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Research Paper

Proteasome inhibitor-induced cleavage of HSP90 is mediated by ROS generation and caspase 10-activation in human leukemic cells

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ABSTRACT

Heat shock protein 90 (HSP90) is a molecular chaperone that supports the stability of client proteins. The proteasome is one of the targets for cancer therapy, and studies are underway to use proteasome inhibitors as anti-cancer drugs. In this study, we found that HSP90 was cleaved to a 55 kDa protein after treatment with proteasome inhibitors including MG132 in leukemia cells but was not cleaved in other tissue-derived cells. HSP90 has two major isoforms (HSP90α and HSP90β), and both were cleaved by MG132 treatment. MG132 treatment also induced a decrease in HSP90 client proteins. MG132 treatment generated ROS, and the cleavage of HSP90 was blocked by a ROS scavenger, N-acetylcysteine (NAC). MG132 activated several caspases, and the activation was reduced by pretreatment with NAC. Based on an inhibitor study, the cleavage of HSP90 induced by MG132 was dependent on caspase 10 activation. Furthermore, active recombinant caspase 10 induced HSP90 cleavage *in vitro*. MG132 upregulated VDUP-1 expression and reduced the GSH levels implying that the regulation of redox-related proteins is involved. Taken all together, our results suggest that the cleavage of HSP90 by MG132 treatment is mediated by ROS generation and caspase 10 activation. HSP90 cleavage may provide an additional mechanism involved in the anti-cancer effects of proteasome inhibitors.

1. Introduction

Heat shock protein 90 (HSP90), a member of the heat shock protein family, is found in most organisms, and its expression is increased when cells are exposed to various stresses [1]. HSP90 is a molecular chaperone that contributes to protein folding and stability. Its client proteins are p53, Bcr-Abl, Raf-1, Akt, HER2/Neu (ErbB2), HIF-1α, etc. [2]. In particular, many onco-proteins (Her2, v-Src, Raf-1, Akt, hTERT, etc.) are also client proteins of HSP90; therefore, most cancer cells express higher levels of HSP90 compared with normal cells [3–5]. In addition, HSP90 contributes to the malignant transition of tumor cells [6]. Therefore, HSP90 is studied as an anti-cancer drug target [3,7–9].

HSP90 is conserved evolutionarily and has two isoforms, HSP90α and HSP90β, in the human cytosol [10]. The HSP90 monomer is comprised of a N-domain, M-domain and C-domain, and the N-domain has an ATP-binding pocket and ATPase activity [11]. HSP90 forms a flexible dimer by C-terminal dimerization, and this structure is important in the ATPase cycle for chaperone activity [12,13]. Binding of ATP to the N-domain promotes dimerization of the two N-domains, and the hydrolysis of ATP to ADP by the ATPase activity of the N-domain promotes N-domain dissociation [14–16]. Co-chaperones recruit client proteins to HSP90 and regulate the ATPase activity of HSP90 by co-chaprone-HSP90 interactions [13,17]. The chaperone function of HSP90 is also regulated by acetylation/deacetylation of K294(HSP90α)/K287(HSP90β) in the M-domain. K294/K287 are deacetylated by histone deacetylase (HDAC) 6, and in this state, HSP90 acts as a molecular chaperone. Acetylation of K294/K287 decreases the affinity of HSP90 to interact with client proteins, and thus, the client proteins are degraded [18,19]. Cleavage of HSP90 is another axis of HSP90 regulation. HSP90 cleavage is induced by various stimuli such as UVB irradiation [20], ascorbate/menadione [21,22], andrographolide [23] and HDAC inhibitors [24]. Even though the cleavage is induced by activating the Fas/Fas ligand axis in the case of UVB irradiation [20], most of the stimuli induce HSP90 cleavage by increasing oxidative stress [21–24]. The cleavage of HSP90 results in 55 kDa [20,23,24] or 70 kDa [21,22] fragments depending on the type of stimulus and involved mechanism. HSP90 down-regulation by cleavage leads to client protein decrease and cell death [20,21].

The extent of oxidative stress that cells face with is generally represented as the levels of reactive oxygen species (ROS) and counteracting anti-oxidants. Cellular ROS are mainly generated in the mitochondrial electron transport chain and are also produced by the NADPH oxidase family and metabolic pathways [25,26]. ROS are important regulators of apoptosis [27]. When cells are exposed to a high

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dose of ROS, they are subjected to apoptosis by caspase activation. [28,29]. On the other hand, ROS promotes cell growth, survival, regulation of cellular signaling and cell number homeostasis depending on the dose [28,30]. Cancer cells maintain an abnormally high level of ROS compared to normal cells by an aberrant metabolism and protein translation for additional mutations and adaptations. At the same time, cancer cells prevent excessively high levels of ROS by regulating antioxidant proteins [31]. Thioredoxin (Trx) is a potent antioxidant regulating intracellular redox in mammalian cells [32]. Vitamin D upregulated Protein 1 (VDUP-1; TBP-2, Txnip) was originally isolated as a negative regulator of Trx [33]. Later, VDUP-1 was proved to be is a multifunctional protein acting as a growth suppressor and a regulator in lipid metabolism [32].

Proteasomes are protein complexes involved in the degradation of ubiquitinated proteins. Proteasomes have been investigated as cancer therapeutic targets [34]. MG132 (carbobenzoxy-Leu-Leu-leucinal) is a substrate analogue and a potent transition-state inhibitor primarily of the chymotrypsin-like activity of the proteasome [35]. Proteasome inhibitors including MG132 inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NK-κB), increase pro-apoptotic proteins, arrest cell cycle, induce ER stress and mitochondria dysfunction, impair DNA-damage responses, and inhibit angiogenesis [34,36]. Most importantly to our hypothesis, they induce apoptosis through ROS generation [37]. Considering that ROS is one of important factors for HSP90 cleavage, we postulated that proteasome inhibitors can also induce cleavage of HSP90 and this function may contribute to the anticancer effect of proteasome inhibitors.

In this study, we found that HSP90 was cleaved after treatment with proteasome inhibitors in leukemia cell lines. We also revealed that the cleavage of HSP90 by MG132 was mediated by VDUP-1 accumulation, ROS generation and caspase 10 activation in K562 cells.

2. Materials and methods

2.1. Cell culture

K562 (chronic myelogenous leukemia) cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO2. KG1a (human acute myelogenous leukemia) cells were maintained in IMDM with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HT-29, HCT-116, LoVo (human colorectal adenocarcinoma), MCF-7, T47D (human breast cancer), Huh-7, and SNU-739 (human hepatocellular carcinoma) cells were maintained in RPMI-1640 with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were treated with proteasome inhibitors at the indicated concentration and for the indicated time. Inhibitors were pretreated 1 h prior to the MG132 treatment, if necessary.

2.2. Chemicals and antibodies

N-acetylcysteine (NAC), dimethyl sulfoxide (DMSO), and z-VAD-fmk were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG132 was purchased from ALEXIS (Lausen, Switzerland). Caspase-family inhibitor set III was from BioVision (Milpitas, CA, USA). The caspase-family inhibitor set includes 8 kinds of inhibitors as followings. Caspase 1 inhibitor, Z-YVAD-FMK; Caspase 2 inhibitor, Z-VDVAD-FMK; caspase 3 inhibitor, Z-DEVD-FMK; caspase 5 inhibitor, Z-WEHD-FMK; caspase 6 inhibitor, Z-VEID-FMK; caspase 8 inhibitor, Z-IETD-FMK; caspase 9 inhibitor, Z-LEHD-FMK; caspase 10 inhibitor, Z-AEVD-FMK.

The monoclonal antibodies anti-HSP90α/β (sc-13119), anti-Bcl-2 (sc-7382), anti-GAPDH (sc-32233), and anti-Raf-1 (sc-7267) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibody anti-VDUP-1(K0205-3) was from MBL International Corporation (Woburn, MA, USA), and anti-HSP90α (AHP1339) was from Bio-Rad (Hercules, CA, USA), respectively. The polyclonal antibodies anti-Akt (#9272) and anti-HSP90β (#7411) were from Cell Signaling Technology (Beverly, MA, USA).

2.3. Western blot analysis

Harvested cells were lysed in a lysis buffer (pH 8.0, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 10 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, protease inhibitor cocktail, and phosphatase inhibitor). Samples were resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% dry milk in phosphate buffered saline-Tween-20 (PBS-T; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.05% Tween-20) and probed with an appropriate primary antibody. Immunoreactive proteins were detected by horseradish peroxidase-conjugated anti-rabbit or anti-mouse (Santa Cruz Biotechnology) secondary antibodies and an ECL solution (iNtRon, Seongnam, Korea or ATTO, Tokyo, Japan).

2.4. Measure of intracellular ROS

For the measurement of the intracellular ROS levels, an oxidation-sensitive fluorescent probe, 2,7-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR, USA), was used as described previously [38] with a minor modification. K562 cells were untreated or treated with 20 mM of NAC for 1 h, followed by treatment with 5 μM of MG132 for 24 h. After treatment, the cells were incubated with 10 μM of DCF-DA for 20 min at 37 °C. The fluorescence images were obtained under fluorescence microscopy (Nikon, Tokyo, Japan).

2.5. Measure of intracellular glutathione (GSH)

The GSH Colorimetric Assay Kit (BioVision) was used to measure the caspase activities according to the manufacturer's instructions. K562 cells were untreated or treated with the indicated doses of MG132 for 24 h. The cells were then incubated with glutathione reductase and the GSH substrate for 5 min. The absorbance values were obtained with a microplate reader (Bio-Rad) at a wavelength of 405 nm.

2.6. Measurement of the caspase activity

The Caspase Colorimetric Substrate Set II Plus (BioVision) was used to measure caspase activities according to the manufacturer's instructions. K562 cells were untreated or treated with 20 mM of NAC for 1 h, followed by incubation with 5 μM of MG132 for 24 h. The cells were then incubated with 200 μM of the indicated caspase family inhibitors for 2 h at 37 °C. The absorbance values were obtained with a microplate reader (Bio-Rad) at a wavelength of 405 nm.

2.7. In vitro HSP90 cleavage assay

K562 cells were dissolved in a lysis buffer. The cell lysates were untreated or treated with 2 μM of the indicated caspase inhibitor for 15 min, followed by incubation with 2U of active recombinant caspase 10 (BioVision) at 37 °C for 4 h in a reaction buffer (50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% Glycerol, and 10 mM DTT). After incubation, HSP90 was detected by Western blot analysis.

2.8. Reverse transcription PCR

Total RNA was isolated with TRI Reagent® according to the manufacturer's instructions (MRC, Cincinnati, OH, USA). Two micrograms of total RNA were reverse-transcribed in the first-strand synthesis buffer containing 6 μg/ml oligo(dT) primer, 50 U M-MLV reverse transcriptase, 2 mM dNTP, 10 mM DTT, and 40 U RNaseOUT recombinant

ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA). The reaction was done at 37 °C for 50 min, and heat inactivated at 70 °C for 15 min. One microliter of synthetic cDNA was subjected to a standard PCR reaction of 20 cycles consisting of denaturation for 40 s at 95 °C, annealing for 40 s at 58 °C, and extension for 40 s at 72 °C. The primer set sequences used are as follows: GAPDH, 5'-TCC ACC ACC CTG TTG CTG TA-3' (sense) and 5'-ACC ACA GTC CAT GCC ATC AC-3' (anti-sense) (product size 452 bp); VDUP1, 5'-CAG CCA ACA GGT GAG AAT GA-3' (sense) and 5'-AGG GGT ATT GAC ATC CAC CA-3' (anti-sense) (product size 223 bp), and Thioredoxin, 5'-GAG AGC AAG CAG CGA GTC TT-3' (sense) and 5'-TTG GCT CCA GAA AAT TCA CC-3' (anti-sense) (product size 371 bp).

2.9. Real-time PCR

For a quantitative analysis of mRNA expression, real-time PCR analysis was done with the iQ™ SYBR® Green Supermix (Bio-Rad) and Rotor-Gene Q Real-Time PCR Detection System (Qiagen, Hilden, Germany). The primers are the same as those for the RT-PCR. The mRNA levels were normalized using GAPDH as an internal control, and the relative expression was determined by dividing all normalized values within a data set by the normalized arbitrary units of untreated K562 cells.

3. Results

3.1. Proteasome inhibitors induce HSP90 cleavage

To determine the effects of proteasome inhibitors on HSP90, the leukemia cell line K562 was treated with proteasome inhibitors (MG132 and epoxomicin). After exposure of the cells to the indicated doses of proteasome inhibitors for 24 h, Western blot analysis was performed. The proteasome inhibitors induced the cleavage of HSP90 to a molecular weight of approximately 55 kDa in a dose-dependent manner (Fig. 1A). The anti-HSP90 antibody that was used in this study recognizes the C-terminal domain of HSP90; therefore, the cleavage site is estimated to be in the middle domain of HSP90. Because MG132 is a typically used proteasome inhibitor, MG132 was used for the rest of the experiments. The cleavage of HSP90 induced by MG132 was also increased in a time-dependent manner (Fig. 1B). We further checked the cleavage of the HSP90 isoforms (HSP90α and HSP90β) using a specific antibody for each isoform. The MG132-induced HSP90 cleavage mainly occurred on HSP90β and partially in the case of HSP90α (Fig. 1C). Because the cleaved HSP90 is presumed to have a defect in its molecular chaperone activity, we examined the stability of HSP90 client proteins after the MG132 treatment. The expression levels of the HSP90 client proteins (Akt, Bcl-2 and Raf-1) were decreased by the MG132 treatment in a dose-dependent manner (Fig. 1D). These data suggest that proteasome inhibitors, including MG132, induce HSP90 cleavage and the instability of HSP90 client proteins.

3.2. Effect of MG132 on various cell lines

To determine whether the MG132 induced HSP90 cleavage is cell-type specific, various cell lines were treated with MG132. KG1a is a human leukemia cell line. HCT-116, HT-29 and LoVo are human colorectal adenocarcinoma cell lines. MCF-7 and T47D are human breast cancer cell lines. Huh-7 and SNU-739 are human hepatocellular carcinoma cell lines. MG132 induced HSP90 cleavage in a dose-dependent manner in the KG1a cells (Fig. 2A); however, there was no change in HSP90 in the other tissue-derived cells (HCT-116, HT-29, LoVo, MCF-7, T47D, Huh-7 and SNU-739) (Fig. 2B–D). These data together with Fig. 1 suggest that the proteasome inhibitor-induced HSP90 cleavage may be dependent on cell type and that the cleavage of HSP90 may occur mainly in leukemia cells.

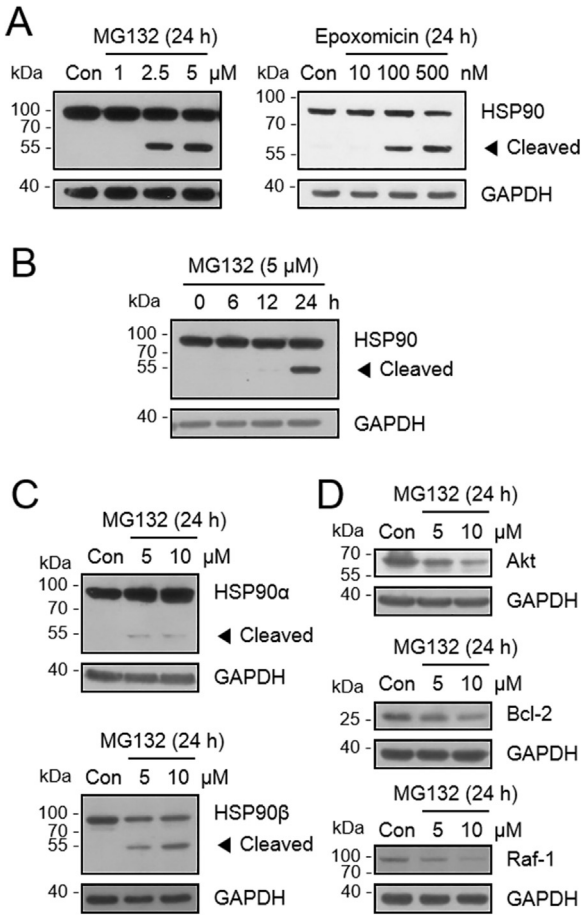

Fig. 1. Proteasome inhibitors induce cleavage of HSP90. A K562 cells were treated with the indicated dose of MG132 and epoxomicin for 24 h. B K562 cells were treated with 5 μM of MG132 for the indicated time periods. C, D K562 cells were treated with the indicated dose of MG132 for 24 h. The cell lysates were subjected to Western blot analysis using the indicated antibodies. The amounts of GAPDH protein are shown as a loading control. The arrowhead indicates the cleaved HSP90 fragment.

3.3. HSP90 cleavage is induced by ROS generation and caspase activation

According to the previous reports published by other investigators, 55 kDa fragment of HSP90 was generated by caspase activation [20,23]. In our previous study, we found that suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, induced HSP90 cleavage to 55 kDa fragment by ROS generation and caspase activation [24]. The HSP90β consists of 724 amino acids and four aspartic acid residues (252th, 259th, 278th and 294th amino acids) are predicted to be cleavable by caspase and to form a 55 kDa fragment. In contrast, approximately 70 kDa fragment of HSP90 was obtained by a non-enzymatic mechanism involving ascorbate/menadione and iron [21,22]. The cleavage site is at the highly conserved N-terminal motif [22]. As shown in Figs. 1 and 2, treatment with proteasome inhibitors in leukemic cells induced cleavage of HSP90 to 55 kDa fragment. Therefore, we examined whether HSP90 cleavage by MG132 treatment was affected by ROS generation and caspase activation.

First, we investigated the generation of ROS after MG132 treatment in K562 cells. The MG132-treated K562 cells generated ROS, and pretreatment with NAC, an antioxidant, decreased the MG132-induced ROS generation (Fig. 3A). To further determine whether ROS mediates the MG132-induced cleavage of HSP90, K562 cells were treated with MG132 after pretreatment with NAC, and then, the cleavage of HSP90 was checked. The MG132-induced HSP90 cleavage was reduced by the NAC pretreatment in a dose-dependent manner (Fig. 3B). Next, to determine whether the MG132-triggered active caspase induces HSP90

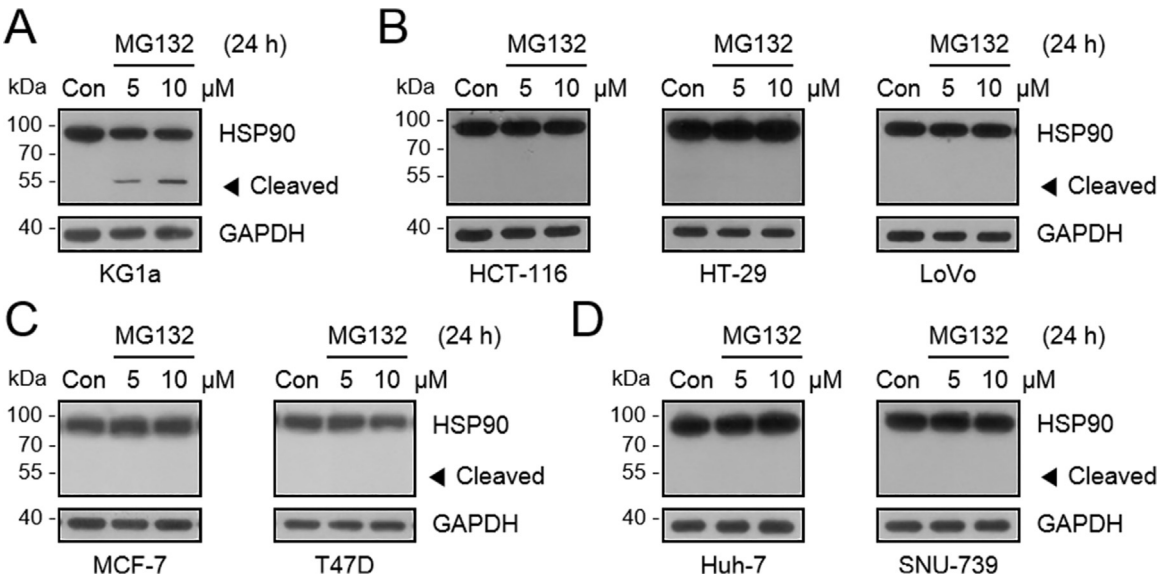

Fig. 2. Effect of MG132 on the cleavage of HSP90 in various tissue-derived cell lines. A Human leukemia cell line. B Human colorectal cancer cell lines. C Human breast cancer cell lines. D Human hepatocellular carcinoma cell lines. The cells were treated with MG132 at the indicated doses for 24 h and analyzed by Western blot analysis.

cleavage, the K562 cells were pretreated with Z-VAD-FMK, a pan-caspase inhibitor, before the MG132 treatment of the cells. As shown in Fig. 3C, the cleavage of HSP90 was blocked by Z-VAD-FMK confirming that the process is caspase dependent. These data suggest that the MG132-induced HSP90 cleavage is dependent on ROS generation and caspase activation.

3.4. MG132-induced ROS generation is mediated by VDUP-1 up-regulation and GSH down-regulation

VDUP-1 and GSH are important factors in ROS regulation [32,39]. VUDP-1 is a negative regulator of Trx, which acts as an antioxidant [40]. MG132 was previously reported to up-regulate the VDUP-1 level and down-regulate the GSH level in other tissue-originated cell lines [41,42]. Therefore, we assessed the level of VDUP-1, Trx and GSH in MG132 treated K562 cells. Based on the results from RT-PCR and quantitative real time PCR, the mRNA expression levels of the VDUP-1 and Trx genes did not significantly change by the treatment with MG132 (Fig. 4A and B). On the other hand, the protein level of VDUP-1 was increased after treatment with MG132 in a dose- and time-dependent manner (Fig. 4C). As shown in Fig. 4D, the GSH level was decreased by the MG132 treatment in a dose-dependent manner. These results suggest that the MG132-induced up-regulation of VDUP-1 and down-regulation of GSH contribute to the generation of ROS and the cleavage of HSP90 in K562 cells.

3.5. Caspase 10 activation by ROS induces HSP90 cleavage

Because we confirmed that HSP90 cleavage is mediated by ROS generation and caspases (Fig. 3B and C), we next determined which members of caspases are involved in the cleavage of HSP90. First, we measured the activity of each caspase family member in the MG132-treated K562 cells using caspase family substrates (Fig. 5A). The activities of caspase 2, 3, 4, 6, 8, 9 and 10 were increased by MG132 treatment, and the activities were decreased by the pretreatment of NAC. Next, we checked the HSP90 cleavage by MG132 in the presence of caspase family-specific inhibitors (Fig. 5B). The MG132-induced HSP90 cleavage was almost completely blocked by the pan-caspase inhibitor and caspase 10 inhibitor suggesting that caspase 10 can be the major player. However, the caspase 1 inhibitor also markedly blocked

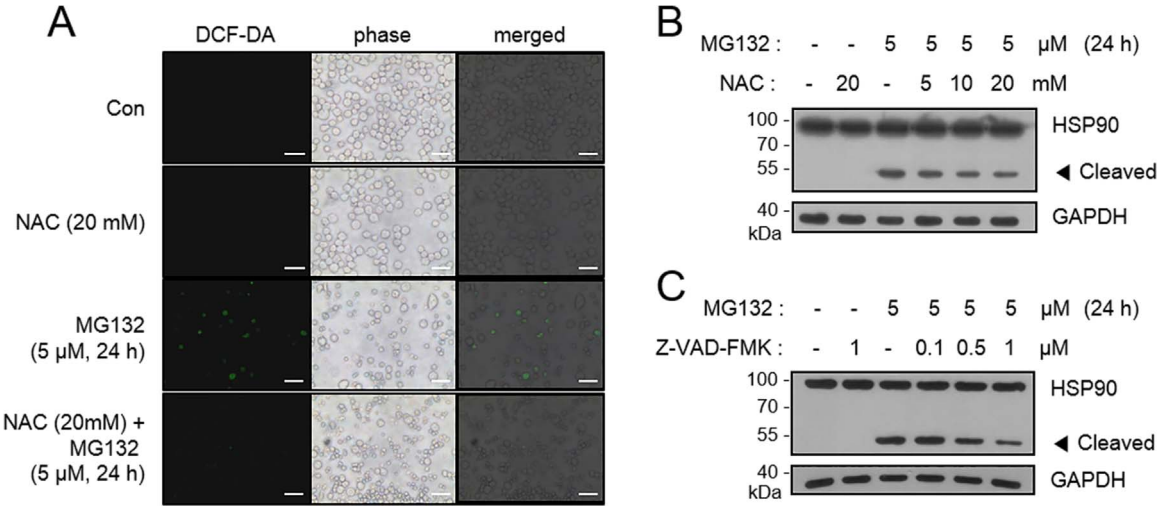

Fig. 3. ROS generation by MG132 induces caspase-mediated cleavage of HSP90. A K562 cells were untreated or treated with 20 mM of NAC for 1 h, followed by treatment with 5 μM of MG132 for 24 h. The cells were incubated with 10 μM of the fluorescent probe DCF-DA for 20 min and visualized with fluorescence microscopy. Scale bar. 300 μm. B, C K562 cells were untreated or treated with the indicated dose of NAC or Z-VAD-FMK for 1 h, followed by treatment with 5 μM of MG132 for 24 h. The cell lysates were subjected to Western blot analysis.

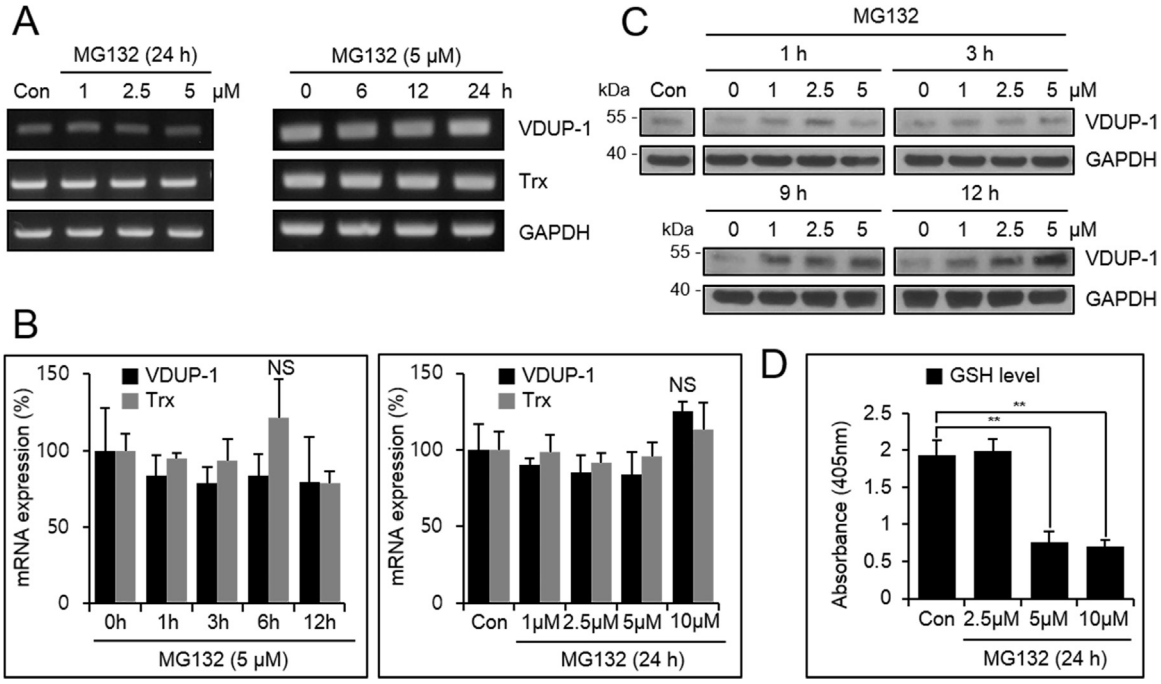

Fig. 4. MG132 induces the up-regulation of VDUP-1 and the down-regulation of GSH. A, B K562 cells were treated with the indicated dose of MG132 for 24 h (left) or 5 μM of MG132 for the indicated time (right). After extraction of the total RNA, the expression of the VDUP-1 and Trx genes were analyzed by RT-PCR (A) and quantified by real-time PCR (B). The levels of the VDUP-1 and Trx mRNA were normalized with respective GAPDH mRNA levels. C K562 cells were treated with the indicated dose of MG132 for the indicated time. The cell lysates were subjected to Western blot analysis. D K562 cells were treated with the indicated dose of MG132 for 24 h. The cell lysates were incubated with glutathione reductase and the GSH substrate, and the absorbance values were measured at a wavelength of 405 nm. Values are the means ± SD. ** $P < 0.01$ vs. control.

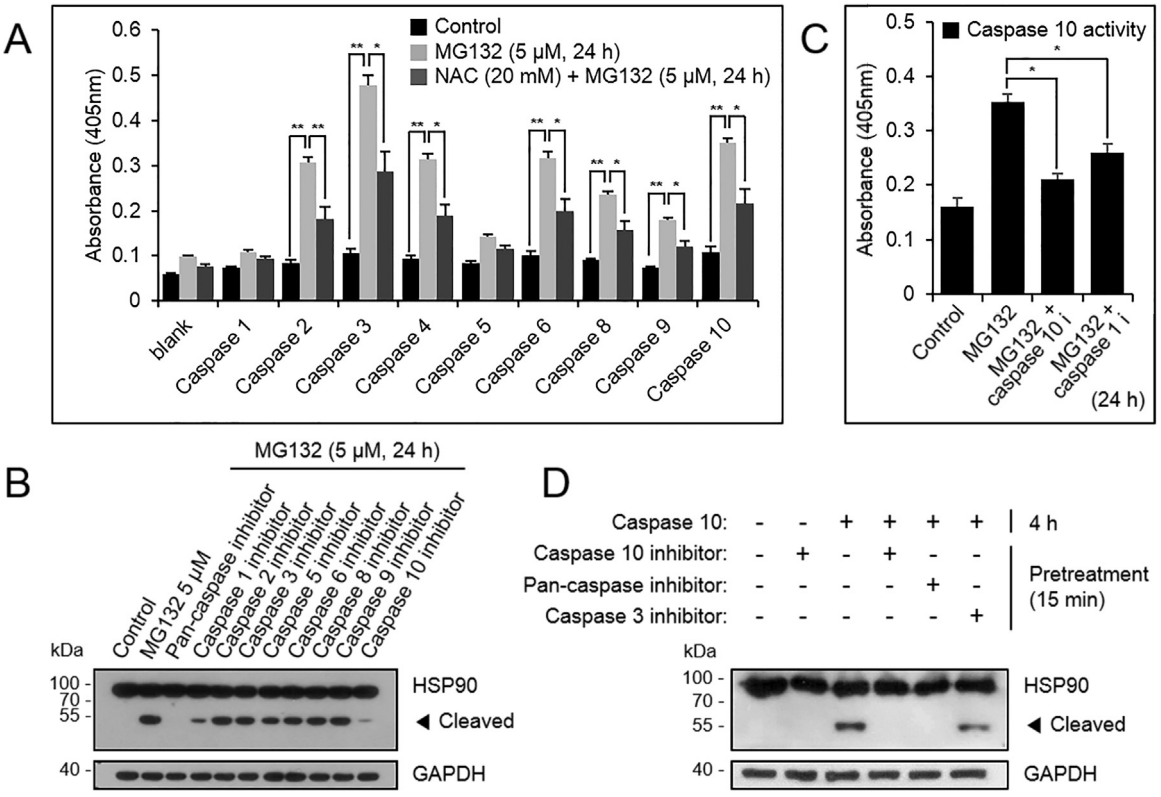

Fig. 5. MG132-induced HSP90 cleavage is mediated by caspase 10 activation. A K562 cells were untreated or treated with 20 mM of NAC for 1 h, followed by treatment with 5 μM of MG132 for 24 h. The cell lysates were incubated with each colorimetric caspase substrate, and activation of each caspase was monitored by measuring color development at a wavelength 405 nm. B K562 cells were untreated or treated with 1 μM of the indicated caspase family inhibitors for 1 h, followed by treatment with 5 μM of MG132 for 24 h. The cell lysates were subjected to Western blot analysis. C K562 cells were untreated or treated with 1 μM of caspase 1 inhibitor (1 i) or caspase 10 inhibitor (10 i) for 1 h, followed by treatment with 5 μM of MG132 for 24 h. The caspase 10 activity was measured with the cell lysates. D K562 whole cell lysates were untreated or treated with 2 μM of indicated caspase inhibitor for 15 min, followed by incubation with 2 U of active recombinant caspase 10 for 4 h. The mixtures were subjected to Western blot analysis. Values are the means ± SD. * $P < 0.05$, ** $P < 0.01$ vs. control.

the MG132-mediated HSP90 cleavage. Caspase 1 is known to be involved in the inflammatory reaction rather than in the apoptosis process [43]. Furthermore, caspase family-specific inhibitors have cross-reactivity among caspases [44]. Therefore, we checked whether caspase 1 inhibitor could reduce caspase 10 activity. As we expected, the caspase 10 activity, increased by MG132, was significantly reduced by the caspase 1 inhibitor as well as caspase 10 inhibitor (Fig. 5C). To further confirm whether caspase 10 is directly involved in the cleavage of HSP90, we incubated untreated K562 cell lysates with active recombinant caspase 10 and found that HSP90 was cleaved by active recombinant caspase 10 *in vitro*. This phenomenon was blocked by caspase 10 inhibitor and pan-caspase inhibitor, but not by caspase 3 inhibitor supporting the specific function of caspase 10 (Fig. 5D). Taken together, these data suggest that the MG132-induced HSP90 cleavage occurs through caspase 10 activation.

4. Discussion

HSP90 is a molecular chaperone that escorts proteins for proper folding and stable structure formation of its client proteins. Because many HSP90 client proteins are necessary for the survival and growth of cancer cells, most cancer cells express higher levels of HSP90 compared with normal cells [2–5]. Therefore, HSP90 cleavage may block the molecular function of HSP90 and result in a decrease of the client proteins and an increase of apoptosis in cancer cells. Here, we found that proteasome inhibitors, including MG132, induce the cleavage of HSP90 in leukemia cell lines and revealed that the MG132-induced cleavage of HSP90 is mediated by ROS generation and caspase 10 activation. We also found that MG132 causes a decrease in the HSP90 client proteins. Based on previous reports, HSP90 cleavage is induced by various stimuli [20–24]. Here, we added proteasome inhibitors as a new stimulus to cause HSP90 cleavage. We speculate that HSP90 cleavage can be induced by a wide spectrum of reagents including commonly used biochemical inhibitors; therefore, we will extend our study to test our hypothesis in the near future.

Proteasome inhibitors are one of the substances studied as anti-cancer drugs. Many studies on the anti-cancer effects of proteasome inhibitors in cancer cells as well as clinical trials using them are underway [34]. Bortezomib (VELCADE®), a proteasome inhibitor that induces apoptosis by ROS generation, was used for chemotherapy to treat myeloma [45–48]. MG132 enhanced the chemotherapeutic sensitivity to DOX-mediated apoptosis in human lymphoma-derived U937 cells [49]. Based on our results, HSP90 cleavage induced by MG132 seems to be cell type-specific found only in leukemic cells. Therefore, the anti-cancer effect of MG132 in leukemic cells may be related with HSP90 cleavage, at least partly. However, the anti-cancer effect of MG132 can also be found in other types of cancer cells. MG132 inhibits the growth of HeLa cells through cell cycle arrest and cell apoptosis. Furthermore, the depletion of the intracellular GSH level and the increase of the ROS level were observed in MG132-treated HeLa cells [37]. Combination of Celecoxib (nonsteroidal anti-inflammatory drug, Celebrex®) and MG132 provided a synergetic effect on anti-proliferation and apoptosis in liver cancer cells [50]. The combination of proteasome inhibitor and HSP90 inhibitor was tried for cancer therapy and the results showed enhanced anti-cancer effects than monotherapy in rhabdomyosarcoma cells and non-small cell lung cancer [51–53]. Considering the effects of MG132 in other types of cancer cells together with our results, it will be helpful to check HSP90 cleavage in cells when MG132 is treated in combination with other anti-cancer reagents for a better understanding of the cell type specificity and perhaps synergistic effect.

VDUP-1, a negative regulator of Trx, inhibits cell growth and metastasis and contributes to apoptosis [32]. VDUP-1 decreases mouse double minute 2 homolog (MDM2)-mediated p53 ubiquitination leading to p53 stabilization and an increaisse of the p53 activity [54]. In human primary tumor tissues and human cancer cell lines, VDUP-1 expression is downregulated or lost, and the abnormal expression of VDUP-1 contributes to tumorigenesis [55]. Trx is one of the major antioxidants regulating the intracellular homeostasis of redox in mammalian cells [32]. In various types of cancers, Trx expression is elevated, and Trx induces hypoxia inducible factor-1α (HIF-1α) and blocks the generation of ROS [32]. VDUP-1 regulates the generation of ROS by direct interaction with the catalytically active center of Trx [56,57]. Overexpression of VDUP-1 was also reported to reduce the expression level of Trx [58]. GSH is an important factor in ROS regulation: it acts as an antioxidant by directly interacting with ROS [59]. Our data revealed that the protein level of VDUP-1 was significantly increased after treatment with MG132 in K562 cells although the mRNA level of VDUP-1 was not changed. Because the VDUP-1 protein level is regulated by E3 ubiquitin ligase Itch-mediated ubiquitination and proteasomal degradation [42], MG132 up-regulates the VDUP-1 protein level by inhibiting the proteasome activity and blocking the proteasomal degradation of VDUP-1. Therefore, we suggest that MG132 induces VDUP-1 accumulation and Trx inhibition accompanied by GSH depletion and ROS increment. The regulation of the VDUP-1/Trx axis may lead to caspase activation and HSP90 cleavage. In conclusion, HSP90 cleavage can be an additional mechanism involved in the anti-cancer effect of proteasome inhibitors in leukemia cells.

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