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Pyrrolidine dithiocarbamate and zinc inhibit proteasome-dependent proteolysis

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Abstract

Proteasomes play important roles in a variety of cellular processes such as cell cycle progression, signal transduction and immune responses. Proteasome activity is important in maintaining rapid turnover of short-lived proteins, as well as preventing accumulation of misfolded or damaged proteins. Alteration in ubiquitin-proteasome function may be detrimental to its crucial role in maintaining cellular homeostasis. Here, we have found that treatment of pyrrolidine dithiocarbamate (PDTC), a zinc ionophore, resulted in the accumulation of several proteasome substrates including p53 and p21 in HeLa cells. The PDTC effect was due to an extended half-life of these proteins through the mobilization of zinc. PDTC and/or zinc also increased fluorescence intensity of Ub^{G76V}-GFP fusion protein that is degraded rapidly by the ubiquitin-proteasome system. Treatment of cells with zinc induced formation of ubiquitinated inclusions in the centrosome, a histological marker of proteasome inhibition. Western blotting showed zinc-induced increase in laddering bands of polyubiquitin-conjugated proteins. In vitro study, zinc inhibited the ubiquitin-independent proteasomal degradations of p21 and α-synuclein. These results suggest that zinc may modulate cell functions through its action on the turnover of proteins that are susceptible to proteasome-dependent proteolysis.

Keywords: Zinc; Proteasomes; p53; p21; α-Synuclein

Introduction

Dithiocarbamates are widely used in agriculture as insecticides and fungicides [1] and in health care for the management of alcoholism and heavy metal poisoning [2]. Dithiocarbamates have been used in the treatment of acquired immune depressive syndrome [3] and cancer [4] and proposed as an adjunct regimen to enhance the therapeutic index of other anticancer drugs [5,6]. Dithiocarbamates have also been applied to prevent atherosclerosis [7]. In addition to its various clinical applications, PDTC, a stable pyrrolidine analogue of dithiocarbamate, has been used widely as a tool in studying the regulation of gene transcription. PDTC is a low-molecular thiol antioxidant and potent inhibitor of NF-κB activation [8]. We have noted the unique inhibitory effect of zinc in NF-κB activation through our previous studies exploring the mechanism of pyrrolidine dithiocarbamate (PDTC) action [9–11]. We have found two novel mechanisms of PDTC inhibition of NF-κB activation. The first is that zinc influx is required for PDTC inhibition of NF-κB activation [9,11,12]. The second is that PDTC inhibited the IκBα degradation that is an essential step for NF-κB activation (C.H. Kim and Y.S. Ahn, unpublished data).

Zinc is an essential element for a great number of proteins, including enzymes involved in signaling processes and transcription factors needed in the regulation of gene expression. Through these actions, zinc plays an important role in cell proliferation, differentiation and death [13]. Besides its universal roles, zinc may also exert its actions in an organ-specific manner. Zinc is abundantly present in pancreatic β-cells [14]. It is co-secreted with insulin. In selected condi-

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tions, zinc may accumulate to a level that interferes with functions of neighboring pancreatic islet cells [15]. Zinc is also abundant in prostate epithelial cells and is believed to exert a pro-apoptotic action against tumorigenesis [16,17]. Zinc is widely distributed in brain. During synaptic activity, vesicular zinc is released into synaptic clefts, but recycled into the synapses via a transporter mechanism [18]. However, zinc may be released in excessive amount, as much as several hundred micromolar, from excited presynaptic neurons into synaptic clefts in stroke, trauma and seizure, to be cytotoxic to neighboring neurons [18–20]. In patients with Alzheimer's disease, the zinc content is abnormally elevated in various brain regions [21,22]. Synaptic zinc contributes to amyloid pathology [23]. Although zinc exerts diverse and dynamic actions in neuronal as well as non-neuronal cells, the molecular mechanisms that underlie its cellular effects remain to be characterized in detail.

Proteasomes are a multicatalytic protease, carrying chymotrypsin-, trypsin- and postglutamyl peptidase-like activities. The 26S proteasome is responsible for the majority of intracellular non-lysosomal proteolysis. It consists of a central 20S proteasome, in which proteins are degraded, and two 19S (also called PA700) complexes, which provide substrate specificity [24]. Ubiquitin is conjugated to specific substrates. Three distinct enzymes, E1, E2 and E3, serve this conjugation process [24,25]. Short-lived normally functioning regulatory proteins as well as midfolded or damaged proteins are degraded by this large protein complex. Through the proteolytic degradation of selected cell cycle regulators such as p21, p27, cyclin B1, cyclin E, proteasomes are involved in many cellular events including the regulation of cell cycle, cell death and gene expression [26,27]. Because proteasome inhibition may result in coordinated alteration of cell cycle, selected proteasome inhibitors are being developed as anticancer drugs [28]. Several proteasome inhibitors exert cytotoxicity in non-neuronal and neuronal cells, suggesting that perturbation of protein degradation mechanisms may cause "proteotoxicity" in various cell types [29]. Proteasomes also catalyze the proteolysis of many transcription factor proteins such as hypoxia-inducible transcription factor 1α (HIF-1α), c-Jun, c-Fos, NF-κB and C/EBPs [30–34]. Intriguingly, zinc modulates activities of several transcription factors including NF-κB, AP-1 and Egr-1 [9,10,35].

In view of the well-known proteasome action in processing p105 precursor of NF-κB subunit p50 and degrading IκBα [34,36], we sought to determine the zinc effect on activity of the ubiquitin-proteasome system. Here, we show that zinc inhibits proteasome-dependent protein degradation.

Materials and methods

Materials

p21, cyclin B1, c-Myc and c-Jun antibodies were purchased form Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal anti-p53 (DO-7) antibody was purchased from NeoMarkers (Fremont, CA). The anti-α-synuclein antibody was purchased from Transduction Laboratories Inc. (Lexington, KY). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin were obtained from Zymed Laboratories (South San Francisco, CA). An antibody to ubiquitin-protein conjugate and a purified 20S proteasome were obtained from Affiniti Research (Exeter, UK). An anti-ubiquitin antibody was obtained from Sigma (St. Louis, MO). Tablets of Complete™ Mini protease inhibitors were purchased from Roche (Mannheim, Germany). All fine chemicals were purchased from Sigma. The ECL detection system was obtained from Amersham Biosciences (Piscataway, NJ). All cell culture products and lipofectamine were purchased from Invitrogen (Grand Island, NY).

Cell cultures and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 incubator. A Ub^{G76V}-GFP construct was generously provided by Dr. Masucci (Karolinska Institute, Stockholm, Sweden). HeLa cells were transfected with this Ub^{G76V}-GFP DNA construct using lipofectamine. HeLa cells stably transfected with the Ub^{G76V}-GFP construct were generated by selection in 500 μg/ml G418 (Invitrogen).

Western blot analysis

Cells were lysed in RIPA buffer [20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% (w/v) SDS, 1.0% (v/v) NP-40], which contained Complete™ Mini protease inhibitors. The amount of proteins was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). An aliquot of 50-μg proteins from a total lysate was electrophoresed on SDS-PAGE gel and then transferred to Immobilon-P (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS), the membrane was incubated with a primary antibody at an appropriate dilution overnight at 4°C and then washed three times in TBST. The membrane was then incubated with a secondary antibody at a dilution of 1:5,000 at room temperature for 1 h and again washed three times in TBST. The blots were visualized with ECL (Amersham).

Fluorescent measurement of proteasome activity

Proteasome activity was monitored by alteration in fluorescence intensity of Ub^{G76V}-GFP. For fluorescence microscopy, the cells stably transfected with Ub^{G76V}-GFP construct were grown on coverslips. After rinsing in PBS, fluorescent images of cells were visualized on a Leica TCS-NT confocal microscope. Following this experiment, cells grown in 10-cm dish were washed with cold PBS and then lysed in 0.5% Triton X-100 in PBS. After centrifugation, GFP fluorescence

on each supernatant aliquot containing 500 μg of proteins was measured in a spectrofluorophotometer. Changes in fluorescent signal intensity were measured at excitation/emission wavelengths of 480/510 nm, respectively.

Immunocytochemistry

Stably transfected HeLa cells attached to glass coverslips were rinsed three times with PBS. Fixation and permeabilization were carried out for 10 min at −20°C in 1 ml ice-cold methanol. After removal of methanol, cells were rinsed three times with PBS. The coverslips were treated with an antibody against ubiquitin (Sigma) in a moist chamber overnight at 4°C, rinsed in PBS and stained with a FITC-conjugated secondary antibody. For double labeling, these primary and secondary incubations were repeated with antibodies against γ-tubulin. Fluorescent images were then visualized on a Leica TCS-NT confocal microscope.

In vitro assay for 20S proteasomal degradation of substrate proteins

The reaction was performed according to the method described by Liu et al. [47]. Purified 20S proteasomes (40 nM) were incubated with recombinant p21-His fusion protein (400 nM) or α-synuclein-GST fusion protein (100 nM) in a 50-μl buffer containing 20 mM Tris–HCl, pH 7.1, 200 mM NaCl, 10 mM $MgCl_2$, 0.25 mM ATP and 1 mM DTT at 37°C. p21 construct was subcloned into pRSET (Clontech, Palo Alto, CA) and histidine-tagged p21 fusion protein was purified. Recombinant α-synuclein protein was purified and generously provided by Dr. J. Kim (Yonsei University College of Medicine, Seoul, Korea). Ten microliters of reaction mixture was subjected to Western blot analysis as described above. Each protein was probed with the anti-α-synuclein monoclonal antibody or anti-p21 polyclonal antibody.

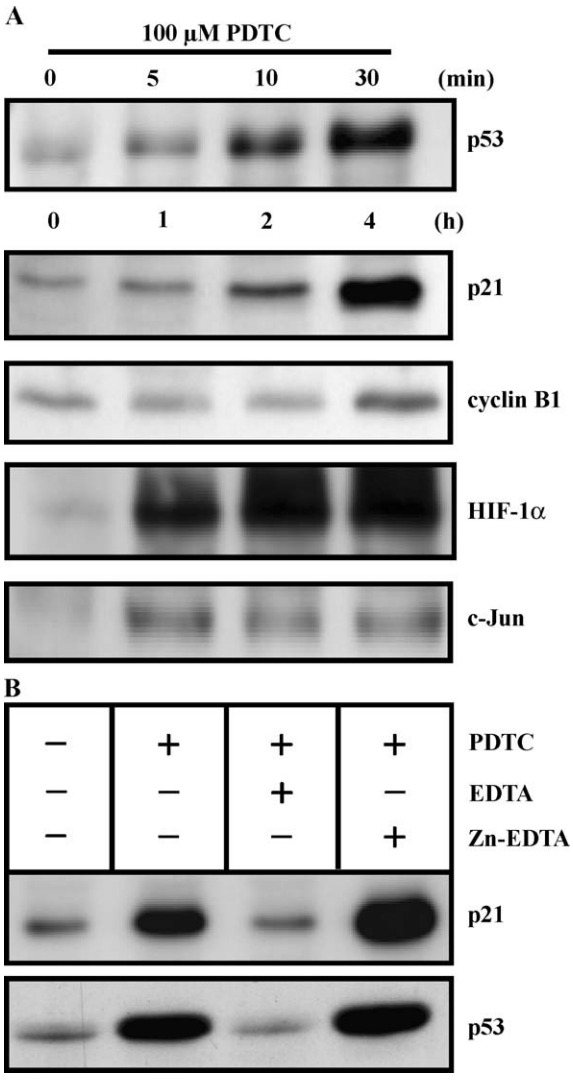

Fig. 1. PDTC increased the stabilities of short-lived proteins in HeLa cells. PDTC (100 μM) increased the cellular levels of short-lived proteins. HeLa cells were treated with PDTC, a zinc ionophore, for indicated times in the presence of 10% FCS in DMEM media. Lysates were processed by SDS-PAGE and then blotted with specific antibodies (A). Free base- or Zn-EDTA (each 10 μM) and PDTC (100 μM) were added together to HeLa cells grown in DMEM containing 10% FCS. After 3 h, p53 and p21 in total cell lysates were visualized by Western blot (B).

Results

PDTC increased the levels of short-lived regulatory proteins

In our earlier studies, PDTC increased the intracellular zinc, reaching plateau within 30 min both in BCECs and HeLa cells [11,12]. Inhibition of NF-κB activity by PDTC was prevented by various metal-saturated EDTAs, but not by zinc-saturated EDTA, indicative of zinc-mediated PDTC action [9,11]. We tested the effect of PDTC on cellular levels of well-known proteasome-governed proteins including p53, p21, cyclin B1, hypoxia-inducible factor 1α (HIF-1α) and c-Jun. These proteins have been shown to accumulate following treatment with proteasome inhibitors [26,31]. Levels of these proteins were all increased within 4 h of PDTC treatment (Fig. 1A). The actions of PDTC on protein levels of p21 and p53 were also blocked by EDTA, but not by Zn-EDTA (Fig. 1B). To know whether the increase of these proteins upon PDTC treatment resulted, at least in part, from inhibition of protein degradation, we performed chase experiment using cycloheximide, a protein synthesis inhibitor (Fig. 2). Chase of protein levels after cycloheximide treatment is a convenient method to follow changes in the half-lives of target proteins. We selected p53 and p21 for this experiment because both showed a robust increase after PDTC treatment (Fig. 1A). The amount of both proteins in cells incubated in the control chase medium declined significantly in the presence of cycloheximide. In contrast, much slower declining rates were observed in HeLa cells if

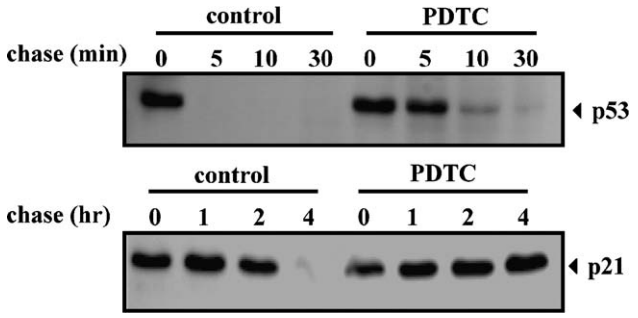

Fig. 2. Chase experiment for determining the half-life of p53 and p21. PDTC prolonged the half-life of both p53 and p21. After pre-incubation of HeLa cells with 100 μM of PDTC for 3 h, chase experiment was performed upon addition of cycloheximide (10 μg/ml), a de novo protein synthesis inhibitor, with a chase intervals of 0–30 min for p53 and 1–4 h for p21. Changes in p21 and p53 protein levels were examined by Western blotting using specific antibodies.

PDTC was added, suggesting a prolongation of the half-life of each protein (Fig. 2).

Effects of various protease inhibitors on the stabilities of p53 and p21

It is well known that p53 and p21 is degraded by the proteasome system [26,37,38]. However, many short-lived proteins are also susceptible to calpain degradation in certain cell types. For example, p53 stability is regulated by calpain in a breast carcinoma cell line [39]. To identify the dominant protease system that regulates the stabilities of p53 and p21 in HeLa cells, we studied the proteolysis of p53 and p21 using inhibitors of the proteasome, calpains and lysosomal proteases. Clasto-lactacystin β-lactone is a highly specific proteasome inhibitor and MG132 is a potent but less specific proteasome inhibitor. Calpeptin, ALLM and ALLN are calpain inhibitors. Chloroquine is a lysosomal acidification inhibitor, which is used as a lysosome inhibitor. Although these protease inhibitors are categorized into three groups, some cross-substrate actions have been noted among these agents [40]. Interestingly, only proteasome, but not calpain or lysosomal protease, inhibitors substantially increased the protein levels of p21 and p53 in HeLa cells (Fig. 3). These findings strengthen the likelihood that the proteasome regulates the stability of p21 and p53 in HeLa cells in basal condition and raise the possibility that PDTC, via its action to induce zinc influx, inhibits proteasomal degradation.

Effects of PDTC and zinc on proteasome-dependent proteolysis

To further confirm that the proteasomal degradation is inhibited by PDTC and zinc, we used a DNA construct that enabled us to measure the activity of ubiquitin/proteasome system specifically in living cells [41]. Ub^{G76V}-GFP expression is driven by this DNA construct that encodes ubiquitin fusion degradation (UFD)-targeted green fluorescent proteins with a mutated (G→V) uncleavable ubiquitin moiety. This GFP reporter is destined to rapid degradation by the ubiquitin/proteasome system in comparison to the wild-type GFP, allowing rapid and convenient measurement of ubiquitin/proteasome system activity. When proteasomes are inhibited, the fluorescent protein quickly accumulates to levels detectable by fluorescence microscopy and spectrofluorophotometer. We prepared cell lines stably expressing this GFP reporter and studied the effects of zinc, PDTC and proteasome inhibitors on the stability of GFP reporter. First, we confirmed that the expression of the ubiquitin-tagged GFP was expressed in stably transfected HeLa cells based on Western blotting (Fig. 4A). In preliminary studies, transfected cells were incubated with 20 μM MG132 for 8 h. A significant increase in fluorescence signal intensity based on fluorescence microscopic assessment confirmed a stably transfected cell line with an appropriate window of expression level of GFP had been selected. In parallel experiments, to know the protease specificity of GFP reporter signal, we added calpain inhibitors and a lysosomal inhibitor to Ub^{G76V}-GFP-transfected HeLa cells. None of these inhibitors altered the fluorescence intensity of GFP signal (Fig. 4B). Treatment with PDTC plus zinc was then carried out to ascertain that zinc influx induced by PDTC inhibited the degradation of Ub^{G76V}-GFP. As expected, PDTC plus zinc increased the fluorescence intensity of GFP in Ub^{G76V}-GFP HeLa cells compared to the control (Fig. 4C). High doses of extracellular zinc also increased the GFP fluorescence signal in a dose-dependent manner (Fig. 5A). GFP fluorescent images of cells were obtained upon treatment with MG132 or various concentrations of zinc using confocal microscopy. In line with the observation based on spectrofluorometry, GFP fluorescence signal was also increased in cells treated with zinc in a dose-dependent manner (Fig. 5B). These results suggest that PDTC mediated by zinc influx or zinc alone inhibited proteasome activity and that the stabilization of p21 and p53 is, at least in part, due to the inhibition of ubiquitin-proteasome activity.

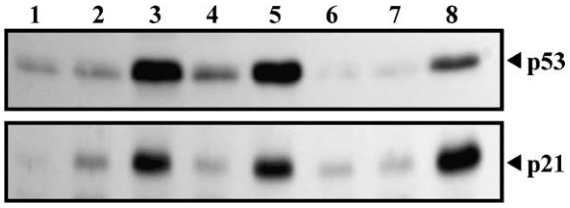

Fig. 3. Effects of various protease inhibitors on the degradation of p21 and p53. HeLa cells were treated with various protease inhibitors for 4 h. Changes in cellular p21 and p53 protein levels in comparison to the controls were assessed by Western blotting. Drug treatments were as follows: 1, control; 2, 1 mM chloroquine; 3, 10 μM clasto-lactacystin β-lactone; 4, 10 μM calpeptin; 5, 20 μM MG132; 6, 20 μM ALLN; 7, 20 μM ALLM; 8, 100 μM PDTC. Clasto-lactacystin β-lactone is a highly specific proteasome inhibitor and MG132 is a potent but less specific proteasome inhibitor. Calpeptin, ALLM and ALLN are calpain inhibitors. Chloroquine is a lysosomal protease inhibitor.

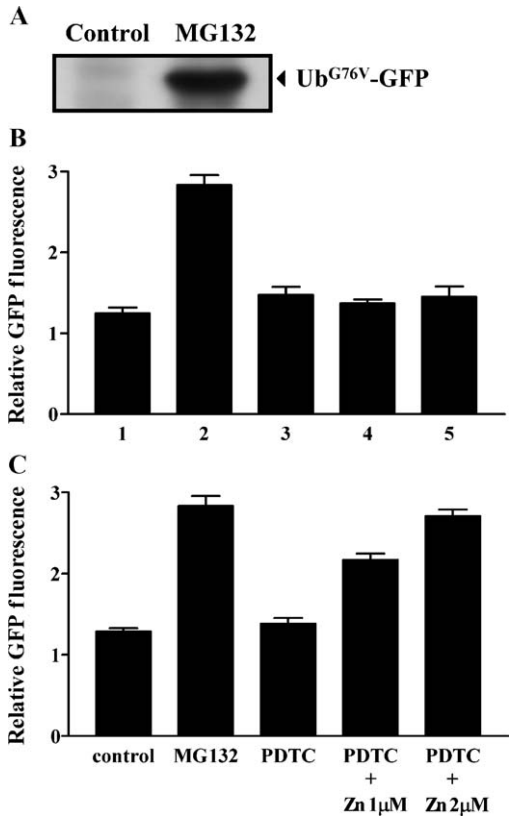

Fig. 4. Zinc-dependent PDTC inhibition of Ub^{G76V}-GFP degradation. HeLa cells were stably transfected with Ub^{G76V}-GFP construct. Expression of GFP protein was assessed by Western blotting using anti-GFP antibody. MG132 (20 μM) was administered to allow detection of unstable Ub^{G76V}-GFP (A). HeLa-Ub^{G76V}-GFP cells were incubated with DMEM containing each of various calpain or lysosomal inhibitors for 8 h. Cells were then lysed with 1% Triton X-100 and centrifuged at 13,000 × *g* for 10 min. GFP fluorescence in the supernatant was measured by spectrofluorophotometry. Drug treatments were as follows: 1, control; 2, 20 μM MG132; 3, 1 mM chloroquine; 4, 10 μM calpeptin; 5, 20 μM ALLN (B). HeLa-Ub^{G76V}-GFP cells were incubated with DMEM containing PDTC (100 μM) with or without $ZnSO_4$ (1 μM or 2 μM) in the absence of serum for 8 h (C). GFP fluorescence was measured using the same method described above. Data are means ± SE (bars) for three independent experiments, each performed in quadruplicate.

Formation of ubiquitinated inclusion upon zinc treatment

We examined the ubiquitin immunoreactivity using an antibody against the ubiquitin to identify ubiquitin-rich cytoplasmic inclusions. Formation of ubiquitinated inclusion is considered a histological marker of inadequate proteasome function. Thus, we investigated possible zinc action in inducing the formation of ubiquitin-rich inclusion structure. Immunostaining with an anti-ubiquitin antibody showed that a single large juxtanuclear inclusion was formed upon treatment with a proteasome inhibitor, MG132 or clasto-lactacystin β-lactone (Figs. 6B and C). Ubiquitinated inclusions were also observed from HeLa cells treated with zinc (Fig. 6D). Recently, it has been reported that nondegradable proteins tend to be deposited around the centrosome with ubiquitin, proteasomes, Hsp70 and Hsp90, and proteasome inhibitors enlarge the centrosome [42,43]. Ubiquitinated inclusions were co-localized with γ-tubulin, a marker of centrosome (Figs. 6D–F). The local build-up of ubiquitinated proteins in centrosome suggests that the proteasome system was not capable of clearing proteins upon treatment with zinc.

Effects of PDTC and zinc on ubiquitination

The failure of proteasomal degradation of proteins prompted a study to determine whether inhibition of ubiquitination is involved in this zinc-mediated event. In fact, ubiquitination can be a potential target of inhibition by zinc in stabilizing short-lived proteins because most proteasomal degradation necessitates the covalent conjugation of ubiquitin to substrate proteins. Increase in Ub^{G76V}-GFP fluorescence can result not only from inhibition of proteasome itself but also from defects

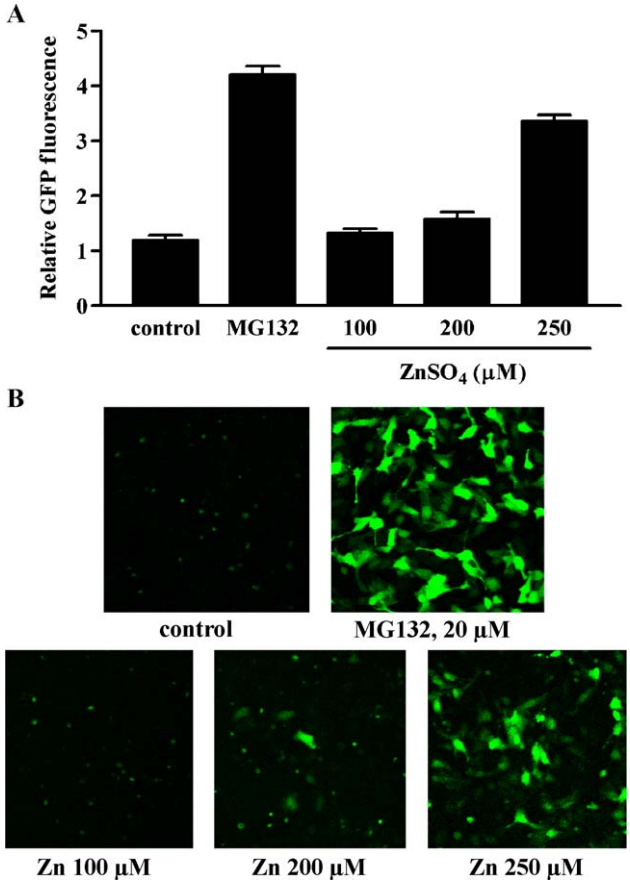

Fig. 5. Zinc inhibition of ubiquitin-proteasome activity in HeLa-Ub^{G76V}-GFP cells. Cells stably transfected with Ub^{G76V}-GFP were incubated with DMEM containing 10% FCS and MG132 (20 μM) or zinc for 8 h. Increases in GFP fluorescence were monitored by spectrofluorophotometry (A) or confocal microscopy (B). Data for spectrofluorophotometry are means ± SE (bars) for three independent experiments, each performed in quadruplicate.

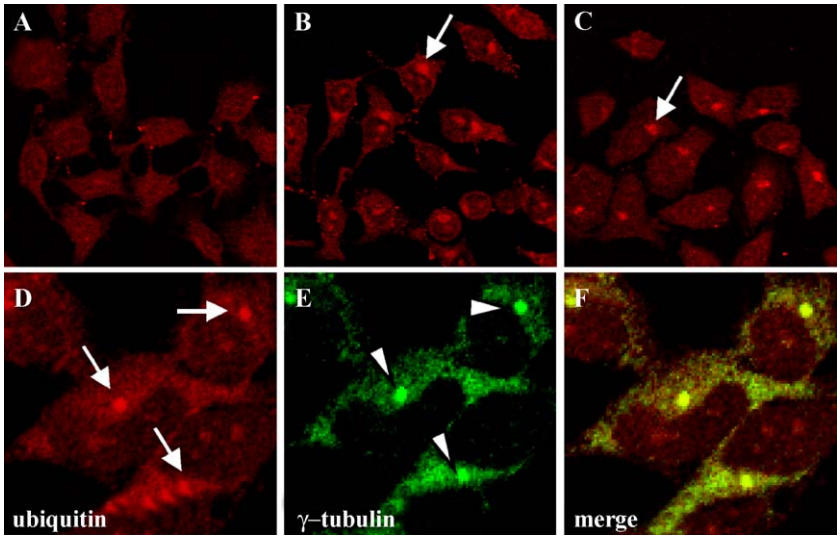

Fig. 6. Immunofluorescence labeling of ubiquitinated inclusions in HeLa cells induced by zinc. HeLa cells were incubated for 12 h with vehicle (A), 20 μM MG132 (B), 10 μM clasto-lactacystin β-lactone (C) or 250 μM zinc (D) in the absence of serum. Note immunocytochemical identification of ubiquitinated inclusions by an anti-ubiquitin antibody. Arrows indicate ubiquitinated inclusion (red) near the nuclei (B–D). In zinc-treated cells, co-localization of ubiquitin (D, arrow) and γ-tubulin, a marker of centromere (E, arrowhead) was evident under confocal microscopy (F). Data shown are representative of four separate experiments with similar results.

in the ubiquitination process [41]. Therefore, possible inhibitions of certain enzymes in the ubiquitin system by zinc may stabilize Ub^{G76V}-GFP. Thus, we performed Western blotting of total ubiquitinated proteins in cells using an anti-ubiquitin conjugate antibody. If zinc inhibition of the ubiquitin system is the major mechanism for zinc to stabilize proteins, no increase in laddering bands of ubiquitinated proteins is expected. As a positive control, MG132 treatment resulted in the accumulation of the ubiquitinated proteins by inhibiting proteasome activity. PDTC or zinc also increased the intensities of laddering ubiquitinated proteins, suggesting that zinc did not exert a global blockade of the ubiquitination machinery to the extent that it could inhibit degradation of proteins (Fig. 7A). On an individual protein, zinc induced accumulation of a polyubiquitinated p53 protein, consistent with the changes noted in the ubiquitination of total proteins upon zinc treatment (Fig. 7B). These findings suggest that zinc affect proteasome-dependent proteolysis mainly by acting on site downstream of ubiquitination processes.

Effect of various metal ions on degradation of Ub^{G76V}-GFP

Metal ions including zinc have been shown to inhibit proteasome activity in vitro enzymatic assays [44,45]. We tested the effects of various metals on ubiquitin/proteasome activity by monitoring changes in fluorescence intensity in HeLa cells stably transfected with Ub^{G76V}-GFP. Copper, zinc, iron and manganese are essential trace metals in humans. Calcium is an important metal in signal transduction. Among metal ions tested, only zinc and copper significantly increased the fluorescence of this GFP reporter (Fig. 8). This finding suggests that zinc and copper may share the similar property in inhibiting proteasome-dependent proteolysis.

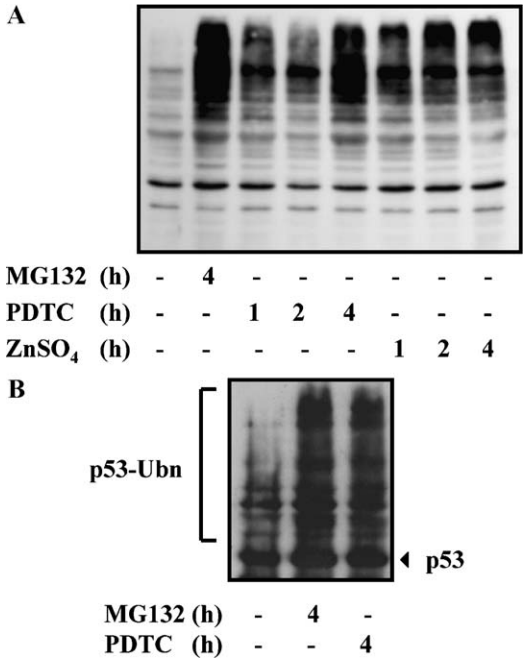

Fig. 7. Effect of MG132, PDTC or zinc on cellular levels of ubiquitinated proteins. HeLa cells were incubated with PDTC or zinc for indicated times. Then, Western blot analysis for ubiquitin-conjugated proteins using an antibody against ubiquitin-protein conjugates (A) or ubiquitinated p53 using an antibody against p53 (B) was performed using total cell lysates. Note substantial increase in cellular levels of ubiquitin-protein conjugates following treatment with MG132 (20 μM), PDTC (100 μM) or zinc (250 μM).

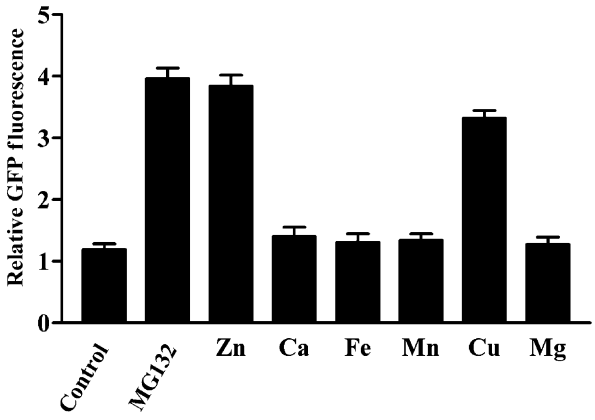

Fig. 8. Effects of various metal ions on ubiquitin-proteasome activity. HeLa-Ub^{G76V}-GFP cells were incubated with various metal ions (each 200 μM) for 12 h. After incubation, cells were lysed with Triton X-100 and then functional GFP in lysates was quantified using spectrofluorophotometer with excitation/emission at 480/510 nm. Data are means ± SE (bars) for three independent experiments, each performed in quadruplicate.

Effect of zinc on proteasomal degradation of p21 and synuclein in vitro

p21 and α-synuclein are the proteins which can be efficiently degraded without ubiquitination [46,47]. Thus, we tested whether zinc can directly inhibit 20S proteasome activity using in vitro assay comprising substrate proteins and 20S proteasomes, but not ubiquitination components. Each fusion protein of p21 and α-synuclein was incubated with purified 20S proteasomes for 1 h. These were subject to Western blot for detecting the change of level of each protein. One-hour incubation of each protein with 20S proteasomes resulted in significant degradation of both proteins (Fig. 9A). Interestingly, addition of zinc inhibited the degradation of p21 and α-synuclein proteins by 20S proteasomes in a dose-dependent manner (Fig. 9B).

Discussion

In the present study, we used HeLa cells stably transfected with Ub^{G76V}-GFP to elucidate the involvement of proteasomal proteolysis system in zinc-induced protein stabilization. In HeLa cells, many of the short-lived proteins accumulated upon treatment with proteasome inhibitors [26]. Thus, we selected this cell line to explore possible zinc actions on proteasomal degradation of proteins. PDTC is the most widely used NF-κB inhibitor. We have previously noted two novel mechanisms that underlie the actions of PDTC: induction of zinc influx [11,12] and prolongation of the half-life of IκBα by inhibiting its degradation (C.H. Kim and Y.S. Ahn, unpublished data). These observations together raise the possibility that zinc may inhibit the ubiquitin-proteasome system. In the present study, PDTC increased the levels of short-lived proteins such as p53, p21, cyclin B1, HIF-1α and c-Jun. An increase in cellular protein levels can result from three different molecular mechanisms, namely, greater stability of protein or mRNA or an increase in mRNA transcription. Results derived from experiments using cycloheximide to suppress de novo protein synthesis suggest that PDTC increased protein stability. However, we cannot exclude the possibility that the increase in cellular protein levels may be a result of a combined effect of the two or three mechanisms described above. PDTC also increased the mRNA level of p21 (I.S. Kim and Y.S. Ahn, unpublished data). Similar findings were observed in human colon cancer cell lines in which an increase in both p21 mRNA expression and p21 protein stability has been noted upon proteasome inhibition [37]. Thus, it is conceivable that an increase in p21 mRNA by PDTC is a consequence of stabilization of certain transcription factors such as c/EBPs or other upstream signaling molecules [5,33]. However, it is evident from the experiments with cycloheximide treatment that PDTC increased the protein levels mainly by prolonging their half-lives.

The ubiquitin-proteasome system, calpains and lysosomal proteases are the three major protease systems that regulate the proteolysis of intracellular proteins in mammalian cells. Experiments using inhibitors of these three major categories of proteases (Fig. 3) revealed that the proteasome was the most probable protease system that governed the degradation of short-lived proteins we have tested in HeLa cells. Although proteasome inhibitors have proved to be valuable for dissecting the cellular roles of the proteasome system, their specificities are not sufficient to delineate the particular specific protease system that is responsible for the degradation of the short-lived proteins in our system. For this reason, we used Ub^{G76V}-GFP-transfected HeLa cell line. This cell line is a useful and specific tool for screening

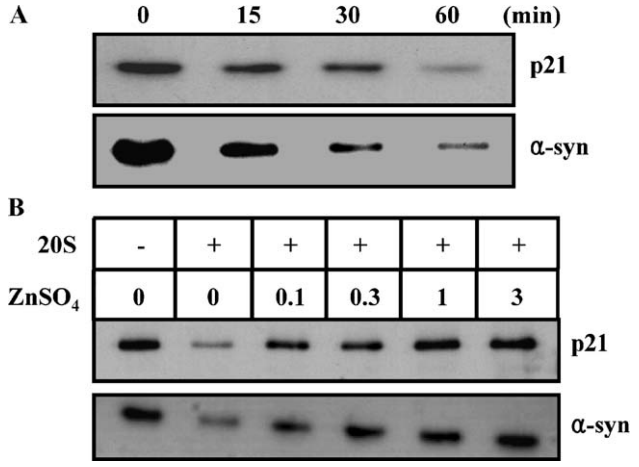

Fig. 9. Effect on zinc on 20S proteasomal breakdown of p21 and α-synuclein in vitro. Recombinant p21 (400 nM) and α-synuclein (100 nM) proteins were incubated with purified 20S proteasomes (40 nM) up to 1 h (A). Indicated concentrations of $ZnSO_4$ (μM) were added to the reaction tubes containing 20S proteasomes and the substrate proteins (B). The reaction was stopped by adding SDS-PAGE sample buffer. Immunoblots were developed with anti-p21 antibody and anti-α-synuclein antibody.

the agents that inhibit ubiquitin/proteasome-dependent proteolysis in living cells [41]. It allowed us to study proteasome activity noninvasively. Previously, in vitro data, which demonstrate that zinc inhibits purified proteasomes, were provided [45]. However, this noninvasive experiment much more strongly suggests that zinc can inhibit proteasome-dependent proteolysis in living cells. This contention is supported by the finding that zinc induced formation of ubiquitinated inclusions in the perinuclear region in HeLa cells. It has been demonstrated that proteasome inhibitors could alter the subcellular localizations of ubiquitinated nondegradable proteins and proteasome machinery [42,48]. In particular, centrosome has been considered to be the subcellular center of protein degradation and is enriched in proteasomal components [42,43]. Upon proteasome inhibition, it enlarges in size and recruits ubiquitin, Hsp70, PA700, PA28 and the 20S proteasome. Based on the characteristic localization of ubiquitinated proteins and expansion of the centrosome in HeLa cells, it appears that zinc inhibit proteasome-dependent proteolysis.

The exact mechanism by which zinc inhibits proteasome-dependent proteolysis remains to be fully elucidated. Accumulation of Ub^{G76V}-GFP reporter upon zinc treatment only reflects that zinc inhibited one of the degradation processes such as ubiquitination, protein unfolding or degradation. Although ubiquitinated proteins were not reduced in amount upon zinc treatment, we cannot exclude the possibility that zinc can also inhibit the ubiquitination system. If the accumulation of ubiquitinated proteins by zinc was faster enough for masking possible inhibition of the ubiquitination system by zinc, there could be no increase in ubiquitinated proteins. However, this does not contradict our hypothesis that the main target of zinc for stabilizing proteins is downstream of ubiquitination processes. It is possible that zinc inhibited isopeptidases that cleave the isopeptide bond between ε-amino groups of lysines in substrate and the C-terminus ubiquitin to upregulate the size of the pool of ubiquitinated proteins. We did not test if zinc inhibits isopeptidases. However, isopeptidase activity is generally considered to be a means of preventing protein degradation by proteasomes. It is plausible to think that possible inhibition of isopeptidases by zinc would rather decrease the stabilities of proteins we tested. Interestingly, a study showed that zinc is required for the catalytic activity of the human isopeptidase T [49].

Recent in vitro data from brain lysates denote that zinc can inhibit proteasome activity through direct interaction with certain subunits of the proteasome system [45]. Results derived from Ub^{G76V}-GFP experiments, histochemical analysis of ubiquitinated inclusions, ubiquitination assays and protease inhibitor experiments in this study suggest that zinc would inhibit the final clearance of ubiquitinated proteins in the ubiquitin-proteasome system. Inhibition of breakdowns of p21 and α-synuclein by zinc in vitro and stabilization of p21 by zinc in cells indicate that 20S proteasomes could be the target of zinc because these substrates are natively disordered and degraded by the proteasome without ubiquitination [46,47].

Very recently, Bloom et al. [50] showed that only free p21 is degraded in the absence of ubiquitination and p21 bound to another protein requires ubiquitination for its degradation. Thus, further research is required to determine whether zinc inhibits both proteasome itself and non-proteasomal modulatory proteins in vivo.

Among various metal ions tested, only zinc and copper showed similar inhibitory effects on proteasome activity. Copper is also capable of inhibiting NF-κB activation under selected conditions, suggesting it may also mediate PDTC actions [9]. This finding raises the possibility that copper may share the same action of zinc in regulating protein stability. In agreement with this contention, copper was also shown to inhibit protease activity of the 20S proteasome derived from brain homogenates in vitro [45]. This suggests that certain common chemical properties of these two metal ions may be involved in proteasome inhibition. Like zinc, copper is also involved in free radical-induced toxicity and amyloid pathology, so its ability to inhibit proteasomes need to be assessed in association with human diseases. In the meantime of our experiment, two studies reported that cadmium and lithium inhibited proteasomes and stabilized proteins such as Nrf2 and C/EBPα [51,52]. Although these studies are also based on in vitro proteasome degradation assay and proteasome inhibitor experiment, they strongly support the notion that there would be a group of metal that can inhibit proteasomes to modulate cellular functions.

From the perspective of pathophysiological significance, any metal ion that has been linked to a variety of disease processes will be of a primary concern. Zinc has been implicated in the pathogenesis of diabetes, prostate cancer and various neurodegenerative diseases. Among these disorders, zinc cytotoxicity is mostly well-established in neuronal degeneration following central nervous system injury. Intraventricular injection of zinc chelator reduced the accumulation of zinc in postsynaptic neurons and resultant neurotoxicity after transient global cerebral ischemia [53]. In traumatic neuronal injury, chelation of zinc before this insult reduced the extent of neuronal death [19]. Zinc has also been implicated in the pathogenesis of Alzheimer's disease. For examples, a copper–zinc chelator reduced the development of β-amyloid (Aβ) plaques in transgenic mice overexpressing APP [54]. Intriguingly, the insight is emerging that proteotoxicity due to disruption of the ubiquitin-proteasome pathway may play an important role in neurodegeneration. It has been reported that processing and degradation of presenilin-1 are regulated by the proteasome [55]. Proteasome inhibition by lipid peroxidation product or paired helical filament-tau contributes to neurodegeneration [56,57]. There are also clinical data supporting this notion. For example, autopsied brains of patients with Alzheimer's disease showed significantly decreased proteasome activities when compared with age-matched control [58]. Proteasome activity in substantia nigra of patients with

Parkinson's disease was reduced [59]. Taken together, inhibition of proteasome-dependent proteolysis by zinc may be of pathophysiological significance in selected neurodegenerative disorders. Understanding zinc inhibition of proteasomal degradation may add in future delineation of the molecular mechanisms that underlie the neurodegenerative processes of diseases such as Alzheimer's disease, Parkinson's disease, stroke or traumatic head injury.

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