cell death; moreover, recent evidence (Kiffin et al., 2006) supports a protective role of the lysosomal system, which can eliminate altered intracellular components through autophagy, at least in the first stages of oxidative injury. So, we suggest that the early activation of autophagy induced by low concentrations of PQ in neuroblastoma SH-SY5Y can be attributed to the cell response like

FIG. 7. Inhibition of PQ-induced autophagy accelerates the apoptotic cell death. (A, B) 3-MA inhibits the cytoplasmatic vacuolization induced by PQ. The human neuroblastoma SH-SY5Y cells were cultured in CM or NF and treated with PQ 10µM in the presence or absence of 3-MA 10mM for 24 h. The cells then were stained with CFMDA and fixed to visualize the presence of cytoplasmatic vacuoles as holes. Representative microphotographs in CM are depicted in A, and the percentage of vacuolated cell area is analyzed in B (*p < 0.05 compared with PQ-treated cells without 3-MA). (C) Quantitative assessment of synergic cell death induction. Cells cultured in CM or NF in the presence or absence of 3-MA 10mM and/or PQ 10µM for 24 h were stained to determine the loss of mitochondrial transmembrane potential (with DiOCl(3)) and viability (with PI). *p < 0.05 compared with PQ-treated cells without 3-MA. (D) Synergic caspase-3 activation induced in combination of PQ and 3-MA treatment. Activation of caspase-3 was determined by Western blot as indicated in “Materials and Methods” section. Actin expression was determined as a loading control. Cells were incubated in complete medium for 24 h with 10µM of PQ in the presence or absence of 3-MA 10mM. Blot is representative of at least three independent experiments.
a defense mechanism against the rapid oxidative stress induced after PQ exposure. Our results indicated that the systems of defense of the cell seemed not to be sufficient because the cell finally succumbed to the apoptotic cell death. In addition, we have seen for the first time in our study that when we inhibited PQ-induced autophagy in presence of 3-MA, we accelerated the apoptotic death process.

In conclusion, our results help to suggest PQ neurotoxicity as an emerging PD model system. This study also provides new molecular bases for the knowledge of the relationship between autophagy and the cell response to stress and apoptosis cell death. However, further studies are required to elucidate the exact mechanism involved in PQ-induced autophagy and its relation with neuronal cell death.

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