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Mitochondrial Impairment as an Early Event in the Process of Apoptosis Induced by Glutathione Depletion in Neuronal Cells: Relevance to Parkinson's Disease

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ABSTRACT. In Parkinson's disease (PD), dopaminergic cell death in the substantia nigra was associated with a profound glutathione (GSH) decrease and a mitochondrial dysfunction. The fall in GSH concentration seemed to appear before the mitochondrial impairment and the cellular death, suggesting that a link may exist between these events.The relationships between GSH depletion, reactive oxygen species (ROS) production, mitochondrial dysfunction and the mode of cell death in neuronal cells remain to be resolved and will provide important insights into the etiology of Parkinson's disease. An approach to determine the role of GSH in the mitochondrial function and in neurodegeneration was to create a selective depletion of GSH in a neuronal cell line in culture (NS20Y) by inhibiting its biosynthesis with L-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase. This treatment led to a nearly complete GSH depletion after 24 hr and induced cellular death via an apoptotic pathway after 5 days of BSO treatment. By using the reactive oxygen species-sensitive probe 2′,7′-dichlorofluorescin, we observed that the rapid GSH depletion was accompanied, early in the process, by a strong and transient intracellular increase in reactive oxygen species evidenced after 1 hr with BSO, culminating after 3 hr when the GSH level decreased to 30% of normal. GSH depletion induced a loss of mitochondrial function after 48 hr of BSO treatment. In particular, the activities of complexes I, II and IV of the respiratory chain were decreased by 32, 70 and 65%, respectively as compared to controls. These results showed the crucial role of GSH for maintaining the integrity of mitochondrial function in neuronal cells. Oxidative stress and mitochondrial impairment, preceding DNA fragmentation, could be early events in the apoptotic process induced by GSH depletion. Our data are consistent with the hypothesis that GSH depletion could contribute to neuronal apoptosis in Parkinson's disease through oxidative stress and mitochondrial dysfunction. BIOCHEM PHARMACOL **56**;5: 645–655, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; glutathione; mitochondria; neuronal cell; oxidative stress; Parkinson's disease

The pathological substrate of the major clinical and pharmacological abnormalities that characterize PD† is the degeneration of midbrain dopaminergic neurons that have recently been shown to die by apoptosis [1]. The cause(s) and mechanism(s) of neuronal degeneration underlying PD is still unknown. However, the intensity of neuronal loss varies from one dopaminergic cell group to another. One hypothesis to explain this selective vulnerability involves formation of highly ROS which may lead to neuronal damage by shifting the cellular oxidation-reduction (redox) equilibrium toward oxidation (so-called oxidative stress) [2–5]. In PD patients, the proportion of dopaminergic neurons with immunoreactive NF-κB (a redox-modulated transcription factor) in their nuclei was more than 70-fold that in control subjects [6]. At a late stage of the disease, the SN exhibits increased lipid peroxidation, suggesting that lipids have been damaged by ROS [7] and defense mechanisms against ROS appear to be compromised. Peroxidase enzyme activity and, more specifically, glutathione peroxidase are said to be reduced [8]. GSH is reported to be markedly reduced in PD, particularly in patients with advanced disease [9,10]. This decrease appeared to be selective for the SN and has led to the speculation that PD may be attributable to nigral GSH deficiency [11]. Following the discovery of inhibition of electron transport complex I by the neurotoxin MPTP [12], which produces a parkinsonian syndrome in humans, monkeys and mice [13],

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† *Abbreviations:* BSO, L-buthionine-(*S,R*)-sulfoximine; CS, citrate synthase; DA, dopamine; H_2DCFDA, 2′,7′-dichlorofluorescin diacetate; ETC, electron transport complexes; GSH, glutathione; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PD, Parkinson's disease; ROS, reactive oxygen species; SN, substantia nigra; and SOD, superoxide dismutase.

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several laboratories have reported abnormalilities of complex I and other ETCs in various tissues of patients with PD [14,15]. Moreover, an inhibition of complexes I and IV by 6-OHDA, an animal "Parkinson's disease"-inducing neurotoxin, was also reported [16,17]. The GSH decrease seemed to appear before ETCs abnormalities and neurodegeneration in presymptomatic PD [11] and was not a consequence thereof. These data suggest that GSH loss may occur early in the disease process. A link may exist between GSH decrease, impaired mitochondrial function and neuronal apoptosis observed in PD although this remains to be fully established.

GSH is a tripeptide (γ-glutamylcysteinyl-glycine) which directly plays a major antioxidant function in the elimination of ROS. It is also a substrate for scavenging ROS through the enzymes GSH peroxidase and GSSG reductase [18]. A major role of GSH is to maintain the intracellular redox state. GSH has recently been implicated in protection against the induction of apoptotic and necrotic cell death in a variety of cell types [19,20]. Neuronal cells may be particularly susceptible to the effects of GSH depletion because these cells, in contrast to glia, have relatively low concentrations of GSH [21]. It is not known whether GSH depletion in the SN in PD represents a primary cause of neurodegeneration or whether it is the end result of a series of reactions precipitated by environmental or genetic factors. However, as GSH is involved in the granular storage of DA in rat pheochromocytoma-derived PC12 cells [22], it is likely that GSH is used to protect susceptible parts of this system against (possibly DA-induced) oxidative damage. GSH depletion induced a death of rat embryonic mesencephalic neurons in culture [23] and produced profound morphological effects, including decreased catecholamine fluorescence, increased levels of lipid peroxidation, lipofuscin accumulation and increased numbers of dystrophic axons [24].

To gain a better understanding of the effect of GSH deficiency on the respiratory chain and to define the role played by this deficiency in neuronal loss, a GSH depletion was realized in a neuronal cell line in culture (NS20Y) by using a specific inhibitor of its *de novo* synthesis (BSO). Our results demonstrated that: 1) a cause/effect relationship existed between GSH depletion and apoptosis in neuronal cells; 2) redox disequilibrium was accompanied by a rapid, strong and transient ROS production; and 3) GSH depletion induced a decrease in ETCs activities preceding DNA fragmentation.

Our data support the view that GSH depletion leading to oxidative stress and mitochondrial respiratory chain impairment may serve as a trigger for apoptosis in neuronal cells. Regulation of neuronal redox status may, therefore, prove to be a useful strategy to prevent mitochondrial impairment and excessive apoptosis in PD.

MATERIALS AND METHODS

Reagents and Cell Culture Supplies

The cell culture supplies fetal calf serum (FCS), culture medium RPMI 1640, penicillin/streptomicin solutions and poly-L-lysine were purchased from Sigma. Plasticware was obtained from Falcon and Nunc; 24-well plastic cultures dishes were from Costar Corporation; coverslip chambers (Lab-Tek) were from Nunc. The *in situ* apoptosis detection kit was from Boehringer-Mannheim. BSO and all other reagents were purchased from Sigma. H_2DCFDA was from Molecular Probes Inc.

Cell Line and Culture Conditions

NS20Y cells are clonal cells derived from mouse neuroblastoma C-1300 [25] and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/L of glutamine, 50 units/mL of penicillin and 25 μg/mL of streptomycin. For cell viability experiments, confluent NS20Y cells were plated at a density of 1.5×10^4 cells/cm^2 in 24-well plastic culture dishes or glass coverslips (Lab-Tek) of 2 cm^2 in 500 μL of RPMI 1640 supplemented with 10% fetal calf serum. Cells were incubated at 37° under a 5% CO_2/95% air atmosphere. For nicked DNA end-labeling and ROS detection, NS20Y were plated at a density of 1.5×10^4 cells/cm^2 on Lab-Tek chambers of 2 cm^2 previously treated with poly-L-lysine at 10 μg/mL. For GSH and antioxidant enzyme activity determinations, confluent NS20Y cells in 175-cm^2 tissue culture flasks were used. Suspension cultures of adherent NS20Y were obtained by seeding cells, harvested from cultures in tissue culture flasks, by agitation without trypsinization. For each experiment, the cells were used before the 30th passage.

Experimental Treatment with BSO

After plating for 48 hr, the culture medium was removed, and cells were supplemented with cultured medium containing 500 μM BSO, a specific inhibitor of γ-glutamylcysteine synthetase [18].

Viability Assays

Morphological changes and photomicroscopy. NS2OY cells were plated on glass coverslips (Lab-Tek). Following the appropriate time of BSO treatment, coverslips were immersed briefly in Dulbecco's PBS (PBS 1×) and then inverted onto glass microscope slides. Morphological changes in the cells were monitored throughout the course of experiments with a Leitz model Laborlux S microscope. Photomicroscopy was carried out with Wild Leitz apparatus.

MTT colorimetric assay. After 2 and 5 days of BSO treatment, the culture medium was removed from the well and replaced by MTT at 0.5 mg/mL in PBS supplemented with glucose (33 mM). After 3 hr of incubation at 37°, this solution was removed and the produced blue formazan crystals were solubilized in 0.5 mL of pure dimethyl sulfoxide. The optical density was immediatly estimated at 550 nm.

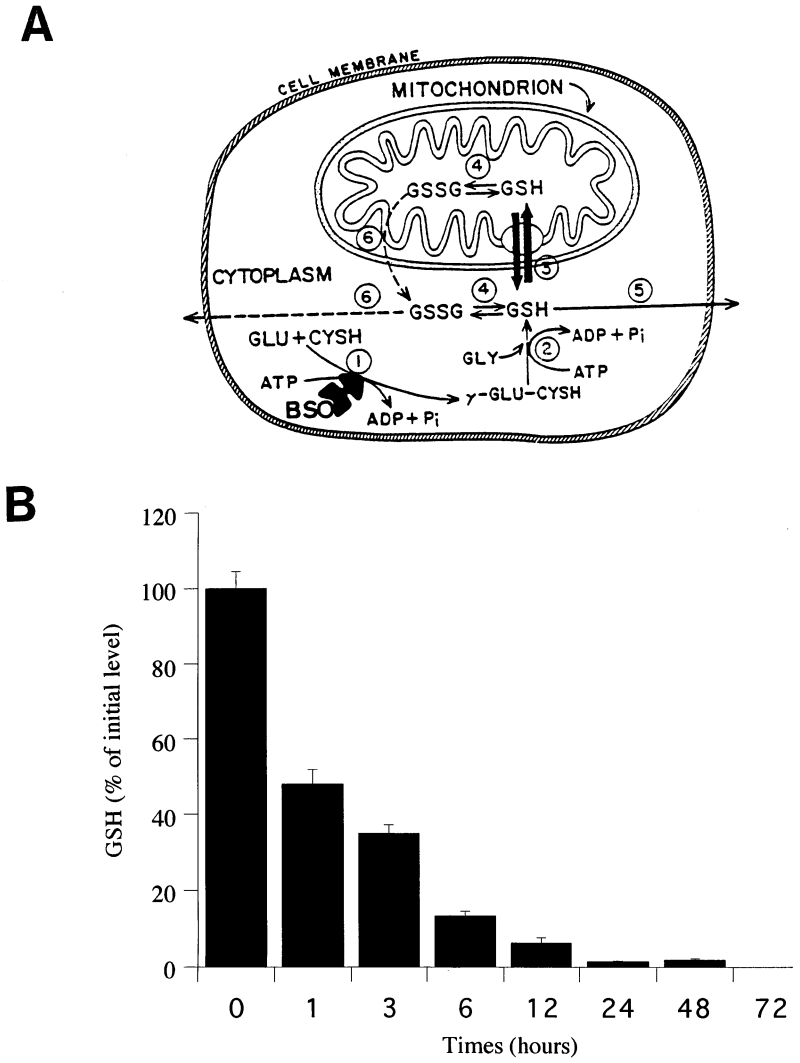

FIG. 1. (A) Scheme for synthesis and transport of GSH in mitochondria and cytoplasm (adapted from [69]. GSH is synthesized only in the cytoplasm (reactions 1 and 2). BSO is an inhibitor of γ-glutamylcysteine synthetase (reaction 1). BSO, after phosphorylation by ATP on the enzyme, binds tightly to its active site, thus inhibiting it irreversibly. The absence of synthetase from mitochondria showed that mitochondrial GSH must arise from the cytosol. Extramitochondrial GSH promotes mitochondrial uptake and exchange, and the intermembranous space appears to function as a recovery zone that facilitates efficient cycling of matrix glutathione. GSH is transported into mitochondria by a system characterized by slow net transport and more rapid exchange transport (reaction 3). Reversible oxidation of GSH to GSSG occurs in both cytoplasm and mitochondria (reaction 4). GSH is exported across the membrane of cells (reaction 5). Under conditions of oxidative stress, GSSG is transported out of the mitochondria and across cell membranes (reaction 6). (B) Time course of glutathione depletion induced by L-buthionine-(S,R) sulfoximine on NS20Y cultures. GSH depletion reached 95% after 24 and 48 hr of BSO treatment and was complete after 72 hr. Data shown are expressed as percentages of control values and are the means of quadruplicate cultures ± SD (bars). The experiment was performed in duplicate.

Apoptosis Assays

In situ determination of DNA fragmentation. Cultures were fixed using buffered paraformaldehyde (4% paraformaldehyde/PBS 1×), washed extensively in PBS 1×, and permeabilized in 0.1% Triton X-100. Cell cultures were processed with the kit based on terminal-deoxynucleotide transferase-mediated dUTP-FITC nick end-labeling (TUNEL, 26) according to the manufacturer's instructions. The specificity of the method was determined by the processing of duplicate samples in the absence of deoxynucleotide transferase. Nuclear labeling was analyzed under epifluorescence optics.

DNA Ladder Analysis

At 24 hr, 48 hr and 5 days of BSO treatment, adherent and nonadherent cells were pooled and total genomic DNA was isolated from cell pellets. Each cell pellet was washed twice with PBS 1×, resuspended in 5 mL of a lysis buffer containing 5 mmol/L of EDTA, 250 mmol/L of NaCl, 10 mmol/L of Tris-HCl (pH 7.5), and 0.2% SDS. RNAse A was added to a final concentration of 10 μg/mL (1 hr at 37°) and proteinase K to a final concentration of 100 μg/mL (2 hr at 56°). Proteins were then extracted with phenol and chloroform/isoamylalcohol (24/1; v/v). The

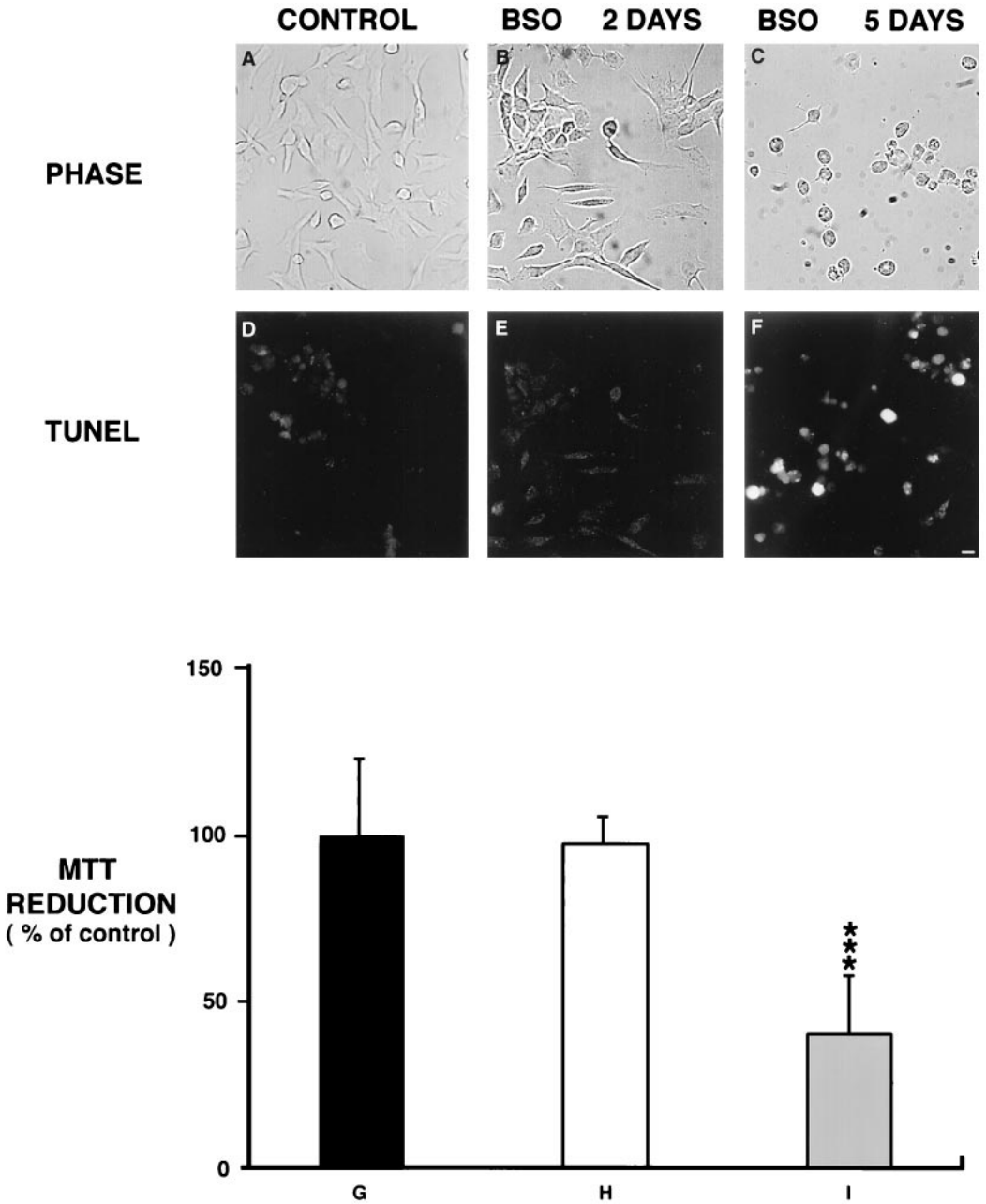

FIG. 2. Cellular mortality of NS20Y neuroblastoma in culture and *in situ* detection of apoptosis during the course of GSH depletion. Morphology of NS20Y cells and cellular death evidenced by phase-contrast microscopy (A, B, C), the FITC-based Tunel technique (D, E, F) and quantification of cellular death by using the MTT assay (G, H, I). A, D, G: untreated cells; B, E, H: 2 days of BSO treatment demonstrated neither morphological changes (B), chromatin fragmentation (E), nor inhibition of MTT reduction (H); C, F, I: 5 days of BSO treatment-induced cellular death and morphological changes (C), a strong fluorescent staining of fragmented chromatin (F) and 62% of inhibition of MTT reduction (I). A–F: Scale bar = 5 μM. G–I: Data are percent of mean ± SD (N = 12); ★★★$P < 0.001$.

DNA was precipitated by 1 volume of 100% ethanol and left overnight at −80°. After centrifugation the DNA was resuspended in 500 μL of TE buffer (10 mmol/L of Tris-HCl, 1 mmol/L of sodium EDTA, pH 7.5) before quantitation by spectrofluorimetry. Electrophoresis was carried out for 6 hr at 80 V through a 2% agarose gel prepared in TBE buffer (80 mmol/L of Tris-borate, pH 8; 2 mmol/L of EDTA) and containing 0.1 μg/mL of ethidium bromide. After electrophoresis, gels were examined under ultraviolet light and photographed.

Monitoring Intracellular ROS Generation

The formation of intracellular ROS was detected using H_2DCFDA, which is a nonfluorescent compound. After entering the cell, H_2DCFDA is de-esterified to form a

compound that can be oxidized by cellular ROS, resulting in its conversion to the fluorescent dye 2′,7′-dichlorofluorescein (DCF-DA) and that can be monitored by microfluorescence in single cells. At appropriate time points during BSO treatment (every hour between 1–6 hr and at 12, 24 and 48 hr), cells were loaded with H_2DCFDA at a final concentration of 30 μm for 30 min at 37° in a humidified 5% CO_2/95% air incubator. After loading, the marker was washed away by several medium changes, and the cells were observed with a Leitz model Laborlux S microscope under epifluorescence optics and on confocal laser microscopy.

Assay of Cellular Antioxidants

Quantification of glutathione. Duplicate aliquots of cells were collected by centrifugation and washed once with PBS 1×. The cell pellets were treated with 5% sulfosalicylic acid (SSA). Supernatants recovered after centrifugation at 17,600 *g* for 15 min were assayed for total GSH as described [27].

Quantification of antioxidant enzymes activities. Cell samples to be assayed for SODs or for GSH-related enzymes were resuspended in 1 mL of 50 mM potassium phosphate buffer (pH 7.0), followed by 1 cycle of freeze-thaw. After centrifugation, supernatants were assayed spectrophotometrically on an automated Cobas-bio (Hoffman Laroche) as previously described [28].

Determination of Respiratory Chain Activities and Mitochondrial Marker

Cell pellet was weighed and homogenized (10% w/v) in buffer containing 320 mM sucrose, 1 mM K_2EDTA, and 10 mM Tris (pH 7,4). The homogenate was centrifugated for 10 min at 1,500 *g* at 4° and the resulting supernatant used for determination of the activities of the respiratory enzymes and CS as previously described [29]. Complex I (NADH:ubiquinone oxido-reductase), complex II (succinate:ubiquinone oxido-reductase), complex IV (ferrocytochrome c:oxygen oxido-reductase or cytochrome oxidase), and CS measurements were performed using an Uvikon 160 Kontron spectrophotometer. All assays were realized in triplicate at different protein concentrations. Protein concentrations were determined using the method of the Bicinchoninic Acid (BCA, Pierce).

Statistical Analysis

Mean measures are presented with standard deviations (mean ± SD). After testing for variance homogeneity (one factor ANOVA), the data were subjected to the unpaired Student's *t*-test between BSO-treated cells and controls. When the variance was heterogeneous, the Mann-Whitney *U*-test was used. A P value < 0.01 was selected as the point of minimal statistical significance.

RESULTS

Time Course of Glutathione Depletion Induced by BSO in NS20Y Cells

GSH is synthesized in two steps from glutamate, cysteine and glycine by the consecutive actions of γ-glutamylcysteine synthetase and GSH synthetase (Fig. 1A; reactions 1 and 2). The absence of synthetases from mitochondria showed that mitochondrial GSH must arise from the cytosol. Extramitochondrial GSH promotes mitochondrial uptake and exchange, and the intermembranous space appears to function as a recovery zone that facilitates efficient cycling of matrix GSH. To elucidate the functions of GSH in neuronal cells, we sought to selectively remove this tripeptide from the cells by using BSO, an inhibitor of γ-glutamylcysteine synthetase.

This treatment led to a progressive decrease in intracellular GSH concentration during the first 24 hr. A GSH depletion of 50% after 1 hr and 98% after 24 and 48 hr was evidenced (Fig. 1B). After 3 days of BSO treatment, the cellular GSH pool was completely depleted (Fig. 1B).

Morphological Changes and Cellular Death Induced by BSO Treatment

The effects of BSO were on cell morphology and cell mortality were studied by using phase contrast microscopy and MTT assays. Incubation of NS20Y cells with BSO for 48 hr did not cause morphological changes (Fig. 2B) nor loss of cell viability (Fig. 2H) as compared to controls (Figs. 2A and G). The effect of GSH depletion on cell morphology and viability was detected after 5 days of BSO treatment. The morphological changes were a loss of cell connections and neurites, a rounding of cell soma (Fig. 2C) as compared to controls (Fig. 2A), and a significant decrease in cell number (−62%, Fig. 2I) as compared to controls (Fig. 2G). This was followed by extensive detachment of the cell lawn. Our experiments demonstrate that simply lowering intracellular GSH levels is sufficient to induce a strong cellular death after 5 days of BSO treatment.

In Situ *Staining of DNA Fragmentation Characteristics of Apoptosis*

To examine the possibility that GSH depletion could initiate apoptosis, we used the TUNEL reaction, at different times with BSO exposure, to identify the nuclei of cells undergoing DNA fragmentation. Nuclei demonstrated chromatin condensation and nuclear fragmentation after 5 days of BSO treatment (Fig. 2F), while no labeling was observed after 2 days (Fig. 2E), as compared to control cultures (Fig. 2D).

Evidence of DNA Ladder on Agarose Gel

Additionally, gel electrophoresis of DNA from GSH-depleted cells for 5 days displayed the presence of 180–200-bp

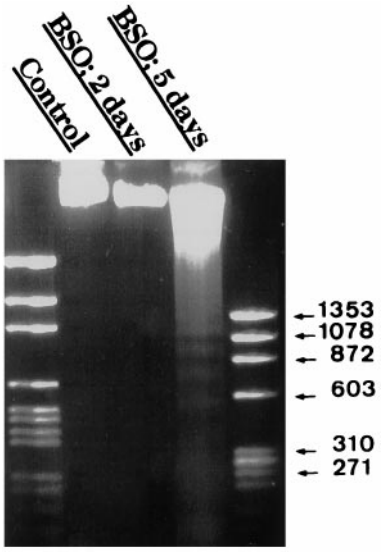

FIG. 3. The DNA ladder of apoptotic cells. DNA was extracted and the oligonucleosomal DNA fragments were resolved by agarose gel electrophoresis as described in Materials and Methods. Lane 1: 1-kilobase DNA ladder marker PGEM (Promega); lane 2: untreated control DNA from NS20Y; lane 3: NS20Y-BSO treated for 2 days; lane 4: NS20Y-BSO treated for 5 days. Low molecular eight DNA from NS20Y-BSO treated for 5 days demonstrates the "apoptotic ladder" of approximately 180-bp multiples characteristic of internucleosomal DNA fragmentation. Lane 5: DNA ladder marker PHI-X174 HAEIII (Life Sciences).

DNA fragments or multiples thereof (Fig. 3). Cleavage of DNA into nucleosomal fragments of 180–200 bps or multiples has often been considered as a major biochemical hallmark of apoptosis. Thus, a direct relationship between GSH depletion and apoptosis is now demonstrated in this neuronal cell type.

Generation of ROS in GSH-Depleted Cells

By using the ROS-sensitive probe H_2DCFDA, we demonstrated that GSH depletion is accompanied, early in the process, by a strong intracellular ROS production evidenced after one hour with BSO (when GSH levels decreased to 50% of normal) and peaking at 3 hr of incubation with BSO when GSH levels decreased to 30% of normal (Fig. 4). At latter stages (4, 5, 6, 12, 24 and 48 hr), this increase in intracellular ROS was not sustained (data not shown), indicating that GSH depletion induced a transient increase in ROS level early in the process.

Modifications of Antioxidant Activities in Cells during the Course of BSO Treatment

To clarify the role of GSH depletion in the apoptotic process in this model, it was important to know if other enzymes implicated in free radical detoxification were affected during the course of BSO treatment. GSH depletion led to secondary changes in the antioxidant system; in particular, a down-regulation of GSH peroxidase in living

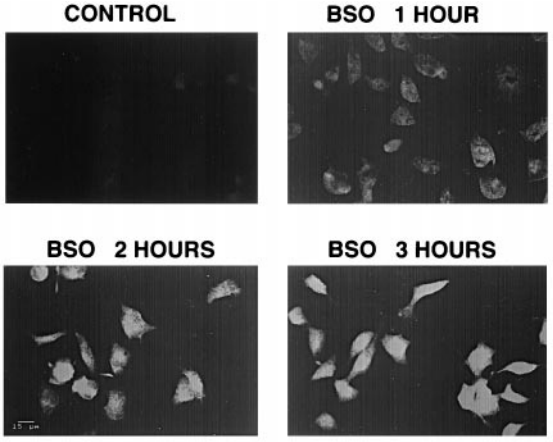

FIG. 4. Time course of ROS generation as a consequence of GSH depletion. Note that BSO induces a time-dependent increase in DCF-DA fluorescence. A maximum was detected after 3 hr of BSO treatment when the GSH level decreased to 30%.

NS20Y after 2 days of BSO treatment was observed (–40%; $P < 0.001$; Fig. 5). This decrease precedes nuclear fragmentation, because no labeling of DNA fragmentation was observed at the cellular level (Fig. 2E) and on agarose gel (Fig. 3). After 5 days of BSO exposure, the GSH-depleted NS20Y cells still living showed a significant increase in CuZnSOD and GSSG reductase activities ($P < 0.01$; Fig. 5), and the significant decrease in GSH peroxidase activity was sustained ($P < 0.01$). Whatever the time of BSO exposure, MnSOD activity was unchanged as compared to control cells.

Drastic Fall in Activities of Complexes II and IV from the Mitochondrial Respiratory Chain as a Result of GSH Depletion

We examined the potential involvement of GSH depletion in mitochondrial function by assessing the activities of ETCs in cellular homogenates. Our results indicate that GSH depletion can cause dysfunction in mitochondrial respiratory chain activity, principally as a result of inhibition of ETCs activities. Complexes II and IV were particularly affected when the activities were expressed as milliunits per milligram of protein (70% and 65%, respectively as compared to controls; $P < 0.001$) (Fig. 6A). A smaller decrease was observed for complex I (–35%) and for CS (–32%), the first enzyme in Krebs' cycle as mitochondrial marker (Fig. 6A). When enzyme activities were reported to the activity of CS, the strong decrease in complexes II and IV persisted (–50% as compared to controls; $P < 0.01$) (Fig. 6B). These results indicate that intracellular GSH status is critical for mitochondrial function. The mitochondrial dysfunction appeared after 48 hr of BSO treatment in cells that were TUNEL-negative and morphologically intact. Thus, dysfunction of ETCs precedes cellular death and nuclear fragmentation and likely contributes to the progression of apoptosis.

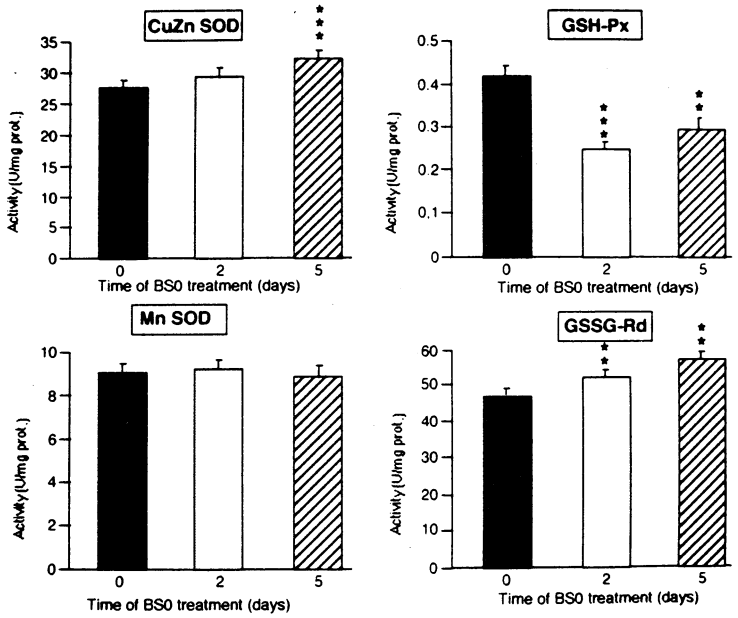

FIG. 5. Basal antioxidant enzyme activities of NS20Y cells before and during the course of GSH depletion. Data are means ± SD (N = 6). The experiment was carried out in duplicate. **$P < 0.01$ and ***$P < 0.001$.

DISCUSSION

Numerous neurodegenerative disorders and mechanisms of neuronal death such as apoptosis are thought to be associated with oxidative stress [2–4,30,31]. Given the constant threat to cell viability imposed by ROS derived from normal cellular metabolism, a down-regulation of antioxidant defense may provide an efficient mechanism by which physiologic and pathologic cell deletion can be achieved. This may play a role in the mechanisms of neurodegeneration and apoptosis in the central nervous system. The relationship between GSH depletion and neuronal degeneration is not well understood but when elucidated will likely provide important insights into neuropathological conditions under which GSH has been demonstrated to be decreased, such as in the SN in PD [7,8]. A mean to analyze the role played by a particular antioxidant in the central nervous system is to selectively modulate it by genetic or metabolic manipulations [32]. Our study indicates that GSH depletion was able to induce by itself a death of a clonal cell line of neuronal origin. This cellular death was characterized as apoptosis. Our results add to the evidence suggesting that direct oxidative damage [33,34] or indirect oxidative stress may cause neuronal death [35] that proceeds through an apoptotic pathway [36]. The glutathione redox cycle is an important component of the antioxidant machinery, with mRNA levels for redox-related genes (CuZnSOD, MnSOD and catalase) falling during apoptosis [37]. Depletion of GSH is an early event in the apoptotic process [38] and has been reported to induce or increase sensitivity to apoptosis in different systems. In particular, glutamate-induced GSH depletion in embryonic cortical neurons [36] and in neuroblastoma and hippocampal neurons [35] results in oxidative stress and apoptotic cell death. Diethylmaleate (DEM) reduces cellular stores of GSH by forming a thioether conjugate in a reaction catalyzed by glutathione *S*-transferase, and as a consequence ROS accumulating in the cells induce neuronal death [39]. Thus, redox modulation seems to play a major role in the regulation of cell death and neuronal apoptosis.

Any new model for the biochemical events defining apoptosis must distinguish between an inducer of apoptosis, of which there are many [40], and a mediator of apoptosis. Such a mediator must satisfy some criteria: 1) demonstrate a change during apoptosis, prior to cell death; 2) modulation of the putative mediator should modulate apoptosis accordingly; and 3) expression of antiapoptotic genes should affect the putative mediator, unless the effect of the antiapoptotic genes are downstream from the putative mediator. GSH fulfills all of these criteria. A decrease in GSH concentration was demonstrated in preapoptotic cells [38,39,41], and its depletion generated cell death via apoptosis in neuronal clones (our data). In addition, the protooncogene *bcl-2*, which inhibits many forms of apoptotic cell death, has been suggested to mediate an antioxidant pathway to prevent apoptosis [39,42]. In particular, neuronal cell lines expressing *bcl-2* exhibit two-fold greater GSH levels than controls [39,43], a 1.7–2.0-fold increase in catalase and SOD enzymatic activities, and a shift in cellular oxidation-reduction potentiel [43]. Other results showed that the up-regulation of cellular GSH evoked by autoxidable agent such as L-DOPA is associated with significant protection of cells [44]. The loss of sensitivity of sympathetic neurons in culture toward MPTP is accompanied by a three-fold increase in the level of GSH [45]. These results also suggest that GSH plays a role in protecting neurons *in vivo* from the toxicity of MPTP. GSH could

be the mediator of a common pathway triggered by both physiologic and pathologic inducers of neuronal apoptosis and could play a key role in inducing naturally occurring neuronal death during development and under pathologic conditions.

The exact mechanism by which GSH depletion induces neuronal apoptosis remains to be established and is of great interest, as it may provide clues as to the mechanisms of cell death and the etiology of PD. We demonstrated, by using a dye oxidized into a highly fluorescent form in the presence of ROS, that ROS were produced transiently and early in the process of GSH depletion. Thus, one may expect a participation of these ROS in an apoptogenic signaling pathway. Decreased levels of GSH would be expected to cause an increase in the production of hydrogen peroxide and hydroxyl radicals from the Fenton reaction, because the competing GSH peroxidase reaction would be diminished. Insight into the mechanism by which GSH depletion leads to ROS production may also be gleaned from the observation that GSH depletion is accompanied by increased neuronal nitric oxide synthase (NOS) activity and NO production [46]. H_2DCFDA can also detect NO and related compounds [47]. It is thus possible, in this model, that ROS detected were NO and related compounds such as peroxinitrite.

Our data showed that GSH depletion induced a profound decrease in ETCs activities before the nuclear fragmentation. GSH depletion could be neurotoxic by inducing ROS production and consequently could induce an apoptogenic signaling pathway in which mitochondria could play an important role. The difference between the model described here and the results described in the Parkinsonian nigra was the strong decrease in complexes II and IV in GSH-depleted cells, since the inhibition of complex I in PD is often described as being highly specific [48]. This discrepancy could be explained by the difference in the level of GSH depletion. A nearly complete GSH depletion was observed after 24 hr of BSO treatment whereas, in PD brains, the GSH depletion was described to be approximately 40% to 50% below the control value [9,49,50]. In the neurotoxin description of MPTP and 6-OHDA action, a GSH depletion of 30% was also described [51,52]. It is possible that the first complex affected both by a slight decrease in GSH level as in PD brains, MPTP and 6OHDA models as well as when a more important GSH depletion occurs, as in the BSO models, is complex I, but complexes II and IV were also affected. However, an inhibition of mitochondrial complex IV by 6-OHDA [16] and MPTP [53] was also described. Other studies have revealed that mitochondrial damage was an important consequence of GSH deficiency in many tissues [54], in the brain [55,56] and in neurons [57]. The intracellular ROS accumulation (H_2O_2 and/or NO) could be responsible for the mitochondrial abnormalities observed. All ETCs are reported to be susceptible to damage by oxygen free radicals *in vitro* [58]. Producing oxidative stress in the rat brain leads to sequential damage to the components of the electron transport chain, with complex IV appearing to be the most susceptible [59]. A relationship betwen oxidative stress and mitochondrial damage has been reported in other model systems: iron loading of PC12 cells leads to loss of cellular GSH, complex I and IV activities [60]. NO-mediated cytotoxicity was associated with inactivation of the ETCs activities in cultured astrocytes and neurons [57]. A decline in mitochondrial transmembrane potential and cellular ATP production following extensive depletion of both cytosolic and mitochondrial pools of GSH occurred prior to the onset of the loss of cell viability in cultured cerebellar astrocytes [61].

A consequence of this impaired mitochondrial function may be cell death due to energy deficiency. Apoptosis has been reported to proceed as a result of damage to the mitochondrial respiratory chain [62,63], and the cellular ATP level has been proposed as an important factor in the control of apoptosis [64]. Moreover, recent studies demonstrate that manipulation of the GSH system influences the susceptibility of dopamine neurons to damage due to energy impairment [65]. Neuronal cells have a relative paucity of GSH and need high energy production, and hence may be particularly vulnerable to the effects of GSH depletion. Thus, we can conclude that with GSH depletion as the

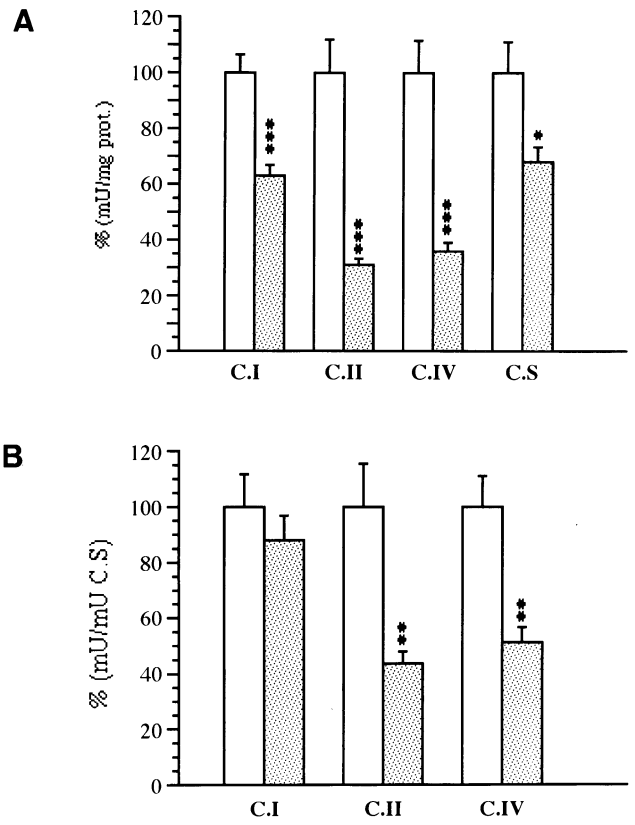

FIG. 6. Activities of respiratory chain complexes after two days of GSH depletion in living NS20 Y cells. (A) Data expressed as milliunits per milligram of proteins; (B) data expressed as milliunits per milliunit of citrate synthase. Data are means ± SD (N = 6). The experiment was done in duplicate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

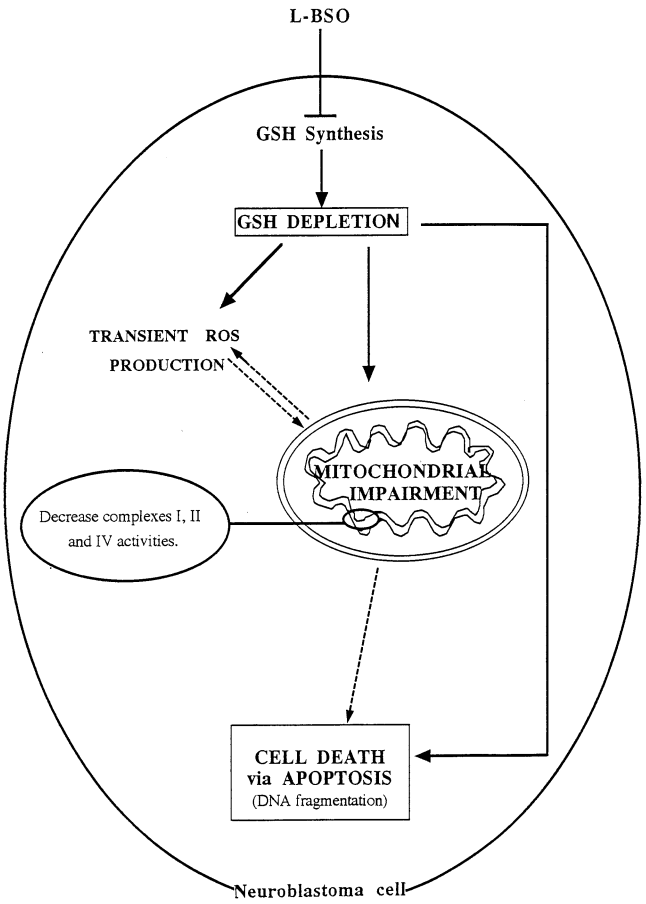

FIG. 7. Scheme summarizing the sequence of events accompanying the apoptotic process induced by GSH depletion.

initial insult, a cascade of events involving a transient increase in ROS production and then mitochondrial dysfunction could contribute to cell injury and finally to apoptosis (Fig. 7). The recent demonstration of a mitochondrial control of nuclear apoptosis also argues in favor of this hypothesis [38,66,67].

Studies in neuronal cultures showed that addition of L-DOPA to cell cultures induced an increase in GSH levels and a protection of the cells [44]. When cultures were pretreated with both L-DOPA and ascorbate, which prevent the rise in GSH level, protection was lost, in accordance with the failure to up-regulate GSH. A relationship between increased GSH levels by basic fibroblast growth factor (bFGF) and its capacity to protect DA neurons from 6-OHDA toxicity [68] also demonstrate that the up-regulation of cellular GSH is associated with significant protection of the cells. This ability to up-regulate GSH may serve a protective role *in vivo*. Thus, the possibility of a prevention of GSH depletion with GSH substitutes (such as *N*-acetylcysteine) might itself contribute to the enhanced neuron survival in dopaminergic neurodegeneration associated with PD. This requires further investigation.

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