

Enhancement of Dopaminergic Neurotoxicity by the Mercapturate of Dopamine: Relevance to Parkinson's Disease

*Jing Zhang, †Vladimir Kravtsov, *Venkataraman Amarnath, *Matthew J. Picklo,
*Doyle G. Graham, and *‡§Thomas J. Montine

Departments of *Pathology, †Hematology, and ‡Pharmacology and §Center for Molecular Neurosciences,
Vanderbilt University Medical Center, Nashville, Tennessee, U.S.A.

Abstract: The mechanisms that underlie dopaminergic neurodegeneration in Parkinson's disease (PD) are not known but have been proposed to involve oxidation of dopamine and related catechols. In other organ systems, cytotoxicity from catechol oxidation is profoundly influenced by mercapturate metabolism. Here we have tested the hypothesis that catechol thioethers produced in the mercapturic acid pathway may act as dopaminergic neurotoxins. A rat mesencephalic/neuroblastoma hybrid (MES) cell line was exposed to dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), or eight different catechol thioethers for up to 24 h, and the extent of apoptosis was quantified by a microculture kinetic assay. Apoptosis also was confirmed morphologically with Giemsa-stained cultures and by demonstration of internucleosomal DNA fragmentation. The results showed that dopamine at 5–50 μM produced concentration-dependent increases in the percentage of apoptotic MES cells. At 25 and 50 μM dopamine, the maximal proportions of apoptotic cells were detected at ~19 ($20.7 \pm 2.0\%$) and 14 h ($30.3 \pm 3.5\%$), respectively. None of the catechol thioethers (up to 5 μM) alone induced significant apoptosis in MES cells. However, when MES cells were incubated with dopamine (25 μM) and catechol thioethers (5 μM) to mimic pathological conditions, 5-S-N-acetylcysteinyldopamine, 5-S-homocysteinyldopamine, and 5-S-homocysteinyldopamine significantly increased the percentage of apoptotic cells compared with dopamine alone. These results suggest that mercapturate metabolism of endogenous catechols may yield products that facilitate dopaminergic neurodegeneration. **Key Words:** Catechols—Thioethers—Mercapturates—Neurodegeneration—Apoptosis—Aging and Parkinson's disease. *J. Neurochem.* **74**, 970–978 (2000).

Idiopathic Parkinson's disease (PD) is a major public health problem for older individuals (Duvoisin and Sage, 1996). The pathological hallmarks of PD are dopaminergic neurodegeneration in the substantia nigra and accumulation of intraneuronal inclusions called Lewy bodies (Lowe et al., 1997). The causes of dopaminergic neurodegeneration in PD remain unclear, but several lines of evidence suggest involvement of oxidative stress in PD

pathogenesis. These include decreased levels of nigral reduced glutathione (GSH) (Perry et al., 1982), increased levels of nigral iron (Dexter et al., 1989), and the accumulation of oxidized products from lipid (Yoritaka et al., 1996), protein (Alam et al., 1997a), and nucleic acids (Alam et al., 1997b; Zhang et al., 1999) in the midbrain of PD patients. It is hypothesized that dopamine metabolism may contribute to oxidative damage in these brain regions. Dopamine is metabolized enzymatically with the generation of hydrogen peroxide (Cohen, 1983). In addition, paramagnetic metal ions catalyze the oxidation (autooxidation) of dopamine and related catechols under physiological conditions, a process that generates superoxide anion and *o*-quinones (Graham, 1978; Graham et al., 1978). Thus, both enzymatic oxidation and autooxidation of catechols have the potential to subject catecholaminergic neurons to especially high levels of oxidative stress (Graham et al., 1978; Cohen, 1983; Hastings and Zigmond, 1994; Ben-Shachar et al., 1995).

Catechol autooxidation is particularly interesting because the resulting *o*-quinones are chemically reactive toward several cellular nucleophiles, with thiolates being greatly favored kinetically. Indeed, adduction by thiolates occurs more rapidly than *o*-quinone reduction by ascorbate (Tse et al., 1976). The products of thiolate

Resubmitted manuscript received October 29, 1999; accepted November 1, 1999.

Address correspondence and reprint requests to Dr. J. Zhang at C3321A Medical Center North, Division of Neuropathology, Vanderbilt University Medical Center, Nashville, TN 37232, U.S.A. E-mail: jing.zhang@mcmail.vanderbilt.edu

Abbreviations used: AcCys-DOPAC, 5-S-N-acetylcysteinyldopamine; AcCys-dopamine, 5-S-N-acetylcysteinyldopamine; CNAT, cysteine-S-conjugate N-acetyltransferase; Cys-DOPAC, 5-S-cysteinyldopamine; Cys-dopamine, 5-S-cysteinyldopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GSH, reduced glutathione; GSH-DOPAC, 5-S-glutathionyl-3,4-dihydroxyphenylacetic acid; GSH-dopamine, 5-S-glutathionyl-dopamine; γGT , γ -glutamyltranspeptidase; hCys-DOPAC, 5-S-homocysteinyldopamine; hCys-dopamine, 5-S-homocysteinyldopamine; KU, kinetic units; MiCK, microculture kinetic; OD, optical density; PD, Parkinson's disease; T_m , time to the maximal response.

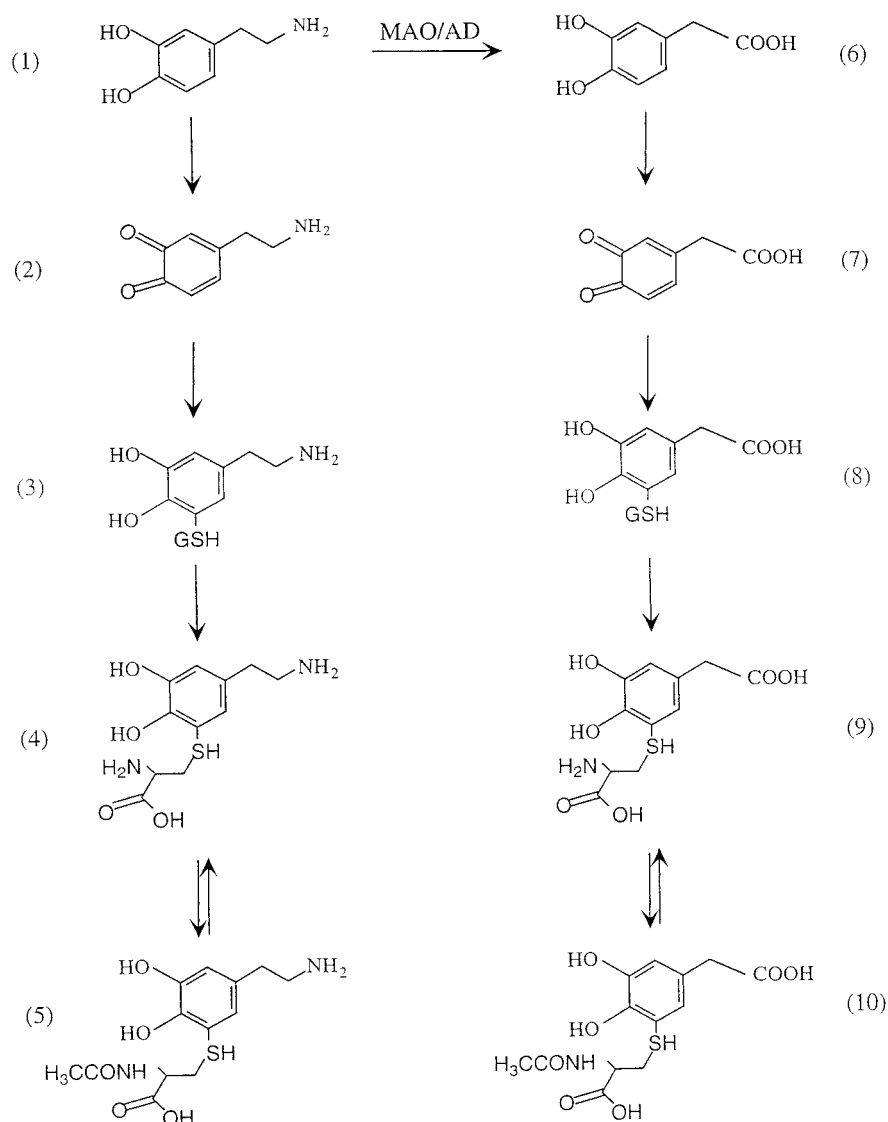


FIG. 1. Formation of catechol thioethers. Autooxidation of dopamine (1) forms dopamine *o*-quinone (2), which leads to formation of GSH-dopamine (3) after intracellular addition of GSH. GSH conjugate is transported out of cells and hydrolyzed by γ GT and dipeptidases to the corresponding Cys-dopamine (4). The cysteine conjugate reenters cells and is metabolized by microsomal CNAT to AcCys-dopamine (5). Cytosolic deacetylases catalyze the reverse reaction, and a balance between CNAT and deacetylase activities determines the concentration of AcCys-dopamine. Dopamine *o*-quinone also reacts with cysteine directly producing cysteinyl (4) catechol thioether. In addition, dopamine may be metabolized by monoamine oxidase (MAO) and aldehyde dehydrogenase (AD) to form DOPAC (6), which undergoes autooxidation to DOPAC *o*-quinone (7) and then a process similar to that for dopamine, forming glutathionyl (8), cysteinyl (9), and mercapturate (10) DOPAC, respectively, i.e., GSH-DOPAC, Cys-DOPAC, and AcCys-DOPAC, respectively.

addition to *o*-quinones are called catechol thioethers (Fig. 1). In biological systems, catechol thioethers form mostly with free cysteine, GSH, or protein-bound cysteine (Graham, 1978; Graham et al., 1978; Montine et al., 1997). Catechol thioethers also may form from norepinephrine by similar chemical reactions (Shen and Dryhurst, 1996); however, most research effort has been directed at catechol thioethers derived from dopamine and its metabolites. Glutathionyl and cysteinyl catechol thioethers derived from dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) have been identified as the major non-protein-bound catechol thioethers in the striatum and midbrain of humans and other mammals. In fact, the cysteinyl catechol thioethers have been studied as biomarkers of catechol oxidation (Fornstedt et al., 1990a,b). Increased levels of 5-*S*-cysteinyl-dopamine (Cys-dopamine) and 5-*S*-cysteinyl-DOPAC (Cys-DOPAC) in midbrain and striatum have been associated with nigral degeneration, advancing age, and oxidant stress

(Fornstedt et al., 1986, 1989, 1990a,b; Fornstedt and Carlsson, 1991; Hastings et al., 1996a,b). Hence, it has been proposed that catechol thioether formation contributes to dopaminergic neurodegeneration by depleting GSH (Hastings et al., 1996a,b; Shen and Dryhurst, 1996; Spencer et al., 1998). Alternatively, catechol thioethers also may act directly or indirectly as endogenous neurotoxins (Li and Dryhurst, 1997; Montine et al., 1997; Picklo et al., 1999).

A few studies have investigated the potential biological activity of catechol thioethers and their products. Dihydrobenzothiazine species produced from cyclization of Cys-dopamine irreversibly inhibit mitochondrial complex I in vitro and induce behavioral responses when administered directly into mouse brain (Shen et al., 1996; Li and Dryhurst, 1997); however, these experiments used dihydrobenzothiazine species at near millimolar concentrations, and neurodegeneration from dihydrobenzothiazine species has not been reported. We have shown that

incubation of organotypic cultures of neonatal rat hippocampus with Cys-DOPAC leads to secondary excitotoxic neurodegeneration; however, this effect also is observed at concentrations orders of magnitude greater than what is expected in vivo (Montine et al., 1997). In a recent in vitro structure–function study we identified the mercapturates of dopamine and DOPAC as the most potent of all the endogenous catechol thioethers at producing oxidative damage (Picklo et al., 1999).

Mercapturate metabolism begins with intracellular addition of GSH to an electrophile such as the *o*-quinones derived from dopamine and DOPAC (Fig. 1) (Wang and Ballatori, 1998). GSH conjugates are transported out of cells and hydrolyzed by γ -glutamyltranspeptidase (γ GT) and dipeptidases to the corresponding cysteine conjugates. Cysteine conjugates reenter cells and are metabolized by microsomal cysteine-*S*-conjugate *N*-acetyltransferase (CNAT) to mercapturate conjugates. Cytosolic deacetylases catalyze the reverse reaction, and a balance between CNAT and deacetylase activities determines mercapturate conjugate concentrations. Although mercapturate formation usually is a means of detoxifying reactive electrophiles, in some instances mercapturate metabolism enhances cytotoxicity (Parkinson, 1996). Perhaps the best example of mercapturate bioactivation is the proneurotoxin 2-bromohydroquinone, a dihydroxybenzene closely related to dopamine (Monks and Lau, 1992). Initially, 2-bromohydroquinone forms GSH conjugates that are then hydrolyzed to the corresponding cysteine conjugates. Bioactivation of 2-bromohydroquinone occurs with cysteinyl adduct formation that significantly lowers the oxidation potential, thereby transforming the parent molecule into a potent oxidant (Parkinson, 1996). Subsequent steps in mercapturate metabolism of 2-bromohydroquinone either maintain or diminish activity of this oxidant (Monks and Lau, 1992).

The entire mercapturic acid pathway, including γ GT, CNAT, and deacetylase activities, exists in rat brain (Miller et al., 1995; Shen et al., 1996). Indeed, the ratio of CNAT to deacetylase activities in rat brain is highest in midbrain (Miller et al., 1995), and γ GT activity is elevated in the substantia nigra of PD patients compared with controls (Sian et al., 1994). However, no investigation yet has been done concerning the effects of mercapturates on dopaminergic neurons. Here we have tested the hypothesis that endogenous dopamine- and DOPAC-derived mercapturates may be dopaminergic neurotoxins at concentrations that may be achieved in vivo and have compared the activities of the mercapturates with the corresponding glutathionyl and cysteinyl catechol thioethers. In addition, the dopaminergic neurotoxicity of homocysteinyll catechol thioethers was examined because of their inability to form benzothiazine species.

MATERIALS AND METHODS

Synthesis of catechol thioethers

Most catechol thioethers used in this study were synthesized as reported (Montine et al., 1997; Picklo et al., 1999). These

include 5-*S*-glutathionyl-dopamine (GSH-dopamine), Cys-dopamine, 5-*S*-*N*-acetylcysteinyldopamine (AcCys-dopamine), 5-*S*-glutathionyl-DOPAC (GSH-DOPAC), Cys-DOPAC, and 5-*S*-*N*-acetylcysteinyll-DOPAC (AcCys-DOPAC). Catechol thioethers were aliquoted and stored in 0.1 *M* HCl under argon at -80°C . The homocysteinyll catechol thioethers of dopamine and DOPAC were synthesized by a slight modification of the above methods. DL-Homocysteine thiolactone hydrochloride (0.77 g, 5 mmol) was added to a mixture of 10 *M* NaOH (1.5 ml) and methanol (8.5 ml) and heated at 65°C with stirring under argon. After 90 min the reaction mixture was cooled, acidified with 6 *M* HCl (1.67 ml), and diluted with methanol (40 ml). A solution of *N*-(*tert*-butoxycarbonyl)dopamine (0.39 g, 1.5 mmol) in methanol (15 ml) containing formic acid (225 μl) was stirred in ice with Ag_2O (0.75 g) and Na_2SO_4 (1 g) for 3 min and filtered through a bed of Celite into homocysteine prepared as above. The red-orange solution slowly turned green with stirring, and the excess homocysteine was removed by filtration. The crude product was first purified by flash chromatography (1:2 methanol/ethyl acetate with 0.1% formic acid). It was further purified by HPLC [column, PRP-1 300 \times 7.5 mm; solvent systems, 5 *mM* HCl (A) and methanol (B); 50% B to 95% B in 10 min; yield, 0.3 mmol (20%); mass spectrometry m/z 386 ($M + 1$)⁺]. The protected adduct was dissolved in 6 *M* HCl to give a 50 *mM* solution that was kept at room temperature for 3 h and evaporated. The final product, 5-*S*-homocysteinyldopamine (hCys-dopamine), confirmed by mass spectrometry [m/z 287 ($M + 1$)⁺], was aliquoted and stored in 0.1 *M* HCl under argon at -80°C . The ethyl ester of 5-*S*-homocysteinyll-DOPAC (hCys-DOPAC) was similarly prepared starting with the ethyl ester of DOPAC. It was purified by flash chromatography (20% methanol in ethyl acetate) followed by HPLC [same conditions as above except acetonitrile as solvent B; yield, 30%; mass spectrometry m/z 330 ($M + 1$)⁺]. The ester was removed in 6 *M* HCl at room temperature for 16 h, and the final product, hCys-DOPAC, was aliquoted and stored in 0.1 *M* HCl under argon at -80°C after confirmation with mass spectrometry [m/z 302 ($M + 1$)⁺].

Cell culture

The hybrid rat mesencephalic/neuroblastoma MES cells, obtained from Dr. Stanley Appel at Baylor University (Le et al., 1995), were maintained at 37°C in logarithmic growth in Dulbecco's modified Eagle's medium without phenol red (GIBCO) containing 50 μM glutamate, 0.8 *mM* magnesium, 1 *mM* calcium, and 2% heat-inactivated newborn calf serum (growth medium). After reaching 50% confluence, cells were plated at a density of 1×10^5 cells/ml in 96-well microtiter plates for quantification of apoptosis by the microculture kinetic (MiCK) assay (see below). Only passage 4–14 MES 23.5 cells were used in the experiments.

Analysis of apoptosis

MiCK assay. The MiCK assay was performed as described previously (Kravtsov and Fabian, 1996; Kravtsov et al., 1998) with minor modifications. In brief, cells were plated in 120- μl aliquots in 96-well microtiter plates at a density of 1×10^5 cells/ml. The plate with cells was incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 18 h to allow attachment of the cells. Dopamine and other chemicals, diluted with the growth medium from stock solutions, were added to the cell microcultures in 5- μl aliquots. The microtiter plate was incubated at 37°C for 30 min in a fully humidified atmosphere of 5% CO_2 in air, after which time 30 μl of sterile mineral oil (Sigma, St. Louis, MO, U.S.A.) was layered on the top of each

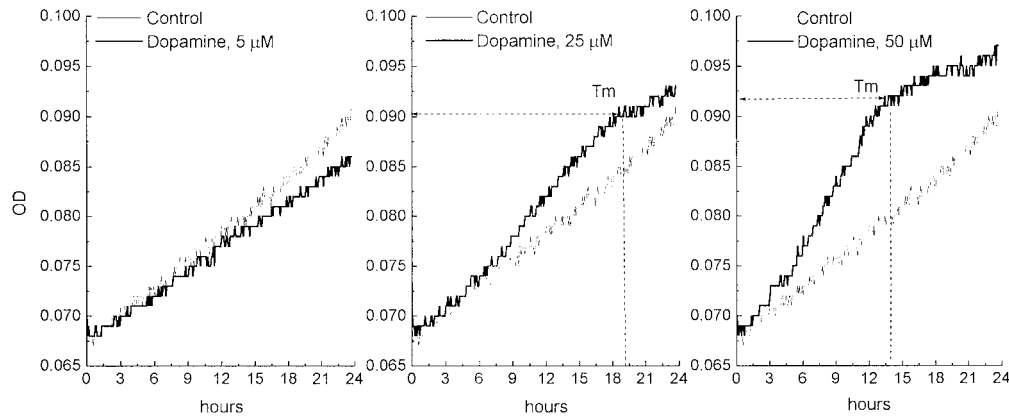


FIG. 2. MiCK assay of dopamine-induced apoptosis in MES cells. Cells were plated in a 96-well plate at 1×10^5 cells/ml (120 μ l per well) and maintained at 37°C in a CO₂ incubator for 18 h. Then, dopamine was added in wells in 5- μ l aliquots to achieve final concentrations of 5, 25, or 50 μ M. Control wells received vehicle only.

microculture. The microtiter plate was placed in the incubated chamber of a spectrophotometer (PowerWave 340; BioTek Instruments, U.S.A.) at 37°C, and the optical density (OD) at 600 nm was read every 5 min for a period of 24 h. The reader was calibrated to zero absorbance using wells containing only complete medium without cells.

Calculation of the extent of apoptosis. In the MiCK assay, extent of apoptosis is measured in kinetic units (KU), which were shown to correlate with the percentage of cells with apoptotic morphology (Kravtsov et al., 1998, 1999). In this study, we compared results of the MiCK assay expressed in KU with results of the measurement of apoptosis in percentage of morphologically apoptotic MES cells in cytospin preparations stained with Giemsa using the same cultures. Apoptotic cells were identified by the condensed and fragmented state of their nuclei and focal protrusions of the cell membrane (see Fig. 3B). The relationship between KU and the percentage of apoptosis for MES cells exposed to various concentrations of dopamine is described by an empirical curve that was used as a nomogram to convert KU into the percentage of apoptotic cells throughout the study (see Fig. 4, inset).

Electrophoretic analysis of DNA fragmentation. Apoptosis in MES cells also was analyzed by agarose gel electrophoresis of DNA as described elsewhere (Kravtsov et al., 1999). Adhered cells were exposed to dopamine for 24 h, harvested, washed twice in cold Tris-buffered saline, and incubated at 37°C for 1 h in lysis buffer [10 mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 20 mg/ml RNase A, and 0.5% sodium dodecyl sulfate]. Cell lysates were incubated at 50°C overnight in the presence of 50 mg/ml proteinase K. DNA was extracted with an equal volume of Tris buffer-saturated phenol/chloroform (1:1 vol/vol). The extraction was repeated three times, and after the third extraction the aqueous phase was mixed with 0.2 volume of 10 M ammonium acetate followed by addition of 2 volumes of absolute ethanol. After 15 min at -70°C, precipitated DNA was dried and resuspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA), and DNA concentrations were determined from the absorbance at 260 nm. Two micrograms of DNA from each sample was separated on 1.5% agarose gels in Tris-phosphate electrophoresis buffer (pH 8.0) containing 0.5 mg/ml ethidium bromide.

Statistical analysis. Statistical analysis was performed with a commercially available software, GraphPad Prism (GraphPad, San Diego, CA, U.S.A.).

RESULTS

Apoptosis in MES cells induced by catechols

The MiCK assay quantifies apoptosis from the slope of the steep OD increase in cultures. This steep OD increase has been shown to be due to augmented side light scattering from the accumulation of morphologically apoptotic cells in the cultures (Kravtsov and Fabian, 1996; Kravtsov et al., 1998, 1999). The maximal rate of increase in the slope of OD (V_{\max}) is used to calculate KU of apoptosis after subtracting the V_{\max} of control cultures using the following formula: $KU = [V_{\max(\text{treated})} - V_{\max(\text{control})}] \times 60 \times 0.03 / (OD_{\text{cell}} - OD_{\text{blank}})$ (Kravtsov et al., 1998). As a real-time kinetic test, the MiCK assay provides continuous monitoring of apoptosis in nondisturbed cell cultures over extended intervals. Therefore, as opposed to end-point assays of apoptosis, the MiCK assay enables both quantification and accurate timing of apoptosis. In particular, the MiCK assay allows for determination of the time at which a maximal proportion of morphologically apoptotic cells can be expected in the culture (time to the maximal response, or T_m) (Kravtsov et al., 1998). Representative results of the MiCK assay of apoptosis in MES cells exposed to 5, 25, or 50 μ M dopamine are shown in Fig. 2. The gradual OD increase in control cultures is explained by cell proliferation resulting in an increased cellularity. Exposure of MES cells to 25 and 50 μ M dopamine produced a steep increase in OD followed by an OD plateau (Fig. 2). At 25 and 50 μ M dopamine, the T_m was found to be ~19 and 14 h, respectively. The calculated apoptotic responses of MES cells to 25 and 50 μ M dopamine exposure were 1.2 and 1.9 KU, respectively. No change in V_{\max} or KU was observed in MES cell cultures exposed to 5 μ M dopamine; however, a deleterious effect from this concentration of dopamine was suggested by a decreased growth rate of the cells as compared with the control cultures (Fig. 2). No change in V_{\max} or KU was observed in MES cell cultures exposed to up to 50 μ M DOPAC (data not shown).

To confirm observations made with the MiCK assay, cells were exposed to 0, 5, 25, and 50 μM dopamine and studied for both morphological evidence of apoptosis in Giemsa-stained preparations (Fig. 3A and B) and for DNA fragmentation (Fig. 3C). Under the conditions tested, <2% of cells had features of necrosis as determined by morphological analysis. The percentage of morphologically apoptotic MES cells was significantly different among the cultures exposed to 0, 5, 25, and 50 μM dopamine ($p < 0.01$ by ANOVA). No significant difference was detected in Giemsa-stained MES cultures exposed to 5 μM dopamine ($5.20 \pm 0.51\%$ apoptotic cells) compared with controls ($7.31 \pm 0.67\%$ apoptotic cells). However, exposure of MES cells to 25 and 50 μM dopamine resulted in 20.72 ± 2.01 and $30.31 \pm 3.54\%$ apoptotic cells, respectively. Repeated-pairs analyses with Bonferroni's correction showed significant differences between controls and cells exposed to 25 or 50 μM dopamine ($p < 0.05$).

Results from the MiCK assay expressed in KU were compared in the same culture with results from the measurement of apoptosis by morphological analysis of cytospin preparations stained with Giemsa. The relationship between KU and the percentage of apoptosis for MES cells exposed to various concentrations of dopamine defined the nomogram in Fig. 4, inset ($R^2 = 0.970$). It is noteworthy that T_m does not factor into the calculation of apoptotic cells. Based on this relationship, dopamine-induced apoptosis as determined by the MiCK assay was quantified on additional six MES cultures (Fig. 4). One-way ANOVA of the MiCK assay data for all exposure groups had $p < 0.01$; post test repeated-paired analyses with Bonferroni's correction had $p < 0.05$ for 25 and 50 μM dopamine versus control. These results are in agreement with other measures of the concentration-response relationship for dopamine-induced apoptosis in dopaminergic neurons, including our earlier study using flow cytometry (Simantov et al., 1996; Zhang et al., 1998).

DNA fragmentation is a relatively late event of apoptosis (Collins et al., 1997; Messam and Pittman, 1998), with maximal DNA fragmentation typically occurring 4–8 h after the maximal percentage of apoptotic cells is determined in the MiCK assay (Kravtsov et al., 1999). In this study, DNA was extracted after a 24-h exposure of the cells to 5, 25, and 50 μM dopamine. The characteristic ladderlike pattern of DNA fragmentation was evident in cells exposed to 25 and 50 μM dopamine (Fig. 3C).

Apoptosis in MES cells induced by catechol thioethers

The above experiments used the well-established model of dopamine-induced neurotoxicity and therefore served to validate the MiCK assay as a means of quantifying neurotoxicity in MES cells. Moreover, data demonstrating the usefulness of the MiCK assay in detecting apoptosis in other cell lines (Kravtsov et al., 1998), the concurrence of our previous flow cytometric studies of

dopamine-induced apoptosis in MES cells (Zhang et al., 1998) with the MiCK assay results reported above, and the confirmatory morphological and DNA laddering analyses in MES cells all indicate that under these conditions the MiCK assay was detecting primarily apoptotic MES cells. Therefore, we used the MiCK assay to quantify apoptosis induced by the six catechol thioethers in the mercapturic acid pathway, viz., glutathionyl, cysteinyl, and mercapturil thioethers of dopamine and DOPAC, as well as the homocysteinyl catechol thioethers of dopamine and DOPAC. The homocysteinyl conjugates of dopamine and DOPAC were synthesized because they lack an *N*-acetyl group yet do not cyclize to form benzothiazine species. The chemical structure of each was verified by mass spectrometry as previously described. MES cells were incubated with each catechol thioether at 5 μM for 24 h and monitored by MiCK assay. One-way ANOVA indicated that there was no significant increase in the percentage of apoptotic cells when MES cultures were incubated with any of these catechol thioethers alone. The extent of apoptosis induced by dopamine and its derivatives at 5 μM was as follows: control, $6.21 \pm 0.23\%$; dopamine, $5.20 \pm 0.51\%$; GSH-dopamine, $7.70 \pm 1.37\%$; Cys-dopamine, $7.27 \pm 0.57\%$; AcCys-dopamine, $5.41 \pm 0.88\%$; and hCys-dopamine, $8.35 \pm 1.73\%$. The extent of apoptosis induced by DOPAC and its derivatives at 5 μM was as follows: control, $6.88 \pm 0.33\%$; DOPAC, $8.67 \pm 1.37\%$; GSH-DOPAC, $8.64 \pm 0.78\%$; Cys-DOPAC, $8.81 \pm 1.17\%$; AcCys-DOPAC, $7.93 \pm 0.90\%$; and hCys-DOPAC, $7.39 \pm 1.23\%$.

We next tested the hypothesis that catechol thioethers may contribute to dopaminergic neurodegeneration under pathological conditions. To model a pathological state, MES cells were coincubated with concentrations of dopamine that induce low-level apoptosis (25 μM) plus a catechol thioether (5 μM) for 24 h (Fig. 5). Under these conditions, apoptosis in MES cultures was enhanced significantly by some, but not all, of the catechol thioethers. One-way ANOVA for the eight catechol thioethers plus dopamine was statistically significant ($p < 0.001$). Post test paired analyses with Bonferroni's correction showed that, of the eight catechol thioethers, only AcCys-dopamine, hCys-dopamine, and hCys-DOPAC when added with 25 μM dopamine produced a significant increase compared with dopamine alone ($p < 0.01$ for each paired comparison). No significant changes in the T_m were noted when cells were exposed to combinations of dopamine and any of catechol thioethers as compared with dopamine alone (data not shown). Additional experiments demonstrated that AcCys-dopamine, hCys-dopamine, or hCys-DOPAC at 5 μM and subcytotoxic concentrations of dopamine (5 μM) did not yield significant apoptosis in MES cells, suggesting that existing significant stress to MES cells was necessary for AcCys-dopamine to display toxic activity.

Although the mechanisms by which AcCys-dopamine, hCys-dopamine, and hCys-DOPAC enhanced the apoptosis induced by dopamine are not known, it is possible

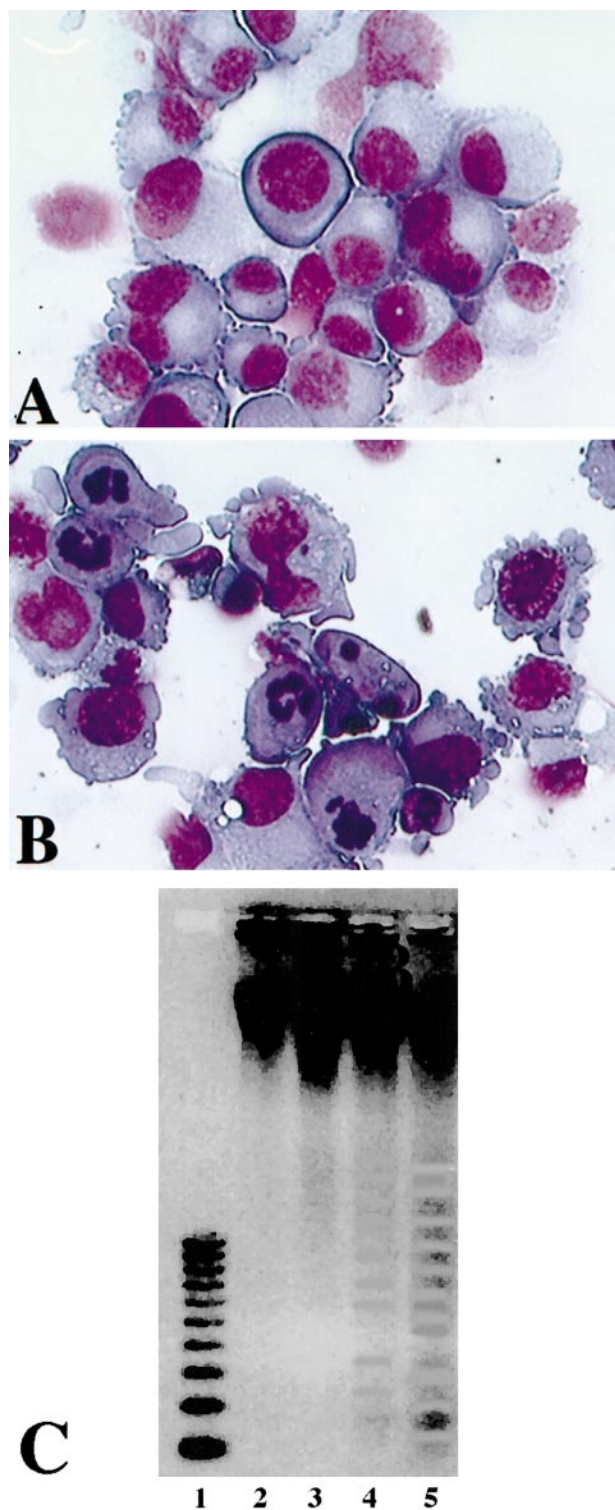


FIG. 3. Characterization of apoptosis of MES cells. **A:** MES cells treated with vehicle. **B:** MES cells treated with dopamine at 25 μM for 19 h, i.e., at T_m as determined by MiCK assay. Cells undergoing apoptosis demonstrated blebbing and condensation of the chromatin. Cells in both A and B were stained with Giemsa. **C:** DNA fragmentation in MES cells. Cells were exposed to increasing doses of dopamine for 24 h, and DNA was isolated and loaded onto a 1.8% agarose gel containing 0.1 $\mu\text{g}/\text{ml}$

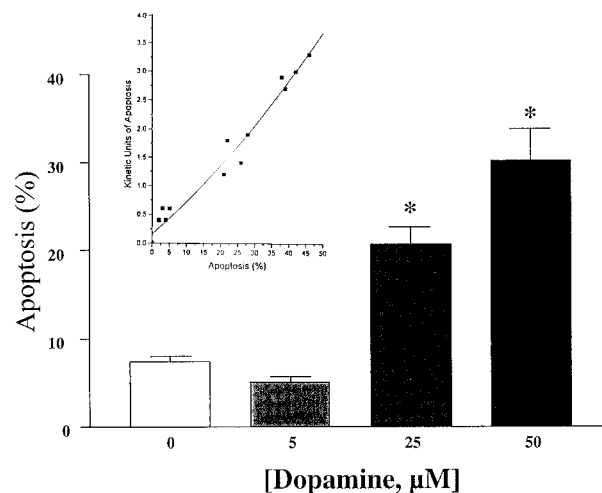


FIG. 4. Quantitative assessment of apoptosis. **Inset:** Relationship between KU of apoptosis and percentage of apoptotic cells. In brief, cells were exposed to increasing doses of dopamine and studied in both the MiCK assay and Giemsa-stained cytopsin preparations at the appropriate time when T_m was detected by MiCK assay. Data are mean \pm SE (bars) percentages of apoptotic cells calculated from the results of the MiCK assay ($n = 6$ for each data point). * $p < 0.05$ for control versus dopamine at 25 or 50 μM .

that these compounds autoxidize in culture medium more rapidly than other catechol thioethers and thereby generate more oxidative stress. Therefore, the half-lives of 5 μM AcCys-dopamine, AcCys-DOPAC, hCys-dopamine, and hCys-DOPAC in culture medium were determined with and without 25 μM dopamine. The results showed that the half-lives for these catechol thioethers were between 2 and 4 h, not significantly different from those of other catechol thioethers (Montine et al., 1997). Moreover, the half-lives of these compounds were not altered by the presence of dopamine.

DISCUSSION

Pathological and biochemical studies have consistently associated endogenous catechol oxidation with dopaminergic neurodegeneration in PD, but the mechanisms are not known. Recent studies have suggested that products of endogenous catechol oxidation, the catechol thioethers, may contribute to the neurodegeneration associated with catechol oxidation; however, there are limited data on the neurotoxicity of endogenous catechol thioethers, and no data exist on the dopaminergic neurotoxicity of this class of molecules. Here we have tested the major endogenous catechol thioethers for their ability to induce apoptosis in a dopaminergic neuronal cell line.

ethidium bromide after treatment with RNase A. Electrophoresis was carried out in 1 mM EDTA in 45 mM Tris-borate buffer (pH 7.8). Lanes 1–5 represent DNA markers and DNA isolated from cells treated with 0, 5, 25, and 50 μM dopamine, respectively.

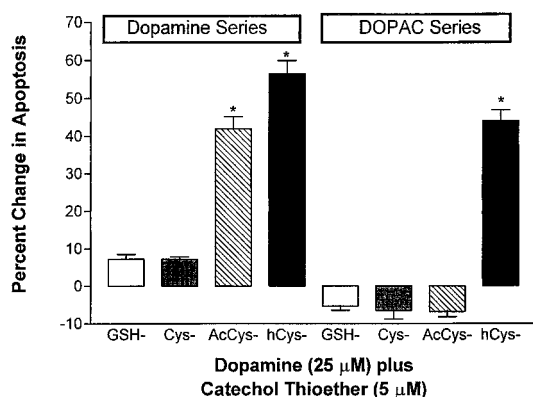


FIG. 5. Catechol thioethers and dopamine toxicity. MES cells were coincubated with dopamine at 25 μ M alone or dopamine at 25 μ M plus a catechol thioether at 5 μ M for 24 h. Data are mean \pm SE (bars) percentages of apoptosis induced by dopamine plus a catechol thioether over dopamine alone ($n = 4$ for each data point). Data not included in the graph are percentages of apoptosis induced by dopamine at 25 μ M alone ($17.63 \pm 0.71\%$). * $p < 0.05$ for control versus dopamine at 25 μ M plus AcCys-dopamine, hCys-dopamine, or hCys-DOPAC at 5 μ M. None of the other catechol thioethers produced changes that were significantly different from controls.

In addition to testing already identified endogenous glutathionyl and cysteinyl catechol thioethers, we included the mercapturates of dopamine and DOPAC because the entire mercapturate acid pathway is present in brain. We also included hCys-dopamine and hCys-DOPAC because they, like AcCys-dopamine, do not cyclize to generate benzothiazine species, a class of putative endogenous neurotoxins in PD. Our results showed that, in contrast to dopamine, none of the endogenous catechol thioethers alone is a dopaminergic neurotoxin at concentrations that may be expected to occur in vivo. However, the mercapturate of dopamine was unique in this series because it significantly enhanced dopamine-induced apoptosis. Moreover, it appeared that the activity of AcCys-dopamine may be related to its inability to cyclize because both hCys-dopamine and hCys-DOPAC were as effective as AcCys-dopamine in enhancing dopamine-induced apoptosis.

These experiments used MES cells, a hybrid cell line derived from rat mesencephalic neurons and a neuroblastoma cell line (Crawford et al., 1992). MES cells display several characteristics of dopaminergic neurons, including (a) tyrosine hydroxylase activity, (b) synthesis and vesicular storage of dopamine but not other catecholamines, and (c) the presence of NMDA receptors. Moreover, we have shown recently that MES cells are sensitive to dopamine-induced apoptosis as determined by flow cytometry, an effect blocked by antagonists of the dopamine transporter (Zhang et al., 1998). Indeed, the results from these earlier flow cytometric studies showing dopamine-induced apoptosis in MES cells are in excellent agreement with the results from the MiCK assay reported here. A limitation of this model system is that MES cells are a homogeneous population, and so

hypotheses concerning specificity for dopaminergic neurons versus other neuron types cannot be tested. Another inherent limitation of studying catechol thioethers is their ease of oxidation in biological systems. In fact, our results indicate that, given the half-lives of the catechol thioethers in culture medium, virtually all of the starting material has been oxidized during the incubation period. Although this oxidation may be related to the toxicity of these molecules, it is entirely likely that new metabolites are formed during incubation and that these may be the ultimate toxins. Therefore, the structure–function study reported here can only address which parent molecules set in motion a series of poorly clarified events that culminate in neurotoxic activity.

The extracellular concentrations of dopamine necessary to achieve significant neurodegeneration in these assays are higher than what is thought to exist in vivo under basal conditions, but it is in the range of extracellular dopamine concentrations that exist under at least some pathological conditions (Globus et al., 1987; Gerlach and Riederer, 1996). The concentrations of catechol thioethers generated under these conditions have not been completely characterized but are only a fraction of dopamine. The concentrations of glutathionyl and cysteinyl catechol thioethers are up to 3% of total dopamine (Fornstedt et al., 1986; Spencer et al., 1998); however, conditions that increase extravesicular dopamine also increase the amount of dopamine converted to Cys-dopamine (Hastings et al., 1996a,b). Moreover, none of these estimates takes into account the possible contribution of AcCys-dopamine used in these experiments (20% of extracellular dopamine) may be a reasonable reflection of what might be expected in vivo, especially under conditions that increase extravesicular dopamine. Finally, although the concentrations of catechol thioethers used in these experiments are at the upper limit of what might be expected to occur under some pathological conditions, they are 100-fold lower than the concentrations of catechol thioethers used in previously published tests of their neurotoxicity (Shen et al., 1996; Li and Dryhurst, 1997; Li et al., 1998).

Thioethers of endogenous brain catechols have significantly lower oxidation potentials than their parent catechols (Shen and Dryhurst, 1996; Picklo et al., 1999). Nonetheless, our previous studies have shown that oxidation potential does not accurately predict the ability of this class of catechol thioethers to produce oxidative damage in vitro under physiological conditions (Picklo et al., 1999). These studies demonstrated that of the endogenous brain catechol thioethers, the mercapturate conjugates were the most potent at producing paramagnetic metal-ion catalyzed oxidative damage to DNA and that AcCys-dopamine and AcCys-DOPAC produced similar amounts of metal-catalyzed oxidative damage in vitro (Picklo et al., 1999). Here we have shown that AcCys-dopamine was much more potent than AcCys-DOPAC at augmenting dopamine-induced apoptosis. These results suggest that enhanced oxidative stress from

exposure to AcCys-dopamine alone cannot account for the proapoptotic activity of this molecule. The basis for the divergence of results in vitro and in MES cells for AcCys-dopamine and AcCys-DOPAC is not understood. It is possible that these molecules have different membrane transport properties or are compartmentalized differently in cells. Alternatively, these molecules may be metabolized differently in cells, and this may modify neurotoxic activity. Finally, AcCys-dopamine and AcCys-DOPAC may have different effects on dopamine metabolism and thereby alter dopamine-induced neurotoxicity.

A central finding of this study is that, based on the results with hCys-dopamine and hCys-DOPAC, the activity of AcCys-dopamine may be attributable to its inability to cyclize to form benzothiazines. The role of cyclized products from catechol thioethers, the benzothiazines, is a complex issue in the cytotoxicity of this class of molecules (Shen et al., 1997). The well-studied example of 2-bromohydroquinone nephrotoxicity suggests that benzothiazine formation is a detoxifying step in the kidney (Monks and Lau, 1992). Similarly, our data showing that homocysteinyl analogues retained proapoptotic effects suggest that cyclization of the cysteinyl conjugates, which occurs rapidly under physiological conditions (Shen et al., 1997), also is detoxifying in dopaminergic neuronal cultures. Of note is that these data from a cell culture system that applied the catechol thioethers extracellularly cannot exclude the possibility that intracellular cyclization of cysteinyl catechols generates neurotoxins. Nevertheless, despite the limitations of this model system, out data with AcCys-dopamine and the homocysteinyl analogues indicate that the noncyclized catechol thioethers also may contribute to dopaminergic neurotoxicity at relatively low concentrations.

In summary, we confirmed that dopamine induced dose-dependent apoptosis in dopaminergic neurons at concentrations that may be achieved under pathological conditions. Acting alone, none of the eight catechol thioethers examined was neurotoxic. However, when combined with dopamine to simulate pathological states of excess extravesicular dopamine, only the mercapturate of dopamine and homocysteinyl analogues significantly increased dopaminergic neurodegeneration. These results are the first to demonstrate neurotoxic activity of catechol thioethers in dopaminergic cells and suggest that the combination of dopamine thioether formation with mercapturate metabolism may produce endogenous molecules that facilitate dopaminergic neurodegeneration.

Acknowledgment: This work was supported by grants ES05842 (to J.Z.), ES05826 (to M.J.P.), ES02611 (to D.G.G.), and AG16835 and AG00774 (to T.J.M.) from the National Institutes of Health.

REFERENCES

Alam Z. I., Daniel S. E., Lees A. J., Marsden D. C., Jenner P., and Halliwell B. (1997a) A generalised increase in protein carbonyls

- in the brain in Parkinson's but not incidental Lewy body disease. *J. Neurochem.* **69**, 1326–1329.
- Alam Z. I., Jenner P., Daniel S. E., Lees A. J., Cairns N., Marsden D. C., Jenner P., and Halliwell B. (1997b) Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. *J. Neurochem.* **69**, 1196–1203.
- Ben-Shachar D., Zuk R., and Glinka Y. (1995) Dopamine neurotoxicity: inhibition of mitochondrial respiration. *J. Neurochem.* **64**, 718–723.
- Cohen G. (1983) The pathobiology of Parkinson's disease: biochemical aspects of dopamine neuron senescence. *J. Neural Transm. Suppl.* **19**, 89–103.
- Collins J. A., Schandi C. A., Young K. K., Vesely J., and Willingham M. C. (1997) Major DNA fragmentation is a late event in apoptosis. *J. Histochem. Cytochem.* **45**, 923–934.
- Crawford G. C., Le W., Smith R. G., Xie W. J., Stefani E., and Appel S. H. (1992) A novel N18TG2×mesencephalon cell hybrid expresses properties that suggest a dopaminergic cell line of substantia nigra origin. *J. Neurosci.* **12**, 3392–3398.
- Dexter D. T., Wells F. R., Lees A. J., Agid F., Agid Y., Jenner P., and Marsden D. C. (1989) Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. *J. Neurochem.* **52**, 1830–1836.
- Duvoisin R. C. and Sage J. (1996) *Parkinson's Disease*. Lippincott-Raven, Philadelphia.
- Fornstedt B. and Carlsson A. (1991) Effects of inhibition of monoamine oxidase on the levels of 5-S-cysteinyl adducts of catechols in dopaminergic regions of the brain of the guinea pig. *Neuropharmacology* **30**, 463–468.
- Fornstedt B., Rosengren E., and Carlsson A. (1986) Occurrence and distribution of 5-S-cysteinyl derivatives of dopamine, dopa and dopac in the brains of eight mammalian species. *Neuropharmacology* **25**, 451–454.
- Fornstedt B., Brun A., Rosengren E., and Carlsson A. (1989) The apparent autooxidation rate of catechols in dopamine-rich regions of human brains increases with the degree of depigmentation of substantia nigra. *J. Neural Transm. Parkinsons Dis. Dement. Sect.* **1**, 279–295.
- Fornstedt B., Bergh I., Rosengren E., and Carlsson A. (1990a) An improved HPLC-electrochemical detection method for measuring brain levels of 5-S-cysteinyl dopamine, 5-S-cysteinyl-3,4-dihydroxyphenylalanine, and 5-S-cysteinyl-3,4-dihydroxyphenylacetic acid. *J. Neurochem.* **54**, 578–586.
- Fornstedt B., Pileblad E., and Carlsson A. (1990b) In vivo autooxidation of dopamine in guinea pig striatum increases with age. *J. Neurochem.* **55**, 655–659.
- Gerlach M. and Riederer P. (1996) Animal models of Parkinson's disease: an empirical comparison with the phenomenology of the disease in man. *J. Neural Transm.* **103**, 987–1041.
- Globus M. Y., Ginsberg M. D., Harik S. I., Busto R., and Dietrich W. D. (1987) Role of dopamine in ischemic striatal injury: metabolic evidence. *Neurology* **37**, 1712–1719.
- Graham D. G. (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol. Pharmacol.* **14**, 633–643.
- Graham D. G., Tiffany S. M., Bell W. R. Jr., and Gutknecht W. F. (1978) Autooxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Mol. Pharmacol.* **14**, 644–653.
- Hastings T. G. and Zigmond M. J. (1994) Identification of catechol-protein conjugates in neostriatal slices incubated with [³H]dopamine: impact of ascorbic acid and glutathione. *J. Neurochem.* **63**, 1126–1132.
- Hastings T. G., Lewis D. A., and Zigmond M. J. (1996a) Reactive dopamine metabolites and neurotoxicity: implications for Parkinson's disease. *Adv. Exp. Med. Biol.* **387**, 97–106.
- Hastings T. G., Lewis D. A., and Zigmond M. J. (1996b) Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. *Proc. Natl. Acad. Sci. USA* **93**, 1956–1961.

- Kravtsov V. D. and Fabian I. (1996) Automated monitoring of apoptosis in suspension cell cultures. *Lab. Invest.* **74**, 557–570.
- Kravtsov V. D., Greer J. P., Whitlock J. A., and Koury M. J. (1998) Use of the microculture kinetic assay of apoptosis to determine chemosensitivities of leukemias. *Blood* **92**, 968–980.
- Kravtsov V. D., Daniel T. O., and Koury M. J. (1999) Comparative analysis of different methodologic approaches to the in vitro studying of drug-induced apoptosis. *Am. J. Pathol.* **155**, 1327–1339.
- Le W. D., Colom L. V., Xie W. J., Smith R. G., Alexianu M., and Appel S. H. (1995) Cell death induced by beta-amyloid 1–40 in MES 23.5 hybrid clone: the role of nitric oxide and NMDA-gated channel activation leading to apoptosis. *Brain Res.* **686**, 49–60.
- Li H. and Dryhurst G. (1997) Irreversible inhibition of mitochondrial complex I by 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (DHBT-1): a putative nigral endotoxin of relevance to Parkinson's disease. *J. Neurochem.* **69**, 1530–1541.
- Li H., Shen X. M., and Dryhurst G. (1998) Brain mitochondria catalyze the oxidation of 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (DHBT-1) to intermediates that irreversibly inhibit complex I and scavenge glutathione: potential relevance to the pathogenesis of Parkinson's disease. *J. Neurochem.* **71**, 2049–2062.
- Lowe J., Graham L., and Leigh P. N. (1997) Disorders of movement and system degeneration, in *Greenfield's Neuropathology: Vol. II* (Graham D. I. and Lantos P. L., eds), pp. 281–366. Arnold, London.
- Messam C. and Pittman R. (1998) Asynchrony and commitment to die during apoptosis. *Exp. Cell Res.* **238**, 389–398.
- Miller R. T., Lau S. S., and Monks T. J. (1995) Metabolism of 5-(glutathion-S-yl)-alpha-methyldopamine following intracerebroventricular administration to male Sprague-Dawley rats. *Chem. Res. Toxicol.* **8**, 634–641.
- Monks T. J. and Lau S. S. (1992) Toxicology of quinone-thioethers. *Crit. Rev. Toxicol.* **22**, 243–270.
- Montine T. J., Picklo M. J., Amarnath V., Whetsell W. O., and Graham D. G. (1997) Neurotoxicity of endogenous cysteinylcatechols. *Exp. Neurol.* **148**, 26–33.
- Parkinson A. (1996) Biotransformation of xenobiotics, in *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Klaassen C. D., ed), pp. 113–186. McGraw-Hill, New York.
- Perry T. L., Godin D. V., and Hansen S. (1982) Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci. Lett.* **33**, 305–310.
- Picklo M. J., Amarnath V., Graham D. G., and Montine T. J. (1999) Endogenous catechol thioethers may be pro-oxidant or anti-oxidant. *Free Radic. Biol. Med.* **27**, 271–277.
- Shen X. M. and Dryhurst G. (1996) Further insights into the influence of L-cysteine on the oxidation chemistry of dopamine: reaction pathways of potential relevance to Parkinson's disease. *Chem. Res. Toxicol.* **9**, 751–763.
- Shen X. M., Xia B., Wrona M. Z., and Dryhurst G. (1996) Synthesis, redox properties, in vivo formation, and neurobehavioral effects of N-acetylcysteinyl conjugates of dopamine: possible metabolites of relevance to Parkinson's disease. *Chem. Res. Toxicol.* **9**, 1117–1126.
- Shen X. M., Zhang F., and Dryhurst G. (1997) Oxidation of dopamine in the presence of cysteine: characterization of new toxic products. *Chem. Res. Toxicol.* **10**, 147–155.
- Sian J., Dexter D. T., Lees A. J., Daniel S., Jenner P., and Marsden C. D. (1994) Glutathione-related enzymes in brain in Parkinson's disease. *Anal. Neurol.* **36**, 356–361.
- Simantov R., Blinder E., Ratovitski T., Tauber M., Gabbay M., and Porat S. (1996) Dopamine-induced apoptosis in human neuronal cells: inhibition by nucleic acids antisense to the dopamine transporter. *Neuroscience* **74**, 39–50.
- Spencer J. P., Jenner P., Daniel S. E., Lees A. J., Marsden D. C., and Halliwell B. (1998) Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: possible mechanisms of formation involving reactive oxygen species. *J. Neurochem.* **71**, 2112–2122.
- Tse D. C., McCreery R. L., and Adams R. N. (1976) Potential oxidative pathways of brain catecholamines. *J. Med. Chem.* **19**, 37–40.
- Wang W. and Ballatori N. (1998) Endogenous glutathione conjugates: occurrence and biological functions. *Pharmacol. Rev.* **50**, 335–356.
- Yoritaka A., Hattori N., Uchida K., Tanaka M., Stadtman E. R., and Mizuno Y. (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc. Natl. Acad. Sci. USA* **93**, 2696–2701.
- Zhang J., Price J. O., Graham D. G., and Montine T. J. (1998) Secondary excitotoxicity contributes to dopamine-induced apoptosis of dopaminergic neuronal cultures. *Biochem. Biophys. Res. Commun.* **248**, 812–816.
- Zhang J., Perry P., Smith M. A., Robertson D., Olson S. J., Graham D. G., and Montine T. J. (1999) Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *Am. J. Pathol.* **154**, 1423–1429.