Ziram Causes Dopaminergic Cell Damage by Inhibiting E1 Ligase of the Proteasome

Received for publication, March 20, 2008, and in revised form, September 24, 2008. Published, JBC Papers in Press, September 25, 2008, DOI 10.1074/jbc.M802210200

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The etiology of Parkinson disease (PD) is unclear but may involve environmental toxins such as pesticides leading to dysfunction of the ubiquitin proteasome system (UPS). Here, we measured the relative toxicity of ziram (a UPS inhibitor) and analogs to dopaminergic neurons and examined the mechanism of cell death. UPS (26 S) activity was measured in cell lines after exposure to ziram and related compounds. Dimethyl- and diethylthiodithiocarbamates including ziram were potent UPS inhibitors. Primary ventral mesencephalic cultures were exposed to ziram, and cell toxicity was assessed by staining for tyrosine hydroxylase (TH) and NeuN antigen. Ziram caused a preferential damage to TH+ neurons and elevated α-synuclein levels but did not increase aggregate formation. Mechanistically, ziram altered UPS function through interfering with the targeting of substrates by inhibiting ubiquitin E1 ligase. Sodium dimethyldithiocarbamate administered to mice for 2 weeks resulted in persistent motor deficits and a mild reduction in striatal TH staining but no nigral cell loss. These results demonstrate that ziram causes selective dopaminergic cell damage in vitro by inhibiting an important degradative pathway implicated in the etiology of PD. Chronic exposure to widely used dithiocarbamate fungicides may contribute to the development of PD, and elucidation of its mechanism would identify a new potential therapeutic target.

Parkinson disease (PD) is a common neurodegenerative disease characterized by relatively selective degeneration of dopaminergic (DA) neurons in the substantia nigra (nigrostriatal neurons). The etiology probably involves both environmental and genetic factors including pesticide exposure (1–3). Hundreds of pesticides are used alone or in combinations making it difficult to separate individual effects. Because no individual pesticide has been established by epidemiologic studies, we chose to perform an unbiased screen of potential toxicants for their ability to interfere with the ubiquitin-proteasome system (UPS), a biological pathway implicated in the etiology of PD. Impaired UPS activity has been reported in the brains of patients with PD, and mutations in two UPS genes, Parkin and UCHL-1, cause rare genetic forms of PD (4). Although these results are not universally reproduced (5–7), in some studies administration of UPS inhibitors to rodents recapitulates many of the clinical and pathological aspects of PD (8–10). We hypothesized that chronic pesticide exposure may increase the risk of developing PD by inhibiting the UPS. We screened several pesticides for their ability to inhibit the UPS and found a number of toxicants that can lower activity at relevant concentrations (11). We then focused on dithiocarbamate fungicides because they were found to be one of the most potent UPS inhibitors and are widely used in crop protection.

In the present study, zinc dimethyldithiocarbamate (ziram) was one of several dimethyl- and diethylthiodithiocarbamates found to inhibit the UPS at 0.15–1 μM. Furthermore, ziram increased α-synuclein expression in DA cells, induced relatively selective DA cell damage in vitro, and inhibited the UPS by interfering with ubiquitin E1 ligase activity. In vivo, systemic administration of the more soluble sodium dimethyldithiocarbamate (NaDMDC) in mice resulted in motor deficits and damage to the nigrostriatal pathway. These findings help explain how chronic pesticide exposure could increase the risk of developing PD.

EXPERIMENTAL PROCEDURES

Chemicals—The test compounds (see Table 1) were from Chem Service (West Chester, PA), Sigma-Aldrich, or other commercial sources as the highest available purity, except for 7 and 8, which were synthesized by Karl Fisher in the Casida laboratory.

Measurement of 26 S Proteasome Activity and Cell Death in Cell Lines—26 and 20 S UPS activity and cell death were measured in human embryonic kidney (HEK) and neuroblastoma SK-N-MC cells by flow cytometry as previously described (12).
Fluorescence of the green fluorescent protein degron fusion protein (GFP-U) was measured and expressed as a percentage of control.

**Rat Primary Ventral Mesencephalic Cultures (VMC)—**VMCs were prepared by using a protocol adapted from Rayport et al. (13). Briefly, the cultures were prepared in two stages. In the first stage, cortical glial feeder cells were established on polystyrene/laminin-coated coverslips, which formed the base of a 10-mm-diameter well cut into 35-mm culture dishes, until they reached confluence in ~6 days. Fluorodeoxyuridine was then added to prevent additional glial proliferation. In the second stage, postnatal day 2 or 3 pups were anesthetized, and 1 mm3 mesencephalic blocks containing the ventral tegmental area were dissected from sagittal sections taken along the midline of the brain. The cells were dissociated and plated onto pre-established glial feeder cells at densities of 1 x 10^5/coverslip. The mixed cultures were grown in chemically defined media containing fluorodeoxyuridine for 10 days before treatment and analysis.

**Immunocytochemistry in VMCs and Cell Counts—**At the conclusion of the experiment, the cultures were immediately washed and fixed in 4% paraformaldehyde for 30 min. The cultures were then incubated with anti-tyrosine hydroxylase (TH) antibodies (1:500; Calbiochem) and anti-NeuN antibodies (1:100; Chemicon) overnight at 4 °C. The cells were stained for 2 h with fluorescein isothiocyanate- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). In some experiments, the cultures were stained with anti-TH and anti-α-synuclein (1:500; Zymed Laboratories Inc.) antibodies. After staining and prior to counting, the coverslips were randomly assigned an identification number, and the experiments were blinded to the experimental conditions. For TH, the entire coverslip was counted but for NeuN—VMCs were subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes and incubated with an anti-α-synuclein antibody (BD Transduction Laboratories; 1:1000) followed by an anti-tubulin antibody for normalization.

**Evaluation of E1 Ligase Activity—**The effects of ziram on E1 ligase activity were investigated using Western blot analysis of treated cellular extracts to determine E1/E1-ubiquitin ratios and using purified enzyme preparations. Ziram-treated SK-N-MC cells were washed with phosphate-buffered saline and then lysed in a thiol stabilizing buffer using the method of Jha et al. (14). The samples were sonicated for 10 s, centrifuged for 15 min at 13,000 x g, mixed with 2 parts thiol gel buffer (33 mM Tris-HCl, pH 6.8, 2.7 mM urea, 2.5% sodium dodecyl sulfate, and 13% glycerol), and boiled for 2 min, and 10 μg of protein/lane was loaded on a 12% SDS-PAGE gel (15). The proteins were electrophoretically transblotted onto nitrocellulose paper, and immunoblots were performed as previously described (16) using anti-E1 ligase antibody (BIOMOL, Plymouth Meeting, PA). Antigen-antibody interactions for immunoblots were visualized using horseradish peroxidase-conjugated secondary antibody and chemiluminesence substrate (Pierce).

For purified enzyme assays, human recombinant ubiquitin-activating E1 enzyme and biotinylated ubiquitin (both from BIOMOL) were incubated for 5 or 10 min in thiolester buffer as per the manufacturer’s protocol. The reactions were stopped using thiol-stabilizing buffer, and the proteins were subjected to SDS-PAGE and transblotted onto nitrocellulose paper. E1-ubiquitin conjugates were determined using streptavidin–horseradish peroxidase and an enhanced chemiluminescence kit. Band densities were measured using a scanning densitometer.

**Animal Studies—**Male C57BL/6 mice were treated for 2 weeks by subcutaneous osmotic minipumps with NaDMDC 50 mg/kg/day in phosphate-buffered saline or with phosphate-buffered saline only. The behavior of the mice was evaluated using the pole test as described by Fleming et al. (17) except that a cut-off time of 60 s was used. One week after the last behavioral testing, the mice were perfused with fixative, and their brains were sectioned for TH staining. Fiber density was measured with a computer-assisted image analysis system as previously described (18).
The SNc was delineated at 5× objective using previously reported criteria (19, 20). After delineation at low magnification, every fourth section throughout the SNc was counted as appropriate. For histochemistry and behavior, statistical analysis was performed with GB-Stat software (Dynamic Microsystems, Inc., Silver Spring, MD, 2000) for Macintosh. A 2×3 mixed design ANOVA was followed by post hoc analysis with Fisher’s least significant difference. To maintain homogeneity of variance, an inverse transform was calculated (21) for each score in a mixed design ANOVA. Ziram Causes Dopaminergic Cell Damage in Primary VMC—Because proteasome inhibition has been implicated in the etiology of PD and ziram causes UPS dysfunction, dopaminergic cells might be selectively vulnerable to the toxicity of ziram. Primary VMC were exposed to ziram for 48 h, and then cellular toxicity was measured by counting TH immuno-positive (TH+) cells and NeuN immuno-positive (NeuN+) cells (a nonspecific neuronal marker). Ziram at 5 and 10 μM was highly toxic to all cells, including the glial bed, causing the cell layers to lift off the coverslip after treatment or during immunostaining.
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| Compound (μM) | TH+ | NeuN+ | TH+/NeuN+ |
|---------------|-----|-------|-----------|
| Ziram         |     |       |           |
| control      | 100±7 | 100±4 | 1.00±0.05 |
| 0.1          | 111±22 | 100±15 | 0.93±0.11 |
| 0.25         | 76±7 | 81±5 | 1.00±0.07 |
| 0.5          | 60±10* | 75±11 | 0.78±0.09* |
| 1            | 59±10* | 81±11 | 0.64±0.07** |
| Lactacystin  |     |       |           |
| control      | 100±7 | 100±4 | 1.00±0.05 |
| 1            | 69±7 | 84±4 | 1.18±0.10 |
| 2            | 84±11 | 81±6 | 1.12±0.14 |
| 5            | 79±15 | 71±6 | 1.19±0.24 |
| 10           | 54±6* | 50±7** | 1.24±0.11 |

* p < 0.05; ** p < 0.01

FIGURE 2. Ziram- and lactacystin-induced tyrosine hydroxylase cell damage in primary mesencephalic cultures. TH+ neurons were selectively vulnerable to ziram but not lactacystin-induced damage. Shown below are representative photomicrographs of TH- (red) and NeuN-stained (green) mesencephalic cultures. A, control. B, ziram (1 μM).

Ziram Toxicity Is Not Dopamine-dependent—One possible mechanism for the relative selective effect to TH+ neurons is that ziram interacts with dopamine metabolism to produce preferential toxicity. To test this hypothesis, ziram toxicity was measured in the presence of α-methyl l-tyrosine, an inhibitor of dopamine synthesis. Treatment of VMSCs with α-methyl l-tyrosine (250 μM) for 48 h resulted in a decrease in dopamine content of 62 ± 6% compared with controls (p ≤ 0.05) but did not significantly alter the number of TH+ cells. Reducing dopamine content with α-methyl l-tyrosine was ineffective in attenuating the toxicity of ziram to TH+ neurons (Fig. 3).

α-Synuclein Expression in VMSCs—Several converging lines of evidence support a role for α-synuclein in the pathogenesis of PD. Mutations in the α-synuclein gene or increased expression of wild-type α-synuclein cause PD in familial PD, and α-synuclein is a major component of Lewy bodies in sporadic PD (22). To determine whether ziram alters the levels of α-synuclein in VMSCs, the neurons were stained for both TH and α-synuclein and relative fluorescence was measured using a blinded manner in cells that were selected randomly (n = 21–97 cells/condition). Representative α-synuclein-stained cells are shown on the right. Scale bars, 10 μm. **, p ≤ 0.01.

An important pathological marker in PD is the formation of α-synuclein inclusions or aggregates. TH+ cells from ziram- and lactacystin-treated VMSCs were determined to be either positive or negative for nuclear, perinuclear, or cytoplasmic α-synuclein inclusions by blinded raters. Aggregates were relatively common in both treated and untreated cells, but surprisingly no significant differences were found with ziram compared with controls. Interestingly, a decrease in nuclear and perinuclear aggregates was seen in the lactacystin-treated cultures (Table 2).

To determine whether ziram treatment results in increased formation of detergent-soluble α-synuclein oligomer formation, we subjected VMSC lysates to Western blot analysis. Both monomeric and oligomeric forms of α-synuclein were apparent...
in detergent-soluble fractions as previously described (25). Ziram treatment resulted in a nonsignificant trend for an increase in oligomeric forms of α-synuclein compared with controls (170 ± 120% optical density units of controls, n = 8 for ziram and n = 5 for controls, p = 0.18). Oligomeric α-synuclein was unchanged in lactacystin-treated VMCs, and monomeric α-synuclein levels were similar in all three conditions (data not shown).

**Ziram Inhibits E1 Ligase Activity**—Ziram was found to inhibit the UPS using an assay that requires the substrate (i.e. degron) to be ubiquitylated via ubiquitin ligases and recognized by the 26 S proteasome before it can be degraded by 20 S proteases (12). Disruption of any of these steps would be detected in the screen. It has been suggested that another dithiocarbamate fungicide, maneb, inhibits the 20 S component of the UPS (26). To determine whether ziram acted in this manner, HEK cells were treated with ziram (1 and 10 μM) for 24 h, but there was no change in 20 S chymotryptic activity (data not shown). The amount of α-subunit of the 20 S proteasome was also measured using Western blot analysis, and no differences were found (data not shown). Furthermore, our earlier study showed that ziram had no effect on 20 S UPS activity when added directly to cell lysate (11). Because 20 S proteolytic activity was not altered by ziram, Western blot analysis on lysates was performed from ziram-treated cells to determine whether ubiquitinated proteins accumulated. As expected, inhibition of the 20 S UPS by lactacystin and rotenone resulted in the accumulation of high molecular weight ubiquitin conjugates (Fig. 5). Conversely, ziram treatment resulted in a significant increase in oligomeric forms of α-synuclein compared with controls (27). Ziram does not appear to act by lowering GSH oxidation (27), and GSH depletion results in the loss of E1 ligases (E1, E2, and E3 ligases). Dithiocarbamates can lead to the formation of high molecular weight ubiquitin conjugates and the reduced formation of E1-ubiquitin conjugates in a purified preparation (Fig. 6).

**DISCUSSION**

Pesticides, Ziram, and PD—The most consistent and strongest association between a group of environmental toxicants and the development of sporadic PD has been with chronic pesticide exposure, although no specific agents have been identified.
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(3). Preliminary data from our population-based study in central California that determined pesticide exposure using a state application registry has revealed some intriguing results. Subjects living within 500 meters of where ziram was applied were at an over 3-fold higher risk of developing PD compared with those with lower exposure.\(^3\) Chronic inhibition of the UPS has been implicated in the pathogenesis of PD, and some pesticides might increase the risk of developing PD by causing UPS dysfunction. The widely used pesticide ziram is one of the most potent inhibitors of 26 S UPS (11). This study further demonstrates that ziram kills TH\(^+\) cells in a relatively selective manner, increases α-synuclein levels, and inhibits El ligase activity, thus interfering with the targeting of proteins destined for UPS degradation. If these preliminary epidemiology findings are confirmed, and taken together with the results of this study, chronic ziram exposure would be a strong candidate as a PD-associated toxicant. The results presented here add a biologically plausible mechanism (at relevant concentrations) by which ziram may increase the risk of developing the disease.

**Mechanism of Ziram Effect on the UPS**—Uncovering how ziram causes UPS dysfunction and cell death might provide important clues to the selective vulnerability of DA neurons. Some of the results in primary cultures were surprising. Although ziram damaged (and probably killed) TH\(^+\) cells in a selective manner, this was not the case for the 20 S proteasome inhibitor lactacystin. Even though TH staining was used as a marker of DA cell survival, it is possible that the cells were still alive but simply lost their TH phenotype. However, this is unlikely because TH levels were actually increased in the remaining cells after ziram exposure (but not for lactacystin). Conflicting results on proteasome inhibitor-induced selective DA cell death may be due to differences in culturing techniques and conditions (28–32). Cultures in the current study contained DA neurons of the ventral tegmental region to increase the number of TH\(^+\) cells/well and power analysis. Because ventral tegmental region neurons are believed to be more resistant to stressors, the results likely underestimate the effects of ziram on SNc DA neurons. Indeed, pilot experiments using predominantly nigral cultures yielded similar results. The fact that different effects were found with ziram compared with

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**TABLE 3**

| Region          | Mean ± S.E. (n = 5) | 2W - Control | Treated | 2 + 9W - Control | Treated |
|-----------------|---------------------|--------------|---------|------------------|---------|
| TH-IR fiber density |                     |              |         |                  |         |
| DL striatum     | 1515 ± 207          | 1456 ± 221   | 1934 ± 299 | 1951 ± 269     |         |
| DM striatum     | 1048 ± 149          | 843 ± 117    | 1435 ± 199 | 1358 ± 130     |         |
| VL striatum     | 1439 ± 142          | 1290 ± 140   | 1833 ± 254 | 1409 ± 166*    |         |
| VM striatum     | 815 ± 103           | 696 ± 62     | 1073 ± 174 | 946 ± 152      |         |
| SNc             | 7980 ± 491          | 7832 ± 574   | 8251 ± 214 | 7897 ± 247     |         |

*\(p \leq 0.05\) compared with corresponding region in control mice at the same time point; ANOVA followed by Fisher PLSD.

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**FIGURE 6.** Effect of ziram on E1-ubiquitin conjugates and E1 ligase activity. A, endogenous E1-ubiquitin conjugates in lysates of ziram-treated HEK cells. Ziram resulted in a dose-dependent reduction in the E1-Ub/E1 ratio compared with untreated controls. The Western blot is shown in the inset. B, E1 ligase activity was inhibited by ziram in purified preparations after 5 and 10 min of incubation (\(n = 4\), \(p \leq 0.05\)).

**FIGURE 7.** Motor deficits in mice determined by the pole test following 2 weeks of subcutaneous minipump treatment with NaDMDC at 50 mg/kg/day followed by an 8-week post-treatment period (10 weeks from the beginning of the experiment). Inverse Transform; reciprocal of the original data point. Baseline, Because untreated mice turn in less than 1 s. **, \(p < 0.01\) compared with saline-treated mice at the same time point. ΔΔ represents \(p < 0.01\) compared with a base line within the same treatment group (2 × 3 Mixed design ANOVA, Fisher’s LSD).

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\(^3\) B. Ritz, J. M. Bronstein, and S. Costello, unpublished data.
lactacystin suggests that they act through different mechanisms. As opposed to lactacystin, a 20 S protease inhibitor, we show that ziram acts upstream by interfering with ubiquitin ligation. Because ubiquitylation is also important in many cellular processes in addition to the UPS, including modification of protein function, facilitation of cell surface receptor turnover, and control of gene transcription, it is possible that some of the actions of ziram may be via alternative pathways (33).

The molecular basis of the ability of ziram to inhibit E1 ligase activity was studied by determining the relative potencies of several of its analogs. The most potent 26 S UPS inhibitors were dimethyl- and diethylthiocarbamates and their salts and disulfides. These compounds may act by copper or iron chelation (34), or undergo oxidative activation to the S-oxides of the dithiocarbamic acids (35) or of the S-methyl thioether carbamates (36), which are reactive with GSH and potentially with a thiol group of the UPS E1 ligase (Fig. 8).

**Maneb and Ethylenebisdithiocarbamate Fungicides**—The effect of ziram on UPS activity has not been observed by others, but the structurally related fungicide maneb is reported to inhibit 20 S protease activity (26). Although ziram and maneb may have many structural features in common, our results with ziram differ significantly from those of Zhou et al. (26) using maneb. In our studies, ziram did not inhibit the 20 S proteasome at concentrations up to 10 μM, did not cause oxidative stress as measured by 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate fluorescence (11), and did not induce α-synuclein aggregates in primary cultures. These observations may be due to differences in the compounds and/or cell assays used. Maneb is an ethylenebis(dithiocarbamate) and contains manganese, which possibly could contribute to some of its effects, but manganese by itself did not alter 26 S UPS activity (data not shown).

Another difference between the effects of maneb and ziram is that maneb is reported to induce α-synuclein aggregates in a rat embryonic mesencephalon murine neuroblastoma-glioma hybrid cell line (26). The present study did not find an increase in aggregate formation in primary mesencephalic cultures but did not evaluate aggregates in immortalized cell lines because neither HEK or SK-N-MC cells expressed significant levels. Several lines of evidence support a mechanism for ziram upstream of the 20 S: inhibition of the formation of high molecular weight ubiquitin conjugates, reduction of the E1-ubiquitin/E1 ratio, and direct inhibition of purified E1 ligase activity. Ziram effects on E2 or E3 ligase activities were not studied because they are dependent on E1 ligase, but because they transfer ubiquitin in a manner similar to that of E1, it is possible that ziram would have similar inhibitory effects on these enzymes.

**Animal Studies on Dithiocarbamate Fungicides**—Further support for the potential role of dithiocarbamates in PD comes from animal studies. Chronic maneb exposure given with paraquat recapitulates many of the behavioral and pathological hallmarks of PD (37). Two week subcutaneous minipump treatment with NaDMDC at 50 mg/kg/day resulted in persistent motor abnormalities typically seen in mice with dysfunction of the nigrostriatal pathway (38). Abnormal motor behavior in our study was associated with a mild, delayed dopamine nerve terminal damage in the ventrolateral region of the striatum but not with nigral cell death. Motor dysfunction without dopamine cell loss may indicate functional damage to these neurons, but we cannot exclude the possibility that the behavioral tests were detecting abnormalities in other areas involved in motor control.

In summary, we show that ziram and structurally related dithiocarbamates induce dysfunction of the UPS by inhibiting E1 ligase. We demonstrate that ziram exposure increases α-synuclein levels and selectively damages dopaminergic neurons in primary cultures. Furthermore, chronic systemic exposure to NaDMDC causes persistent motor deficits and mild injury to the nigrostriatal pathway. This study, along with emerging epidemiological investigations, suggests that chronic exposure to dithiocarbamate fungicides can contribute to the pathogenesis of PD.

**Acknowledgment**—We thank Dr. Sheila Fleming for help with the statistical analysis.

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DECEMBER 12, 2008 • VOLUME 283 • NUMBER 50
JOURNAL OF BIOLOGICAL CHEMISTRY • 34703