Cystamine Inhibits Caspase Activity

IMPLICATIONS FOR THE TREATMENT OF POLYGLUTAMINE DISORDERS*

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Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder caused by an abnormally expanded polyglutamine domain. There is no effective treatment for HD; however, inhibition of caspase activity or prevention of mitochondria dysfunction delays disease progression in HD mouse models. Similarly administration of cystamine, which can inhibit transglutaminase, prolonged survival of HD mice, suggesting that inhibition of transglutaminase might provide a new treatment strategy. However, it has been suggested that cystamine may inhibit other thiol-dependent enzymes in addition to transglutaminase. In this study we show that cystamine inhibits recombinant active caspase-3 in a concentration-dependent manner. At low concentrations cystamine is an uncompetitive inhibitor of caspase-3 activity, becoming a non-competitive inhibitor at higher concentrations. The IC₅₀ for cystamine-mediated inhibition of caspase-3 activity in vitro was 23.6 μM. In situ cystamine inhibited in a concentration-dependent manner the activation of caspase-3 by different pro-apoptotic agents. Additionally, cystamine inhibited caspase-3 activity to the same extent in cell lines stably overexpressing wild type tissue transglutaminase (tTG), a mutant inactive tTG, or an antisense for tTG, demonstrating that cystamine inhibits caspase activity independently of any effects it may have on the transamidating activity of tTG. Finally, treatment with cystamine resulted in a robust increase in the levels of glutathione. These findings demonstrate that cystamine may prolong neuronal survival and delay the onset of HD by inhibiting caspasess and increasing the level of antioxidants such as glutathione.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder caused by an abnormal expansion of a CAG repeat in the gene encoding for huntingtin, a protein of unknown function (1). Although, the precise mechanisms responsible for the pathogenesis of HD remain to be elucidated, there is evidence to suggest that the genetic defect in huntingtin is associated with mitochondrial dysfunction leading to an increase in oxidative stress conditions (2). Further, recent studies support a role for caspases in the progression of HD (3, 4), and aberrant protein-protein interactions related to the abnormally expanded polyglutamine stretches likely play a critical role in the etiology of HD (5, 6). Indeed, the presence of neuronal intranuclear and cytoplasmic inclusions composed of mutant huntingtin in HD brain constitutes a striking neuropathological hallmark of the disease (7, 8). However, the role of the aggregates in HD pathogenesis remains to be elucidated. Another feature of HD brain is a significant increase in tissue transglutaminase (tTG) (9, 10), and it has been hypothesized that tTG may contribute to the etiology of several neurodegenerative disorders such as HD (9).

Tissue TG (also called type 2 TG) belongs to a family of thiol-dependent transamidating enzymes that catalyze a calcium-dependent acyl transfer reaction between the γ-carboxamide group of a polypeptide-bound glutamine and the ε-amino group of a polypeptide-bound lysine residue to form an ε-(γ-glutamyl)lysine isopeptide bond (for review see Ref. 9). Transglutaminases can also catalyze the incorporation of a polypeptide leading to the formation of a γ-(γ-glutamyl)lysine isopeptide bond (11). The recent resolution of the x-ray crystallographic structure of human tTG has defined its structural and functional domains (12). Tissue TG is organized into four domains: a β-sandwich domain, followed by a transamidation catalytic core domain, and two carboxyl-terminal β-barrel domains (12, 13). The catalytic core of tTG comprises a catalytic triad constituted of the residues cysteine, histidine, and aspartic acid (Cys-277, His-335, and Asp-358 in human tTG) (14). This catalytic triad is conserved in all members of the TG family and presents remarkable similarity with the papain-like catalytic center (13, 15), suggesting a common evolutionary lineage (13), and providing the structural features suggesting that the mechanism of the TGs is similar to the reverse mechanism of the cysteine proteinases (15). Given the fact that a polypeptide-bound glutamine is the primary determining factor for a tTG-catalyzed reaction it has been suggested that tTG may contribute to the formation of huntingtin aggregates in HD (16). Indeed, tTG levels and TG activity are significantly elevated in brain area affected in HD (10, 17). These findings have raised the hypothesis that inhibition of TG may be an effective therapeutic strategy for HD. However, in a recent study we demonstrated that tTG is not necessary to the formation of mutant huntingtin aggregates (18). Further, treatment of HD mouse with the TG inhibitor cystamine did not affect the appearance or the frequency of neuronal inclusions (19). Nonetheless, cystamine treatment has beneficial effects in an HD mouse model (19). Because cystamine inhibits tTG, likely by forming a mixed disulfide, it has been suggested that, in addi-
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EXPERIMENTAL PROCEDURES

Materials—Cystamine was obtained from Sigma, the glutathione reductase (lot B43049) and MG132 were purchased from Calbiochem, and the caspase substrate AC-DEVAMC was obtained from Alexis. The purified recombinant active human caspase-3 (CPP32) (lot MO62912) and the monoclonal antibody for poly(ADP-ribose) (PARP) (PARP) were obtained from BD Pharmingen/Transduction Laboratories. The monoclonal tTG antibody TG100 was from Neomarkers. Human neuroblastoma SH-SY5Y cells were maintained in RPMI 1640 media supplemented with 20 mM glutamine, 10 units/ml penicillin, 100 μg/ml streptomycin, 5% fetal clone II serum (HyClone), and 10% horse serum. Cells were maintained in a humidified 5% CO₂ incubator with 5% CO₂. SH-SY5Y cells that stably overexpress tTG, mutated inactive tTG (C277S), or antisense tTG were described previously (22, 23). Stably transfected cells were maintained in the same media containing 100 μg/ml G418 (Geneticin). SH-SY5Y cells were plated at a density of ~10⁵ cells/60-mm dish 48 h before apoptosis-inducing treatments. To determine the effects of cystamine on caspase-3 activity, cells were placed in serum-free media and incubated in the absence of cystamine or in the presence of various concentrations of cystamine for 10 h prior to treatment with apoptotic stressors. Cells were subsequently treated with the indicated concentrations of the proteosome inhibitor MG132 for 16 h, which previously has been shown to induce apoptosis in various cell types (24, 25), or with 100 μM hydrogen peroxide for the time indicated prior to measurement of caspase-3 activation or PARP cleavage.

Cell Viability—The release of the intracellular enzyme lactate dehydrogenase (LDH) into the medium was used as a quantitative measurement of cell viability. The measurement of LDH was carried out as described previously (26). The percentage of LDH released was defined by LDH activity in the medium divided by total LDH activity.

Collection of Cell Lysates—Cells were collected in the media and spun at 2000 × g at 4°C for 10 min. Cell pellets were washed twice in ice-cold phosphate-buffered saline prior to being resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml cocktail of each of aprotonin, leupeptin, and pepstatin. The pellets were then sonicated on ice and spun at 2000 × g at 4°C for 10 min. Protein concentration of the supernatant was determined using the bicinchoninic acid assay (BCA) method (Pierce).

Immunoblotting—Cell lysates were diluted to a final concentration of 1 mg/ml in 2× reducing stop buffer (25 mM Tris-HCl (pH 7.5), 2% SDS, 25 mM dithiothreitol, 5 mM EDTA, 10% glycerol, and 0.01% bromophenol blue as tracking dye) and incubated in a boiling water bath for 5 min. Proteins were separated on 7.5 and 10% for immunoblotting and 460 nm emission.

In Vitro Caspase Activity—In situ caspase-3 activity was measured using a previously described protocol (27). In brief, 200 μl of assay buffer (20 mM Hepes, pH 7.5, 10% glycerol, and 2 mM dithiothreitol) containing the peptide substrate for caspase-3 (AC-DEVAMC) was added to 10 μg of each final concentration of of 55 μg/ml) of a 200-well clear bottom plate (Corning). Cell lysate (20 μg of protein) was added to start the reaction. Triplicate measurements were done for each sample. Background fluorescence was measured in wells containing assay buffer, substrate, and lysis buffer without the cell lysate. Assay plates were incubated at 37°C for 1 h, and fluorescence was measured on a fluorescence reader (Bio-Tek, Winooski, VT) set at 360 nm excitation and 460 nm emission.

Effect of Cystamine on MG132-induced Caspase Activation—To examine the effects of cystamine on caspase-3 activity in situ, SH-SY5Y cells were pre-incubated with or without cystamine (31.2–500 μM) for 10 h prior to incubation in the absence or presence of the proteasome inhibitor MG132 (200 nM) for 16 h. Treatment of SH-SY5Y cells with MG132 alone resulted in a significant increase in caspase-3 activity compared with cells treated with vehicle alone (Fig. 3A). The MG132-mediated increase in caspase activity was further confirmed by the caspase-dependent proteolysis of PARP (Fig. 3B).

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1. Cystamine Inhibits Caspase-3 Activity in Vitro—Cystamine is known to inhibit TG activity (29), probably by forming a mixed disulfide, raising the possibility that cystamine may react in a similar manner with other thiol-dependent enzymes such as the caspases (20). To test this hypothesis, the activity of human recombinant caspase-3 (5–20 ng) was measured in the absence or presence of various concentrations of cystamine (0–500 μM) (Fig. 1, A and B). Under these experimental conditions, recombinant caspase-3 in the absence of cystamine exhibited a concentration-dependent increase in activity (Fig. 1A) and the caspase-3-mediated cleavage of AC-DEVAMC curve was within a linear range (r = 0.97) over a time course of at least 60 min (Fig. 1B). To examine the effects of cystamine the activity was measured using different amounts of recombinant active caspase-3 (5–20 ng) incubated in the presence of increasing concentrations of cystamine (31.2–500 μM). The results from a typical experiment are shown in Fig. 1 and revealed that cystamine inhibited caspase-3 activity in a concentration-dependent manner (Fig. 1, A and B). Caspase-3 activity was significantly decreased (~60%) in the presence of 31.2 μM cystamine and almost completely inhibited in the presence of 500 μM cystamine (Fig. 1, A and B). The IC₅₀ for cystamine-mediated inhibition of caspase-3 activity was 23.6 μM. A Lineweaver-Burk analysis (Fig. 2) demonstrated that cystamine is an uncompetitive inhibitor of caspase-3 activity, with a Kᵣ of 38.5 μM; however, at the highest concentration (500 μM) cystamine became a non-competitive inhibitor of caspase-3 activity.

2. Cystamine Does Not Alter Cell Viability—The effects of cystamine on caspase-3 activity were examined next in situ. Prior to measuring the effects of cystamine on caspase-3 activity in situ, the effects of cystamine on cell viability were analyzed by measuring LDH release. Incubation of SH-SY5Y cells with cystamine (31.2–500 μM) for 24 h did not result in a significant increase in LDH release compared with cells exposed to the vehicle alone, revealing that cystamine did not induce a significant loss in cell viability at these concentrations (data not shown).

3. Effect of Cystamine on MG132-induced Caspase Activation—To examine the effects of cystamine on caspase-3 activity in situ, SH-SY5Y cells were pre-incubated with or without cystamine (31.2–500 μM) for 10 h prior to incubation in the absence or presence of the proteasome inhibitor MG132 (200 nM) for 16 h. Treatment of SH-SY5Y cells with MG132 alone resulted in a significant increase in caspase-3 activity compared with cells treated with vehicle alone (Fig. 3A). The MG132-mediated increase in caspase activity was further confirmed by the caspase-dependent proteolysis of PARP (Fig. 3B).

4. Measurement of Glutathione Levels—Cells (60-mm plate, ~0.2–0.4 mg of protein) were washed twice with ice-cold phosphate-buffered saline and deproteinized with 200 μl of ice-cold 5% (w/v) 5-sulfosalicylic acid (SSA). Forty microliters of a 1 M HEPES/1 M KOH solution was added to protein-free SSA, and the pH was adjusted to 7.0 with 1 M KOH. Total glutathione (GSH + GSSG) was measured by an enzymatic recycling procedure in which it was sequentially oxidized by 5,5’-dithio-bis-(2-nitrobenzoic acid) and reduced by NAPDH in the presence of glutathione reductase (28). The rate of 2-nitro-5-thiobenzoic acid formation was monitored at 412 nm, and the concentration of glutathione determined by comparison of that result with a standard curve generated with known amounts of GSH.

5. Statistical Analysis—Data were analyzed using Student’s t test. Values were considered significantly different when the two-tailed p value was <0.05. Results are expressed as means ± S.E.
Incubation of SH-SY5Y cells with cystamine (31.2–500 μM) prior treatment with MG132 prevented in a dose-dependent manner the activation of caspase-3 (Fig. 3A). The MG132-mediated increase in caspase-3 activity was significantly attenuated by 125 μM cystamine and completely inhibited by 500 μM cystamine (Fig. 3A). The finding that cystamine inhibited caspase-3 activity induced by MG132 was further supported by a reduced degree of PARP proteolysis in cells incubated with cystamine prior to treatment with MG132 (Fig. 3B).

**Effect of Cystamine on H2O2-induced Caspase Activation**—To further examine the effect of cystamine on caspase-3 activity another apoptotic paradigm was used: H2O2-induced increases in caspase-3 activity. In the absence of cystamine, 100 μM H2O2 treatment resulted in an increase in caspase-3 activity starting at 2 h after H2O2 treatment and reaching a maximum after 6 h.

**FIG. 1.** Cystamine inhibits caspase-3 activity in vitro. The activity of recombinant human caspase-3 incubated in the absence or presence of various concentrations of cystamine was measured using the caspase-3 substrate DEVD-AMC. A, recombinant active caspase-3 (5–20 ng) was incubated for 1 h at 37 °C in the absence or presence of cystamine (0–500 μM) prior to measuring caspase-3 activity. Incubation in the presence of cystamine resulted in a concentration-dependent inhibition of caspase-3 activity; the IC50 for cystamine inhibition of caspase-3 activity was 23.6 μM. Results are expressed as arbitrary units of fluorescence (AUF), and representative data from a typical experiment are shown (n = 4 independent experiments and each measurement was done in triplicate). B, recombinant caspase-3 (20 ng) was incubated in the absence or presence of various concentrations of cystamine (0–500 μM) for 0–60 min. In the absence of cystamine, caspase-3 activity was within the linear range (r = 0.97) over a time course of at least 60 min. Representative data from a typical experiment are presented (n = four independent experiments, and each measurement was done in triplicate). The concentration of cystamine is indicated at the right of each line.

**FIG. 2.** Lineweaver-Burk plot of data from the cystamine-mediated inhibition of recombinant caspase-3 activity. Cystamine acts as an uncompetitive inhibitor of caspase-3 activity, becoming a noncompetitive inhibitor at higher concentrations (n = 4 independent experiments; representative data of a typical experiment are presented). The concentration of cystamine is indicated for each line.

**FIG. 3.** Cystamine inhibits the MG132-mediated activation of caspase-3. A, cells were incubated in the absence or presence of the indicated concentrations of cystamine for 10 h before treatment with MG132 (200 μM) for 16 h; cells were then collected, and caspase-3 activity measured. Treatment with MG132 resulted in a significant increase in caspase-3 activity as compared with cells exposed to the vehicle alone. Pretreatment of the cells with cystamine resulted in a concentration-dependent inhibition of the MG132-mediated increase in caspase-3 activity. Results are expressed as a percent of caspase-3 activity induced by MG132 alone. B, the caspase-dependent proteolytic cleavage of PARP was measured by immunoblotting lysates from cells that have been incubated in the absence or presence of cystamine (500 μM) for 16 h. Pretreatment of SH-SY5Y cells with cystamine potently prevented the MG132-mediated caspase cleavage of PARP to the 85-kDa fragment. Representative immunoblot from a typical experiment are shown (n = four independent experiments).
Cystamine Inhibits Caspase Activity Independently of tTG—
Cystamine is known to inhibit tTG activity (29); therefore we next examined the potential contribution of the cystamine-induced inhibition of tTG on the cystamine-prevented activation of caspase-3. For this, the effects of cystamine on caspase-3 activity were examined in cell lines stably overexpressing the wild type tTG, mutated inactive C277S tTG, an antisense for tTG, and as a control, the empty vector. These different cell lines have been extensively characterized in previous studies (22, 23). The levels of tTG expression in the different cell lines used in this experiment are shown in Fig. 5A. The level of tTG expression was low in cells stably transfected with the empty vector (pcDNA) and not detected in cells stably transfected with the antisense tTG (Fig. 5A). However, in cells stably expressing wild type tTG (tTG cells) or mutant inactive tTG (C277S cells), tTG expression was significantly higher, and similar expression levels were detected in both cell lines (Fig. 5A). In the four cell lines pretreatment with cystamine inhibited the MG132-mediated increase in caspase-3 activity compared with cells treated with the vehicle alone. Data are presented as means ± S.E. (n = five to six independent experiments, each in triplicate; *, p < 0.05).

FIG. 4. Cystamine inhibits the H2O2-mediated activation of caspase-3. SH-SY5Y cells were incubated in the absence or presence of cystamine (250 μM) for 10 h before treatment with H2O2 (100 μM) for 0–8 h. A, cells were then collected, and caspase-3 activity measured. Results are expressed as a percent of caspase-3 activity in cells exposed to the vehicle alone; means ± S.E. for three to five independent experiments; *, p < 0.05 compared with cells exposed to H2O2 alone at each time point. B, PARP proteolysis was examined in cell lysates by immunoblotting. The H2O2-mediated proteolytic cleavage of PARP was greatly decreased in cells incubated with cystamine prior to exposure to H2O2.

FIG. 5. Cystamine inhibits caspase-3 activity in a tTG-independent manner. A, representative immunoblot of the levels of tTG in the different cell lines. In SH-SY5Y cells stably transfected with the empty vector (Vec) or with an antisense for tTG (Anti) the level of tTG protein was low or undetectable, respectively. However, in cell stably transfected with the wild type human tTG (tTG) or with the mutant inactive tTG (C277S) a significant and equivalent increase in tTG expression was observed. B, to determine the contribution of tTG to the cystamine-mediated inhibition of caspase, caspase-3 activity was measured in the different cell lines that were incubated in the absence or presence of cystamine (250 μM) prior to incubation in the absence or presence of MG132 (200 nM, 16 h). In the four cell lines pretreatment with cystamine inhibited the MG132-mediated increase in caspase-3 activity to the same extent revealing that cystamine inhibits caspase-3 activity independently of tTG. Data presented are means ± S.E. (n = five to six independent experiments, each in triplicate; *, p < 0.05).

FIG. 6. Treatment with cystamine results in an increase in glutathione levels. SH-SY5Y cells were incubated in the presence (+) or absence (−) of cystamine (250 μM) for 10 h, and the level of glutathione (GSH + GSSG) was measured. Cystamine treatment resulted in a significant increase in glutathione levels as compared with cells treated with the vehicle alone. Data are presented as means ± S.E. (n = five independent experiments; *, p < 0.05).
prevented the MG132-mediated activation of caspase-3 activity (Fig. 5B), and cystamine inhibited caspase-3 activity to the same extent for all the cell lines demonstrating that cystamine inhibits the activation of caspase-3 independently of any effects it may have on the transamidating activity of tTG.

**Treatment with Cystamine Results in an Increase in Glutathione Levels**—Because it has been previously suggested that cystamine treatment may affect the state of the glutathione system (30, 31), the effects of cystamine on the level of glutathione in the cell were examined. SH-SY5Y cells were incubated in the absence or presence of 250 μM cystamine for 8 h prior to collection and measurement of the levels of glutathione (GSH + GSSG) (Fig. 6). In the absence of cystamine the level of glutathione in the cells was 12.7 ± 4.2 nmol/mg of protein. Treatment with cystamine significantly increased the levels of glutathione to 40.2 ± 11.04 nmol/mg of protein (Fig. 6).

**DISCUSSION**

Tissue TG is a multifunctional enzyme that has been implicated in several physiological processes and pathological conditions (for review see Ref. 9). Indeed, recent evidence suggests that a dysregulation of tTG may contribute to the pathology of polyglutamine disorders such as HD (9, 16, 32). The potential role of tTG in the etiology of HD and other polyglutamine diseases is particularly intriguing as it has been suggested that tTG may contribute to the formation of insoluble nuclear and cytoplasmic aggregates that constitute a striking neuropathological hallmark of many of these diseases including HD (7, 8). However, in a recent study we demonstrated that modulating the level and activity of tTG did not affect the frequency or localization of huntingtin aggregates, revealing that tTG was not necessary for their formation (18). In support of these findings, ablation of tTG in an HD mouse model (R6/1 mice) resulted in a significant increase in the frequency of huntingtin aggregates (33). Further, administration of cystamine, initially characterized as a TG inhibitor, to 7-week-old transgenic mice expressing exon 1 of huntingtin containing an expanded polyglutamine repeat (R6/2 mouse) did not influence the appearance or frequency of neuronal nuclear inclusions (19). These results strongly suggest that tTG is not necessary for aggregation formation in HD; however, in the R6/2 mice cystamine treatment was beneficial as it prolonged survival of the mice and decreased the abnormal movements and associated tremor (19). Further in a cell culture system, cystamine decreased the apoptotic cell death induced by the expression of a truncated dentatotuberal-pallidolysian atrophy protein containing an expanded polyglutamine repeat domain (21). Based on the effects of cystamine, it has been suggested that inhibition of tTG may provide a new treatment strategy for HD (19). However, it has been hypothesized that cystamine inhibits tTG by forming a mixed disulfide in the active site, raising the possibility that a similar reaction may also occur with other thiol-dependent enzymes such as caspases (20). Indeed, the results of this study unequivocally demonstrate that in vitro and in situ cystamine inhibits caspase-3 activity in a tTG independent manner. However, at low concentrations cystamine acts as an uncompetitive inhibitor of caspase-3, revealing that cystamine preferentially inhibits caspase-3 after formation of the complex enzyme-substrate, rather than preventing interaction of the substrate with caspase-3. This suggests that the interaction of caspase-3 with a substrate causes a conformational change in caspase-3 allowing cystamine to interact with the target thiol group. Cystamine by inhibiting caspase-3 may contribute to the observed neuroprotective effects of cystamine in the HD mouse model. Several studies have demonstrated that caspases may play a critical role in the etiology of HD. For example, lymphoblasts derived from HD patients showed increased stress-induced apoptotic cell death associated with caspase-3 activation (34). Similarly treatment of R6/2 mice with the tetracycline-derivative drug minocycline inhibits caspase-1 and caspase-3 expression and delays disease progression (3). Further, the combined inhibition of both caspase-1 and caspase-3 is required for effective pharmacotherapy in the R6/2 mouse model of HD (3). Finally, crossingbreeding the R6/2 mice with transgenic mice expressing a dominant-negative mutant of caspase-1 in the brain extended the survival and delayed neurotransmitter receptor alterations and onset of the symptoms (4). These studies strongly suggest that in the R6/2 mice caspasas may play an important role in the etiology of the disease (4). Considering the potential implication of caspases in HD etiology, it is likely that the cystamine-mediated inhibition of caspase-3, and potentially other caspasas, could contribute to the prolonged survival observed in HD mouse models.

Our results show that cystamine treatment of SH-SY5Y cells induces a robust increase in the intracellular level of glutathione, which plays a key role in protecting cells against oxidative damage by reacting with hydrogen peroxide and organic peroxides. There is compelling evidence to demonstrate that the HD gene defect is associated with impaired oxidative phosphorylation, resulting in an increase in oxidative damage (35–37). Indeed, biochemical analysis of postmortem brain tissues provided evidence of impaired mitochondrial enzyme activity in brain regions affected in HD (37). Further, increased oxidative damage is also well documented in HD brains (38); these include increased evidence of DNA strand break and increased levels of oxidative damage products such as 8-hydroxydeoxyguanosine, 3-nitrotyrosine, and malondialdehyde in brain regions affected in HD (38). These findings suggest that a neuroprotective strategy for HD might include the prevention of mitochondria dysfunction and the resulting oxidative damage. Indeed, dietary creatine supplementation significantly improved survival and slowed the formation of huntingtin-positive aggregates in HD mouse models (39, 40). Considering these and others findings, it can be hypothesized that by increasing the level of antioxidants such as glutathione and inhibiting caspases, cystamine may prolong neuronal survival and contribute to the delay in the onset of the disease.

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