Cadmium is a potent cell poison known to cause oxidative stress by increasing lipid peroxidation and/or by changing intracellular glutathione levels and to affect the ubiquitin/ATP-dependent proteolytic pathway. However, the cellular mechanisms involved in cadmium toxicity are still not well understood, especially in neuronal cells. To investigate the relationship between cadmium-induced oxidative stress and the ubiquitin/ATP-dependent pathway, we treated cultures of neuronal cells with different concentrations of the metal ion. In addition to decreases in glutathione levels, we observed marked increases in protein-mixed disulfides (Pr-SSGs) after exposure of HT4 cells (a mouse neuronal cell line) or rat primary mesencephalic cultures to Cd²⁺. The increases in intracellular levels of Pr-SSGs were concordant with increases in the levels of ubiquitinated proteins (Ub proteins) when the HT4 cells were subjected to lower (25 µM or less) concentrations of cadmium. However, higher concentrations of cadmium (50 µM), which were toxic, led to increases in Pr-SSGs but inhibited ubiquitination, probably reflecting inhibition of ubiquitinating enzymes. The cadmium-induced changes in Pr-SSGs and Ub proteins were not affected when more than 85% of intracellular glutathione was removed from the cells by the glutathione synthetase inhibitor l-buthionine-(S,R)-sulfoximine. However, the reducing agent dithiothreitol, which prevented the build up of Pr-SSGs in the cell, also blocked the accumulation of Ub proteins induced by cadmium. In addition, dithiothreitol blocked the effects of the higher toxic (50 µM) concentrations of cadmium on cytotoxicity and on glutathione, Pr-SSGs, and Ub proteins. Together, these results strongly suggest that changes in the levels of intracellular Pr-SSGs and ubiquitin-protein conjugates in neuronal cells are responses closely associated with the disruption of intracellular sulfhydryl homeostasis caused by cadmium-mediated oxidative stress.

One of the hallmarks of neurodegeneration is the appearance of intraneuronal inclusions consisting of ubiquitin-protein conjugates (1, 2). The mechanisms generating such abnormal inclusions remain unknown. Ubiquitination of proteins occurs posttranslationally and is a complex ATP-dependent process in which ubiquitin is sequentially activated, transferred to ubiquitin-conjugating enzymes, and then ligated to protein substrates (reviewed in Ref. 3). Very often, more than one ubiquitin is attached to the target proteins, forming polyubiquitin chains (4). Ubiquitin can be removed from the ubiquitin-protein conjugates by deubiquitinating enzymes (5).

Covalent binding of ubiquitin to proteins in the cytosol and in the nucleus is frequently viewed as a means by which proteins are marked for subsequent degradation by the ubiquitin/ATP-dependent proteasome, commonly known as the 26 S proteasome (reviewed in Refs. 6 and 7). In general, ubiquitinated proteins (Ub proteins) do not accumulate in healthy cells. They are rapidly degraded by the 26 S proteasome (reviewed in Ref. 7). The failure to eliminate the ubiquitin-protein deposits in the degenerating neurons may result either from a malfunction of the ubiquitin/ATP-dependent proteolytic pathway or from structural changes in the protein substrates rendering them inaccessible to the proteolytic machinery. The accumulation of Ub proteins can then lead to proteotoxicity.

Oxidative stress is one of the mechanisms that contributes to structural changes or misfolding of proteins. Substantial evidence has accumulated showing that oxidative stress may play an important role in neurodegeneration (reviewed in Refs. 8–10). The reactive oxygen species resulting from episodes of oxidative stress promote the modification of cellular proteins (11). Cells possess a protective mechanism to overcome the potentially toxic accumulation of oxidatively modified proteins, namely an increase in proteolysis (12, 13). More recently, Davies and co-workers (14) demonstrated that oxidative stress in cultured liver epithelial cells led to measurable changes in intracellular proteolysis. The degradation of oxidatively modified proteins was postulated to occur via ubiquitin-independent and ATP-independent mechanisms (15, 16).

On the other hand, studies with yeast showed that overexpression of the polyubiquitin gene conferred resistance to oxidative stress in cells grown by respiration (17). In addition, Taylor and co-workers (18–20) detected significant increases in ubiquitin-protein conjugates, ubiquitin-activating and ubiquitin-conjugating enzyme activity, and intracellular proteolysis in lens epithelial cells recovering from episodes of oxidative stress induced by H₂O₂. Together, these studies suggest that the ubiquitin/ATP-dependent proteolytic system may play a role in the removal of oxidatively modified proteins.

To further investigate the mechanisms underlying the removal of oxidatively modified proteins in mammalian cells, we chose to induce oxidative stress with cadmium in a neuronal cell line (HT4)
and in rat mesencephalic primary cultures. Cadmium is a potent cell poison known to cause oxidative stress (reviewed in Ref. 21) and to affect the ubiquitin/ATP-dependent proteolytic pathway (22, 23). Our results show that the heavy metal decreased intracellular glutathione concentrations and increased the levels of protein-mixed disulfides (Pr-SSGs) and of ubiquitin-protein conjugates in a time- and concentration-dependent manner. In addition, we demonstrate that only a small pool of glutathione (less than 15% of the total) is sufficient to produce significant increases in Pr-SSGs levels in response to cadmium. Most importantly, we show that the thioredoxin-reducing agent dithiothreitol blocks the increases in Pr-SSGs and Ub protein levels produced by cadmium, indicating that one of the mechanisms responsible for cadmium toxicity is the perturbation of intracellular sulfhydryl homeostasis.

**EXPERIMENTAL PROCEDURES**

**Cells Cultures—**HT4 cells were derived from a mouse neuroblastoma cell line containing a recombinant temperature-sensitive mutant of SV40 large T antigen. When grown at 39 °C (nonpermissive temperature), HT4 cells differentiate with neuronal morphology, express neuronal antigen s, synthesize and secrete nerve growth factor, and express receptors for nerve growth factor (24) and for glutamate (25). The cells were maintained at 33 °C in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO2. To induce differentiation, the temperature was changed to 39 °C, at which the cells were kept for 3 days. Following the period of differentiation, the cells were maintained at 37 °C, at which they were kept for at least 7 h prior to treatment with the heavy metal.

Cultures of embryonic rat mesencephalon were prepared as described in Mytilineou et al. (26). Briefly, on day 14 of gestation, the mesencephalon was surgically removed, and the cells were dissociated mechanically and plated at a density of 100,000 cells/cm2 on polyornithine-coated 35-mm tissue culture dishes.

**Glutathione Assay—**Total glutathione was quantified as described previously (26) by a modification of the standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) in the presence of glutathione reductase and NADPH. The assay measures both GSH and GSSG. Typically, GSH constitutes less than 5% of the total glutathione in control cell cultures. The medium was first aspirated, and then the cells were rinsed twice with PBS and harvested in 500 μl of a solution of 0.04 M HCl in isopropanol was added and gently shaken to dissolve the precipitated dye. The solution was transferred into 1.5-ml microcentrifuge tubes and centrifuged at 16,000 × g for 5 min, and the absorbance of the supernatant was read at 550 and 620 nm with a plate reader (ATOC model 540; SLT Laboratory Instruments, Hillsborough, NC). The results were expressed as the difference between the values obtained at the two wavelengths.

**Preparation of Cell Extracts for Western Blotting—**Following the indicated treatments, cell extracts were prepared as described previously (26). Proteins were separated by SDS-polyacrylamide gel electrophoresis (following the method of Laemmli (30) on 8% gels for ubiquitin-protein conjugate detection. Identification of the Ub proteins was by Western blotting, and the antigens were visualized by a horseradish peroxidase method (Bio-Rad) utilizing the substrate 3,3’,5,5’-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Quantitative analysis of the immunostaining was by image analysis (as described previously (26)).

**Antibodies—**Ubiquitin-protein conjugates were detected with a rabbit polyclonal antibody (1:600) against ubiquitin conjugated to γ-globulins with glutaraldehyde, obtained from Dako Corp. (Carpinteria, CA).

**Protein Determination—**Protein determination was by a bichinchoninic acid assay kit (Pierce) and by the method of Lowry et al. (32) using bovine serum albumin as a standard.

**Statistical Analysis—**Statistical comparisons were performed with the Tukey-Kramer multiple comparison test (Instat 2.0, Graphpad Software, San Diego, CA).

**RESULTS**

**Cadmium Induces a Time- and Dose-dependent Decrease in Intracellular Glutathione—**To determine the time and concentration dependence of the effect of the metal on the intracellular levels of glutathione, we treated confluent differentiated HT4 cells with a range of concentrations between 1 and 100 μM CdSO4. After incubations for 1–8 h, the cells were harvested, and intracellular concentrations of glutathione were measured as described under “Experimental Procedures.” The results are presented in Table I and represent the mean ± SE of three experiments. Time course of the changes in glutathione levels measured after treatment with 10, 25, and 50 μM CdSO4 is shown in Fig. 2A. It shows that the decrease in glutathione induced by all three concentrations of the metal ion achieved statistical significance after 2 or 4 h of treatment. At the highest concentration tested in these experiments (50 μM), the glutathione level was decreased to almost zero after 8 h of treatment.

**Cadmium Induces a Time- and Dose-dependent Increase in the Levels of Protein-Mixed Disulfides in HT4 Cells—**To determine the time and concentration dependence of the effect of cadmium (10 μM) on intracellular levels of protein-mixed disulfides by de-
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Fig. 1. Cadmium decreases glutathione levels in a time-dependent (A) and dose-dependent (B) fashion in HT4 cells. Intracellular glutathione levels were determined as described under “Experimental Procedures.” Data represent the mean and S.E. from at least eight determinations in A and six determinations in B. All levels are expressed relative to no cadmium treatment, which corresponds to an average of 28.9 ± 2.6 nmol of glutathione/mg of protein (100%). The cadmium concentrations (A) were as follows: ■, 10 μM; ○, 25 μM; ◆, 50 μM. The times of incubation (B) were as follows: △, 1 h; ▼, 4 h. The * identifies the values that are significantly different from control, with p < 0.05 or less for all values marked.

Fig. 2. Cadmium increases Pr-SSG levels in a time-dependent (A) and dose-dependent (B) fashion in HT4 cells. Intracellular Pr-SSGs levels were determined as described under “Experimental Procedures.” Data represent the mean and S.E. from at least eight determinations in A and six determinations in B. The intracellular levels of Pr-SSGs were expressed as pmol of Pr-SSGs/mg of protein. The cadmium concentrations (A) were as follows: ■, 10 μM; ○, 25 μM; ◆, 50 μM. The times of incubation (B) were as follows: △, 1 h; ▼, 4 h. The * identifies the values that are significantly different from control, with p < 0.05 or less for all values marked.

Cadmium Induces Changes in Glutathione and Protein-Mixed Disulfides in Primary Cultures of Fetal Rat Mesencephalon—To establish that the Cd2⁺ effect was not restricted to transformed neuronal cells, such as the HT4 cell line, we studied the effect of the heavy metal on primary cultures of embryonic rat mesencephalon. As with HT4 cells, incubations for 1 h with all Cd²⁺ concentrations tested led to no statistically significant changes in the cellular levels of glutathione in the mesencephalic cultures (Fig. 3A). However, longer (4 h) incubations with the divalent metal induced significant changes in the cellular glutathione levels. The lowest concentration of CdSO₄ tested (5 μM) produced a transient increase in intracellular glutathione, but the highest concentration tested (100 μM) caused a drop to 45% of control levels (Fig. 3A).

The dose-dependent elevation in Pr-SSGs induced by the heavy metal in the mesencephalic cultures (Fig. 3B) was parallel to but not as great as that detected in HT4 cells. After 4 h of treatment, the highest concentration tested (100 μM) induced an 8-fold increase in Pr-SSGs in the HT4 cells as compared with controls, but only a 4-fold increase was observed in the treated mesencephalic cultures (compare Figs. 2B and 3B).

Cadmium Decreases HT4 Cell Viability—The time-dependent cytotoxicity of 10, 25, and 50 μM cadmium was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Fig. 4). Treatment with 10 and 25 μM Cd²⁺ was not cytotoxic up to 8 and 6 h of incubation, respectively. However, there was a sharp drop in viability in cells treated for 8 h with 25 μM heavy metal. Treatment with 50 μM Cd²⁺ significantly reduced cell viability even after 1 h of incubation (Fig. 4).

Cadmium Induces a Time- and Dose-dependent Change in the Levels of Ubiquitin-Protein Conjugates in HT4 Cells—The cadmium-induced thiolation of protein-SH groups leading to the formation of Pr-SSGs may provoke the misfolding of proteins, which may then be targeted for degradation by the ubiquitin/ATP-dependent proteolytic pathway. Therefore, we examined whether cadmium ions led to an accumulation of Ub proteins in the treated cells. As shown in Figs. 5 and 6, the levels of ubiquitin conjugates were significantly increased after 1 and 2 h of treatment with 25 and 10 μM CdSO₄, respectively. The greatest levels (4.3-fold of control) were detected after 8 h of exposure to 10 μM heavy metal. The highest dose of cadmium tested (50 μM) caused no detectable increases in the accumulation of Ub proteins. In fact, the levels of ubiquitin-protein conjugates in cells treated with 50 μM Cd²⁺ for 6 and 8 h were significantly below the control levels (Figs. 5 and 6). The latter immunoblot was overstained to show this decrease (Fig. 5, right panel).

Comparison of the Effects of Glutathione Depletion and Protein Thiol Reduction on the Intracellular Changes Induced by Cadmium—Glutathione is considered the most important intracellular thiol involved in the formation of protein-mixed disulfides. Therefore, we determined whether depletion of intracellular GSH would interfere with the cadmium-induced
formation of protein-mixed disulfides. HT4 cells were incubated with 5 μM glutathione synthetase inhibitor L-buthionine-(S,R)-sulfoximine (L-BSO) for 24 h followed by an additional 4 h of incubation with fresh reagent, preceding the cadmium treatment. Under these conditions, the concentrations of glutathione (20–30 nmol/mg of protein), as well as those of Pr-SSGs (0.1–0.2 nmol of GSH equivalent/mg of protein), were reduced to 14 and 30% of control levels, respectively (Fig. 7A). In contrast, the increase in the levels of Pr-SSG resulting from treatment with cadmium was not blocked by depletion of more than 85% of glutathione (Fig. 7A). Total depletion of intracellular glutathione was never accomplished, even when the cells were treated with higher concentrations of L-BSO for longer periods of time (results not shown). Therefore, the data demonstrate that the residual 3–4 nmol of glutathione/mg of protein left in the cell are sufficient for the formation of Pr-SSGs in response to the cadmium treatment. The cadmium-induced accumulation of Ub proteins was also not affected by the glutathione deficiency (Fig. 7A).

When, in separate experiments, all of the intracellular glutathione was removed by subsequent addition of N-ethylmaleimide (60 μM for 1 h), no Pr-SSGs were formed in response to cadmium (not shown).

One of the mechanisms involved in protein S-thiolation is the oxidation of sulfhydryl groups of cysteine residues in proteins by glutathione disulfide (GSSG). The protein-bound glutathione (Pr-SSG) should be reduced by dithiothreitol (DTT), an effective reducing agent useful in the study of thiol-disulfide exchange reactions. To determine whether there was an association between Pr-SSG formation and the accumulation of ubiquitinated proteins, we incubated cells with DTT (1 mM). Exposure of the neuronal cells to the reducing agent alone significantly (p < 0.05) decreased the control levels of Pr-SSGs, without affecting the control levels of glutathione and Ub proteins. However, addition of the reducing agent to cadmium-
As noted earlier, incubations with 50 μM DTT—significantly different from control, with p < 0.05 or less for all values marked. Data represent the mean and S.E. of three identical experiments for each condition tested. The * identifies the values that are significantly different from control, with p < 0.05 or less for all values marked.

Prevention of the Cytotoxic Effect of Cadmium by the Thiol-reducing Agent DTT—As noted earlier, incubations with 50 μM cadmium significantly diminished cell viability (Fig. 4) and suppressed Ub proteins (Figs. 5 and 6). To determine whether one of the mechanisms involved in cadmium toxicity is the oxidation of protein thiolis, we attempted to block the toxic effect of 50 μM cadmium by treating cells with increasing concentrations of DTT.

As shown in Fig. 8, concentrations of the reducing agent up to 1 mM prevented the decreases in glutathione (Fig. 8A) and Ub proteins (Fig. 8C) and the increases in Pr-SSGs (Fig. 8D) observed in the presence of 50 μM Cd^{2+}. Nevertheless, Pr-SSG was still increased by 37% over control under these experimental conditions.

The highest DTT concentration tested (10 mM) was as effective as 1 mM in reversing the decrement in glutathione (Fig. 8A). However, it was less effective than the lower concentration in blocking the changes in the other three parameters tested, namely Pr-SSGs, Ub proteins, and cytotoxicity, perhaps due to its inhibition of protein synthesis (34).

**DISCUSSION**

Cadmium accumulates in humans throughout their lives because of its very long half-life (35). The heavy metal is a substantial industrial and environmental pollutant that seriously injures a variety of organs, such as the brain, liver, testis, and kidneys (for a review, see Ref. 36). Recent studies demonstrated that cadmium toxicity was mediated by the oxidative damage of essential cellular macromolecules (reviewed in Ref. 21). For example, the heavy metal was shown to increase lipid peroxidation in the brain, an organ particularly sensitive to cadmium toxicity (37), and in hepatocytes and testicular Leydig cells (38, 39). In addition, Cd^{2+} increased cellular levels of hydrogen peroxide in Leydig cells (39) and inhibited SOD in the liver and kidneys (40). Cadmium ions were also shown to cause changes in intracellular glutathione concentrations and to induce the synthesis of metallothioneins, cysteine-rich proteins that avidly bind the metal ion (reviewed in Ref. 21).

The cellular mechanisms involved in cadmium toxicity are still not well understood. The heavy metal interacts with thiol groups of proteins with a greater affinity than Zn^{2+} and may therefore disrupt the structure of certain cellular proteins (41). In addition, Cd^{2+} forms complexes with reduced glutathione (GSH), binding mostly to the sulphydryl group of the cysteinyli moiety (42). The heavy metal may therefore contribute to an imbalance of the sulphydryl homeostasis in the cell.

To test this hypothesis, we investigated the effect of cadmium on a mouse neuronal cell line (HT4 cells) and on rat mesencephalic primary cultures. Our studies are the first to show that cadmium induced a time- (up to 8 h) and dose-dependent increase in protein-mixed disulfides reflecting decreases in glutathione. Many other studies with nonneuronal
cells report similar decreases in glutathione (reviewed in Ref. 21) but did not measure protein S-thiolation.

To explain our findings we propose the following mechanism (Scheme 1) mediating cadmium action.

$$\text{Cd}^{2+} + \text{GSH} \rightarrow \text{GSSG}$$

$$\text{GSSG} + \text{Pr-SH} \rightarrow \text{Pr-SSG} + \text{GSH}$$

**SCHEME 1**

Although Cd$^{2+}$ does not by itself facilitate the aerobic oxidation of GSH in solution at neutral pH,$^2$ it induced oxidation of GSH to GSSG within cells by a yet unidentified mechanism. Cadmium may therefore stimulate other intracellular events leading to the oxidation of GSH to GSSG, which in turn promotes the oxidation of protein thiol groups.

A second possibility is that cadmium induces intracellular oxidation of GSH or protein thiols to thyl radicals (43), which may in turn generate mixed disulfides.

$$\text{GSH or Pr-SH} \rightarrow \text{Cd}^{2+} \rightarrow \text{GS- or Pr-S} \rightarrow \text{Pr-SSG}$$

**SCHEME 2**

The cadmium-induced elevations in Pr-SSGs were not blocked by depletion of more than 85% of glutathione by the glutathione synthetase inhibitor (L-BSO), suggesting that less than 15% of intracellular GSH can sustain significant increases in Pr-SSGs. This small cellular pool of GSH consistently failed to be depleted by the L-BSO and cadmium treatment.

The effect of cadmium could also be mediated by a third mechanism, described in Scheme 3. The divalent metal may form complexes directly with the thiol groups of proteins, leading to oxidized aggregates, such as Pr-SS-Pr (disulfide-linked proteins).

$$\text{Pr-SH} \rightarrow \text{Cd}^{2+} \rightarrow \text{Pr-SS-Pr}$$

**SCHEME 3**

Both Pr-SSG and Pr-SS-Pr products were identified in cells treated with iodoacetamide, an alkylating reagent known to decrease glutathione levels and to induce oxidative stress (34).

Our study also shows that decreases in glutathione and accumulation of Pr-SSGs were detected in cadmium-treated mesencephalic cultures. These primary cultures contain neurons and glial cells, possibly explaining why the changes may not be as great as those observed in the pure neuronal HT4 cell cultures. For example, astroglia cells were shown to tolerate low levels of lead exposure, which could be toxic to neuronal cells (44).

In addition to producing increments in Pr-SSGs, we found that cadmium has a biphasic effect on the ubiquitin/ATP-dependent proteolytic pathway. Although low concentrations of Cd$^{2+}$ (25 $\mu$M or less) increase the intracellular levels of Ub proteins, higher concentrations (50 $\mu$M or more) have the opposite effect. The accumulation of Ub proteins observed in the presence of low cadmium concentrations could result from: (i) a direct inhibition of the activity of the 26 S proteasome; (ii) an overload of the ubiquitin/ATP-dependent pathway due to an increase production of structurally damaged proteins, such as the Pr-SSGs or Pr-SS-Pr; and/or (iii) inhibition of the activity of deubiquitinating enzymes. The latter are thiol isopeptidases (45), and the essential active site sulphydryl groups may be come oxidized in the presence of Cd$^{2+}$.

Higher concentrations of cadmium (50 $\mu$M) decreased the intracellular levels of Ub proteins. This result may be explained by a decline in the catalytic activities of the ubiquitinating enzymes, including ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes. These enzymes contain sulphydryl groups in the active sites (reviewed in Ref. 46) and may be oxidized in the presence of high cadmium concentrations. This hypothesis is supported by recent studies demonstrating that ubiquitin-activating and ubiquitin-conjugating enzymes are inactivated by S-thiolation under conditions of oxidative stress induced by exposure to hydrogen peroxide (19, 20). In addition, others have shown that in yeast, expression of the ubiquitin-conjugating enzymes and of the poly ubiquitin gene is highly increased after exposure to 100 $\mu$M cadmium for 30 min and that strains defective in proteasome activity are more susceptible to cadmium toxicity (22). However, ubiquitin overexpression did not increase yeast tolerance to cadmium toxicity (47).

Two effects of cadmium discussed above, namely increases in Pr-SSGs and Ub proteins, could be reversed by the thiol-reducing agent DTT (Fig. 7). These results conclusively show that the heavy metal perturbs the thiol-disulfide redox status of intracellular proteins.

In summary, cadmium induces the loss of glutathione, the oxidation of protein thiols to Pr-SSGs, and the accumulation of ubiquitinated proteins in the neuronal cells. These effects can be reversed by a thiol-reducing agent, indicating that they result from perturbations of the thiol-disulfide redox status of intracellular proteins.

The formation of Pr-SSGs appears to be linked to inactivation of the Ub/ATP-dependent pathway, which leads to the accumulation of ubiquitinated proteins. This general mechanism may reflect cellular responses to other agents that promote the modification of the structure of intracellular proteins and inhibit the Ub/ATP-dependent pathway. Failure to overcome this inhibition may result in the proteotoxic accumulation of ubiquitinated proteins in intracellular inclusions and may lead to cell degeneration.

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