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Rapid Communication

A New Inhibitor of the Chymotrypsin-Like Activity of the Multicatalytic Proteinase Complex (20S Proteasome) Induces Accumulation of Ubiquitin–Protein Conjugates in a Neuronal Cell

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Abstract: Exposure of HT4 cells (a mouse neuronal cell line) to a new potent permeable peptidyl aldehyde inhibitor of the chymotrypsin-like activity of the multicatalytic proteinase complex (MPC) causes accumulation of ubiquitinylated proteins. In contrast, inhibition of calpain or treatment with a lysosomotropic agent failed to produce detectable ubiquitin–protein conjugates. The appearance of such conjugates is not a nonspecific phenomenon because incubation with the peptidyl alcohol analogue of the inhibitor does not produce accumulation of ubiquitinylated proteins. The MPC inhibitor may therefore be a useful tool for identification and study of physiological pathways involving MPC. Furthermore, the inhibitor may help develop a model for the study of neurodegeneration where accumulation of ubiquitin–protein conjugates is commonly detected in abnormal brain inclusions. **Key Words:** Multicatalytic proteinase complex inhibitor—Ubiquitin–protein conjugates—20S proteasome—Chymotrypsin-like activity—Neuronal cells.
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The ubiquitin/ATP-dependent proteolytic system, also known as the 26S proteasome, plays a major role in the removal of abnormal and denatured proteins as well as short-lived proteins (for review, see Ciechanover and Schwartz, 1994). The "catalytic core" of the 26S proteasome is the multicatalytic proteinase complex (MPC; 20S proteasome) (Hough et al., 1988; Eytan et al., 1989), an extralysosomal, high-molecular-mass neutral proteinase (≈700 kDa) composed of 24–28 low-molecular-mass subunits and found in all eukaryotic cells examined (for review, see Orlowski, 1990). MPC has multiple catalytic activities, including components that hydrolyze peptide bonds on the carboxyl side of basic (trypsin-like activity), acidic (peptidylglutamyl-peptide hydrolyzing activity), and hydrophobic (chymotrypsin-like activity) amino acids (Wilk and Orlowski, 1980, 1983*a*). Direct evidence that changes in the catalytic activities of MPC induce the accumulation of ubiquitinylated proteins was provided by experiments in yeast. Under stress conditions, yeast strains carrying mutants of MPC displaying a specific reduction in chymotrypsin-like activity, exhibited decreased protein degradation, and accumulation of ubiquitin–protein conjugates (Heinemeyer et al., 1991, 1993).

Thus far, no reports exist in the literature showing that an inhibitor of MPC can cause an accumulation of ubiquitinylated proteins. We present evidence that a peptide aldehyde developed as a potent inhibitor of the chymotrypsin-like activity of MPC induces the accumulation of ubiquitin–protein conjugates in HT4 cells, a mouse neuronal cell line. In addition, we show that neither inhibition of calpain nor treatment with a lysosomotropic agent produces detectable accumulation of ubiquitinylated proteins. These results strongly suggest that in the HT4 cells, a decrease in the chymotrypsin-like activity associated with MPC is responsible for the observed accumulation of ubiquitinylated proteins.

MATERIALS AND METHODS

Cell Culture

The HT4 neuronal cell line derived from mouse neuronal tissue and immortalized by treatment with a temperature-sensitive conditional oncogene (Whittemore et al., 1991) was used. Cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 100 U/ml of penicillin/streptomycin at 33°C in 5% CO_2. For all experiments, cells were seeded at a density of 4.4×10^3 cells/cm^2 in six-well (35-mm-diameter wells) plates coated with 5 μg/ml of poly-L-ornithine. Following a 24-h plating period at 33°C, the cells were placed at 39°C in 5% CO_2 for 3 days to eliminate oncogene action. Cells were re-fed with serum-free medium [Dulbecco's modified Eagle's medium/

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Abbreviations used: MPC, multicatalytic proteinase complex; PAGE, polyacrylamide gel electrophoresis; PNA, nitroanilide; SDS, sodium dodecyl sulfate; Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-methylcoumarylamide; Z-GGL-pNA, *N*-benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide; Z-IE(OtBu)AL-CHO, *N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal; Z-IE(OtBu)AL-OH, *N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinol.

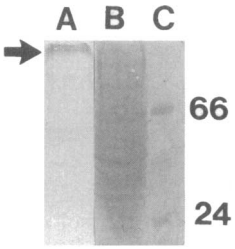

FIG. 1. Lane A, detection of ubiquitinylated proteins in HT4 cell extracts (12 μg) by western blotting as described in Materials and Methods. The immunoblot was overstained to allow visualization of ubiquitinylated proteins (arrow) in untreated cells. Lane B, Coomassie Brilliant Blue staining of the protein content of HT4 cell extracts (12 μg). Lane C, molecular mass markers, in kDa.

F12 (1:1) with 5 μg/ml of insulin, 5 μg/ml of transferrin, 30 n*M* selenium, 20 n*M* progesterone, and 100 μ*M* putrescine] 24 h before experiments.

Preparation of cell extracts and western blotting

Following the indicated treatments, cells were washed twice with phosphate-buffered saline and lysed with 300 μl of hot (80–90°C) 10 m*M* Tris-EDTA (pH 7.5) containing 1% sodium dodecyl sulfate (SDS), 4% β-mercaptoethanol, sucrose, and bromophenol blue. After scraping to free adherent cells, samples were boiled for 5 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 6% gels and probed by western blotting with a rabbit polyclonal antibody (diluted 1:600; DAKO Corp., Carpinteria, CA, U.S.A.) that recognizes protein conjugates of ubiquitin (the same antibody is routinely used for immunohistochemical identification of ubiquitinylated filamentous inclusions in neurodegenerating brains under the manufacturer's specification). For visualization, an affinity-purified goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad, Hercules, CA, U.S.A.) and the substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD, U.S.A.) were used following the recommendations of DAKO.

Protein concentration per 35-mm-diameter well averaged 0.4 ± 0.05 mg, and the inhibitor did not alter protein content or cell viability as determined by trypan blue exclusion (95 ± 2%, n = 3).

Quantitative analysis of the immunoblots

The intensities of individual bands were quantitated by image analysis (Pereira et al., 1992).

Enzyme assays

Purified MPC was prepared from bovine pituitaries (Orlowski and Michaud, 1989). MPC (2 μg) and 400 μ*M* substrate were incubated at 37°C for 1 h with inhibitors in dimethyl sulfoxide or with vehicle alone (control) and 0.05 *M* Tris-HCl (pH 7.5) in a total volume of 100 μl. The chymotrypsin-like activity of MPC measured with succinyl-Leu-Leu-Val-Tyr-methylcoumarylamide (Suc-LLVY-AMC) was assayed colorimetrically (Wilk and Orlowski, 1980, 1983*a*).

To measure catalytic activities associated with MPC in cell extracts, HT4 cells were grown in 15-cm-diameter plates as described above and incubated in serum-free medium with inhibitors (5 μ*M* in dimethyl sulfoxide) or vehicle (control) for 3 h at 37°C. Cells were then washed twice with phosphate-buffered saline, scraped off, and lysed in buffer I (0.01 *M* Tris-EDTA, pH 7.5) or in buffer II [0.01 *M* Tris-EDTA (pH 7.5) with 2 m*M* ATP and 20% glycerol], which maintains the integrity of the 26S proteasome (Hough et al., 1987). The samples were then homogenized and centrifuged at 16,000 g at 4°C for 10 min. Enzyme activities against Suc-LLVY-AMC and *N*-benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide (Z-GGL-pNA) were measured in the crude cytosol (63 μg) as described (Wilk and Orlowski, 1980, 1983*a*). Protein concentration per 15-cm-diameter dish averaged 1.26 ± 0.05 mg/ml.

Other procedures

SDS-PAGE was conducted on 6% gels as described (Laemmli, 1970). Protein determination was by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

We first tested whether ubiquitin–protein conjugates could be detected in a neuronal cell line (HT4). As shown in Fig. 1, lane A, an antibody that recognizes protein conjugates of ubiquitin reacted with a heterogeneous population of high-molecular-weight proteins in western blots of cell extracts, a pattern characteristic of ubiquitinylated proteins.

Because peptidyl aldehydes are believed to act as transition-state analogue inhibitors of serine and cysteine proteinases (Thompson, 1973), we synthesized the peptidyl aldehyde *N*-benzyloxycarbonyl-Ile-Glu(*O*-*t*-butyl)-Ala-leucinal [Z-IE(OtBu)AL-CHO] as an inhibitor of the chymotrypsin-like activity of MPC [synthesis and characterization of Z-IE(OtBu)AL-CHO will be published elsewhere]. The chymotrypsin-like activity of MPC determined by hydrolysis of Suc-LLVY-AMC is potently inhibited (IC_{50} = 0.25 μ*M*) by Z-IE(OtBu)AL-CHO (Fig. 2A). Hydrolysis of Z-GGL-pNA, another substrate used to measure the chymotrypsin-like activity, is less strongly inhibited (IC_{50} = 6.5 μ*M*). At the highest inhibitor concentration tested (65 μ*M*), hydrolysis of *N*-benzyloxycarbonyl-D-Ala-Leu-Arg-2-naphthylamide (trypsin-like activity) and of *N*-benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide (peptidylglutamyl peptide activity) was suppressed by 14 and 9%, respectively (details to be published elsewhere).

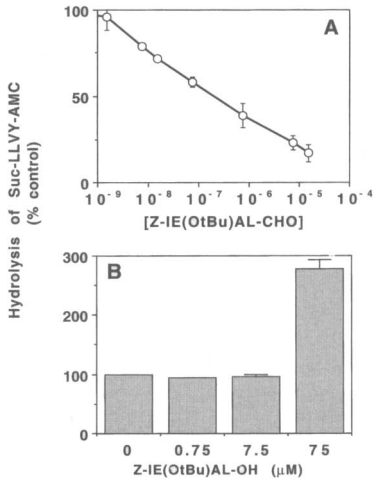

FIG. 2. Effect of (**A**) Z-IE(Ot-Bu)AL-CHO and of (**B**) Z-IE(Ot-Bu)AL-OH on purified MPC assayed with Suc-LLVY-AMC as substrate as described in Materials and Methods. Data are shown as percentages of the activity measured with no added aldehyde or alcohol (control) and are means ± SE (values) of three experiments.

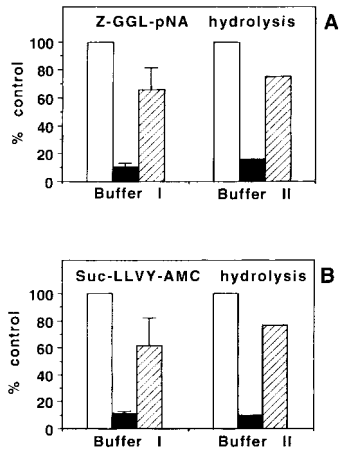

FIG. 3. Chymotrypsin-like activity of HT4 cells assayed with (**A**) Z-GGL-pNA or (**B**) Suc-LLVY-AMC in the absence (□) or presence of 5 μM Z-IE(Ot-Bu)AL-CHO (■) or 5 μM Z-IE(Ot-Bu)AL-OH (▨) as described in Materials and Methods. Data are shown as percentages of the activity measured with no added aldehyde or alcohol (control) and are mean ± SE (bars) values of four experiments (buffer I) and the means of two experiments (buffer II). Buffer I, 0.01 *M* Tris-EDTA (pH 7.5); buffer II, 0.01 *M* Tris-EDTA (pH 7.5) containing 2 m*M* ATP and 20% glycerol.

We also synthesized the peptidyl alcohol *N*-benzyloxycarbonyl-Ile-Glu(*O-t*-butyl)-Ala-leucinol [Z-IE(OtBu)AL-OH]. The alcohol should be a much less potent inhibitor than the aldehyde (Wilk and Orlowski, 1983*b*) and therefore can be used to rule out nonspecific effects. Hydrolysis of Suc-LLVY-AMC by purified MPC is not inhibited by Z-IE(OtBu)AL-OH. In fact, at a high concentration (75 μM) the alcohol stimulates Suc-LLVY-AMC hydrolysis almost threefold (Fig. 2B). Hydrolysis of Suc-LLVY-AMC is known to be stimulated by other compounds (Ishiura et al., 1985).

To investigate if the MPC inhibitor was cell permeable, we incubated HT4 cells for 3 h with 5 μM Z-IE(OtBu)AL-CHO and measured MPC-associated catalytic activities of cell homogenates. To assure removal of extracellular inhibitor before homogenization, the medium was aspirated and the cells were washed twice with phosphate-buffered saline. The cells were homogenized either in 0.01 *M* Tris-EDTA (pH 7.5; buffer I) or in the same buffer containing (final concentrations) 2 m*M* ATP and 20% glycerol (buffer II) to preserve the integrity of the 26S proteasome (Hough et al., 1987). Similar results were obtained under both conditions of homogenization. Z-IE(OtBu)AL-CHO treatment strongly decreased the chymotrypsin-like activity measured with either Z-GGL-pNA or Suc-LLVY-AMC (Fig. 3). The trypsin-like and peptidylglutamyl peptide components were less affected (data not shown). The peptide aldehyde can therefore effectively suppress the intracellular chymotrypsin-like activity associated with MPC.

Hydrolysis of Z-GGL-pNA or Suc-LLVY-AMC by homogenates of HT4 cells exposed to the peptidyl alcohol at 5 μM for 3 h was slightly inhibited (Fig. 3). This may be due to some biotransformation of the peptidyl alcohol to the aldehyde. The resulting aldehyde concentrations are most likely too low to produce a major decrease in chymotrypsin-like activity.

Finally, we examined the effect of Z-IE(OtBu)AL-CHO on accumulation of ubiquitinylated proteins. Incubations for as little as 30 min with 50 μM Z-IE(Ot-Bu)AL-CHO (Fig. 4A) or for 3 h with 5 μM Z-IE(OtBu)AL-CHO (Fig. 4B) produced accumulation of ubiquitin–protein conjugates. The accumulation of the detected ubiquitinylated proteins peaked after incubations of 1 h with the peptide aldehyde at 50 μM (Fig. 4D).

We also tested whether incubation of HT4 cells with the peptidyl alcohol would lead to accumulation of ubiquitinylated protein. Exposure of HT4 cells for 3 h to Z-IE(Ot-Bu)AL-OH at concentrations up to 100 μM did not cause accumulation of ubiquitinylated proteins (Fig. 4C). This finding strongly suggests that the appearance of ubiquitin–protein conjugates resulting from treatment of the neuronal cells with the peptidyl aldehyde is not a nonspecific phenomenon, but must result from inhibition of the chymotrypsin-like component of MPC. Only the peptidyl aldehyde and not the peptidyl alcohol can react with the active site residue to form a catalytically inactive transition-state analogue (Thompson, 1973; Wilk and Orlowski, 1983*b*).

We tested the effect of inhibitors of other proteolytic enzymes on the degradation of ubiquitin–protein conjugates in HT4 cells (Fig. 4B and C). Because calpains are inhibited by the peptidyl aldehydes calpeptin (*N*-benzyloxycarbonyl-Leu-norleucinal) and calpain inhibitor 1 (*N*-benzyloxycarbonyl-Leu-Leu-norleucinal) (Tsujinaka et al., 1988;

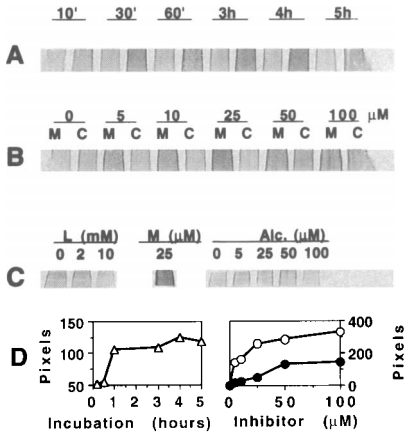

FIG. 4. Detection of ubiquitinylated proteins in HT4 cell extracts (12 μg per lane) by western blotting as described in Materials and Methods. Cells were incubated at 37°C (**A**) without (−) or with (+) 50 μM Z-IE(Ot-Bu)AL-CHO for the times indicated, (**B**) with increasing concentrations of the MPC inhibitor Z-IE(Ot-Bu)AL-CHO (M) or of the calpain inhibitor *N*-benzyloxycarbonyl-Leu-leucinal (C) for 3 h, and (**C**) with several concentrations of the lysosomotropic agent NH_4Cl (L) or of the peptidyl alcohol analogue Z-IE(Ot-Bu)AL-OH (Alc.) or with 25 μM Z-IE(Ot-Bu)AL-CHO (M) for 3 h. The immunoblots shown are representative of one of at least three identical experiments for each condition tested. Because ubiquitinylated proteins are identified at the top of the gel (see Fig. 1A), only the top sections of the western blots are shown. **D:** Quantitative analysis of the accumulation of ubiquitinylated proteins in HT4 cells incubated with 50 μM Z-IE(Ot-Bu)AL-CHO for different times (△) or with different concentrations of Z-IE(Ot-Bu)AL-CHO (○) or *N*-benzyloxycarbonyl-Leu-leucinal (●) for 3 h as described in Materials and Methods. The values show the relative number of pixels above control levels (cells grown in the absence of inhibitors). D represents different experiments from those of A and B.

Wang, 1990), it was not unexpected to find that Z-IE(Ot-Bu)AL-CHO is also an inhibitor of calpain (IC_{50} = 4 μM). Therefore, we determined whether the observed accumulation of ubiquitin–protein conjugates could be attributed to calpain inhibition. Recently, we demonstrated that calpeptin, a potent cell-permeable calpain inhibitor, is a relatively weak inhibitor of MPC activities (Figueiredo-Pereira et al., 1994). Calpeptin concentrations of 0.5–1.0 μM almost totally inhibit the degradation of β-casein by calpain, whereas the catalytic activities of MPC are virtually unaffected. We synthesized a calpeptin analogue, *N*-benzyloxycarbonyl-Leu-leucinal, and found it to have the same potency as calpeptin on calpain and MPC activities (data not shown). Incubations of HT4 cells for 3 h with *N*-benzyloxycarbonyl-Leu-leucinal concentrations up to 25 μM produced negligible accumulation of ubiquitin–protein conjugates (Fig. 4B and D). Only when cells were exposed for 3 h to 50–100 μM *N*-benzyloxycarbonyl-Leu-leucinal was an accumulation detected (Fig. 4B and D); however, at these high concentrations the chymotrypsin-like activity of MPC is inhibited by >50% (data not shown). These findings demonstrate that the accumulation of ubiquitin–protein conjugates induced by Z-IE(Ot-Bu)AL-CHO is not due to inhibition of calpain. This was not unexpected because calpain is not known to play a role in the degradation of ubiquitinylated proteins. Treatment of HT4 cells for 3 h with the lysosomotropic agent NH_4Cl (Seglen, 1983) did not lead to accumulation of ubiquitin–protein conjugates (Fig. 4C). Together, these results indicate that the proteinase responsible for the degradation of ubiquitinylated proteins that accumulate in HT4 cells on treatment with Z-IE(Ot-Bu)AL-CHO is neither a lysosomal enzyme nor calpain.

This report documents the development of an inhibitor of the chymotrypsin-like activity of MPC that unequivocally induces the accumulation of ubiquitinylated proteins in a neuronal cell line. In addition to its ability to degrade ubiquitinylated proteins such as cyclins, oncogenes, and transcription factors as the "catalytic core" of the 26S proteasome, MPC was also proposed to generate intracellular antigenic peptides for presentation by the major histocompatibility complex-restricted class I pathway, to degrade oxidatively modified proteins, and to serve a general housekeeping role in extralysosomal protein metabolism (for review, see Ciechanover and Schwartz, 1994). Z-IE(Ot-Bu)AL-CHO should be a valuable tool for the definition of cellular pathways directly associated with MPC either alone or as part of the 26S complex. Furthermore, it may provide the means for developing an in vivo model to study the cellular events leading to neurodegeneration.

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