Biochemical and Biophysical Research Communications 430 (2013) 616–622

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Zerumbone, an electrophilic sesquiterpene, induces cellular proteo-stress leading to activation of ubiquitin–proteasome system and autophagy

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ARTICLE INFO

Article history:
Received 17 November 2012
Available online 5 December 2012

Keywords:
Zerumbone
Ubiquitin–proteasome system
Autophagy
p62
Protein quality control
Hormesis

ABSTRACT

Zerumbone, a sesquiterpene present in *Zingiber zerumbet* Smith, has been implicated as a promising chemopreventive agent. Interestingly, a number of studies have revealed that its potent bioactivities are dependent on the electrophilic moiety of its α,β-unsaturated carbonyl group, while our recent findings showed its chemical potential for binding to cellular proteins through a Michael reaction. In the present study, modifications of proteins by zerumbone led to their insolubilization *in vitro*. In living cell models, zerumbone induced ubiquitination and aggregation of cellular proteins, which demonstrated its substantial proteo-toxicity. On the other hand, it was also revealed that zerumbone possesses potential for activating intracellular proteolysis mechanisms of the ubiquitin–proteasome system and autophagy. Furthermore, it up-regulated expressions of pro-autophagic genes including p62, which is known as a cargo receptor of aggrephagy, the selective autophagic process for protein aggregates. Pretreatment of Hepa1c1c7 cells with zerumbone conferred a phenotype resistant to cytotoxicity and protein modifications by 4-hydroxy-2-nonenal, an endogenous lipid peroxidation product, in a p62-dependent manner. Together, these results suggest that protein modifications by zerumbone cause mild proteo-stress, thereby activating intracellular proteolysis machineries to maintain protein homeostasis. We consider these effects on proteolysis mechanisms to be hormesis, which provides beneficial functions through mild biological stresses.

1. Introduction

Zerumbone (ZER) is a sesquiterpene found in the tropical zingiberaceous plant *Zingiber zerumbet* Smith, which has been used as folk medicines for anti-inflammatory purposes in Southeast Asia. In 1999, we identified ZER as a potent inhibitor of tumor promoter-induced Epstein-Barr virus activation [1]. Thereafter, accumulated evidence has shown it to be a promising chemopreventive agent [2]. Although the molecular mechanisms underlying its chemopreventive activity have not been fully elucidated, nuclear factor erythroid 2-related factor-2 (Nrf2)-dependent xenobiotics detoxifying activity is considered to play significant roles [3]. Interestingly, α-humulene, an analog of ZER lacking a carbonyl group, was shown to possess lower levels of these activities [2]. Based on the fact that an α,β-unsaturated carbonyl group reacts with a nucleophilic group *via* a Michael reaction, covalent binding of ZER to cellular proteins might be essential for its bioactivities. Our previous *in vitro* study using ZER-immobilized Sepharose gel demonstrated that ZER covalently binds to thiol groups in cellular proteins including Kelch-like ECH-associated protein 1 (Keap1), the key regulator of Nrf2 activation [4].

We have also identified other proteins including β-actin as ZER binding molecules [4]. Since the chemical structure of ZER is simple and small, its binding selectivity is not expected to be high. In support of this notion, several isothiocyanates, bioactive electrophiles present in cruciferous plants, were shown to bind to unidentified multiple proteins in cultured cells [5,6]. Given that such non-selective protein modifications by chemicals are presumed to be potentially proteo-toxic, ZER might cause denaturing stress to cellular proteins. Generally, denatured proteins are recognized and thereby repaired by molecular chaperones such as heat shock proteins (HSPs) [7]. Additionally, excessively denatured proteins are occasionally ubiquitinated by carboxy terminus of Hsc70 interacting protein (CHIP), a chaperone-dependent E3 ligase [8]. Ubiquitinated proteins readily form cellular aggregates, whose accumulation has been known to promote ageing and have associations with onset of various diseases, such as cancer and neurodegenerative disorders [9]. Abnormal proteins are normally degraded

Abbreviations: CHIP, carboxy terminus of Hsc70 interacting protein; D3T, 3*H*-1,2-dithiole-3-thione; HNE, 4-hydroxy-2-nonenal; HSP, heat shock protein; HUM, α-humulene; Keap1, Kelch-like ECH-associated protein 1; LC3, microtubule-associated protein 1 light-chain 3; LPS, lipopolysaccharide; NEM, *N*-ethylmaleimide; Nrf2, nuclear factor erythroid 2-related factor-2; UPS, ubiquitin–proteasome system; ZER, zerumbone.

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http://dx.doi.org/10.1016/j.bbrc.2012.11.104

through intracellular proteolysis mechanisms such as ubiquitin–proteasome system (UPS) and autophagy in order to maintain protein homeostasis.

The 26S proteasome, a multi-catalytic protease complex, is responsible for degradation of monomeric proteins. Ubiquitinated proteins are recognized by the 19S regulatory subunit including Rpt1-6, and proteolysis occurs in the 20S catalytic core subunit consisting of α1-7 and β1-7, which exhibits chymotrypsin, trypsin, and caspase-like activities [10]. Nrf2 has been reported to induce proteasome activity in response to oxidative and electrophilic stress [11]. Microarray analysis showed that Nrf2 is responsible for induction of both 19S (Rpt2, Rpt5, Rpt11, etc.) and 20S (α3, α6, β3, β5, β7, etc.) subunits [12]. On the other hand, autophagy is a bulk degradation system by which cytoplasmic materials are engulfed by double-membrane vesicles, known as autophagosomes, and delivered to lysosomes for degradation, It is known as an essential step in autophagy induction that conjugation of phosphatidylethanolamine to microtubule-associated protein 1 light-chain 3 (LC3) in generation of LC3-II associated with autophagosomes [13]. Recently, there is growing evidence that aggrephagy, the selective autophagic process for protein aggregates, is a highly selective protein quality control mechanism [9]. In aggrepahgy, p62 binds to both LC3 and ubiquitinated proteins to execute proteolysis [14].

In the present study, we evaluated the proteo-toxicity of ZER and its effects on proteolysis machineries. ZER induced insolubilization, CHIP-dependent ubiquitination, and aggregation of cellular proteins. Meanwhile, ZER activates UPS and autophagy, and regulates the expressions of pro-autophagic genes including p62, which confers a phenotype resistant to proteo-stress by 4-hydroxy-2-nonenal (HNE), an endogenous lipid peroxidation product. Collectively, our results show that ZER, a substantially proteo-toxic electrophile, enhances intracellular proteolysis machineries for activation of protein quality control systems. We consider this phenomenon to be hormesis, an adaptation mechanism against proteo-toxic chemical stresses.

2. Materials and methods

2.1. Cell culture

Hepa1c1c7 mouse hepatocytes were provided by Dr. Norio Yamamoto (House Wellness Foods Co., Ltd). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS and streptomycin (100 μg/mL) at 37 °C under a humidified atmosphere of 95% air and 5% CO_2.

2.2. Reagents

ZER was purified as previously reported [1]. Antibodies were obtained from the following sources: mouse anti-ubiquitin, rabbit anti-CHIP, rabbit anti-p62, and HRP-conjugated anti-rabbit immunoglobulin G were purchased from Cell Signaling Technology (Beverly, MA); rabbit anti-β5 was from Abcam (Cambridge, MA); mouse anti-LC3 was from MBL (Nagaya, Japan); rabbit anti-histone H1 was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-4-hydroxy-2-nonenal Michael adduct was from Calbiochem (La Jolla, CA); mouse anti-α-tubulin was from EMD Bioscience (La Jolla, CA); and HRP-conjugated anti-mouse immunoglobulin G was from DAKO (Tokyo, Japan). HRP-conjugated streptavidin was obtained from Thermo Scientific (Waltham, MA). siRNAs and Lipofectamine™ RNAiMAX were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless specified otherwise.

2.3. Fractionation of cell lysates

Extraction of insoluble cell lysates was performed using a ReadyPrep™ Protein Extraction Kit (Soluble/Insoluble) (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's protocol with some modifications. Hepa1c1c7 cells were lysed with lysis buffer from the kit, which were incubated with ZER, α-humulene, or *N*-ethylmaleimide (0, 1, 5, 25 mM, 2.5% DMSO, *v*/*v*) for 6 h at room temperature. After centrifugation (16,000*g*, 30 min), the pellets were extracted with lysis buffer for insoluble proteins [10 mM Tris, pH 7.4, 2% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM dithiothreitol, 1 mM sodiummetavanadate (V)].

2.4. Western blot analysis

Hepa1c1c7 cells were treated with the sample or vehicle (0.5% DMSO, *v*/*v*) for various times, then lysed in BioPlex cell lysis buffer, and centrifuged at 10,000*g* for 10 min. Denatured proteins were separated using SDS–PAGE on a 10% or 15% polyacrylamide gel, then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). After blocking with 2% Block Ace for 1 h, each membrane was treated with the appropriate specific primary Ab (1:2000), followed by the corresponding HRP-conjugated secondary Ab (1:2000). The blots were developed using ECL western blot detection reagents (GE Healthcare, Buckinghamshire, UK).

2.5. Observation of cellular aggresome

Fluorescent staining of cellular aggresome was performed using a ProteoStat Aggresome Detection Kit (Enzo Life Science, New York, NY), according to the manufacturer's protocol. Hepa1c1c7 cells were treated with ZER or lactacystin (50 μM each, 0.5% DMSO, *v*/*v*) for 6 h. Cells were fixed in 4% formaldehyde, then permeabilized by 0.1% Triton X-100. Cells were incubated with Proteostat Aggresome Detection Reagent (1:4000) and Hoechst 33342 (1:2000). Images of cellular immunofluorescence were acquired using a UFX-35A fluorescent microscope (Nikon, Tokyo) (original magnification: 400×).

2.6. Determination of 20S proteasome activity

Determination of 20S proteasome activity was performed using a 20S Proteasome Assay Kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's protocol. Hepa1c1c7 cells were treated with ZER (0, 2, 10, 50 μM, 0.5% DMSO, *v*/*v*) for 6 h, then washed with PBS and cultured in DMEM supplemented with 10% FBS for another 18 h. Cells were lysed with lysis buffer from the kit, then the lysates were incubated with a 20S proteasome substrate (SUC-LLVY-AMC) for 1 h at 37 °C. Fluorescent products generated by cleavage of the substrate were measured using a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific, Wilmington, DE) (excitation 355 nm, emission 485 nm).

2.7. Detection of acidic vesicular organelles

Hepa1c1c7 cells treated with ZER (0, 10, 50 μM, 0.5% DMSO, *v*/*v*) for 24 h were stained with 1 μg/mL acridine orange for 15 min, then examined under a UFX-35A fluorescent microscope (Nikon, Tokyo) (original magnification: 400×).

2.8. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Hepa1c1c7 cells were treated with the sample or vehicle (0.5% DMSO, *v*/*v*) for 12 h. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's specifi-

cations. The amount and purity of RNA were assessed by spectrophotometry using a SmartSpec® 3000 Spectrophotometer (Bio-Rad Laboratories). cDNA was synthesized using 1 μg total RNA with an RNA PCR Kit (AMV). Thermal cycling was performed using a 7300 real time PCR system with SYBR green PCR mix (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The PCR conditions were as follows: 95 °C for 3 min, 95 °C for 10 s, and 60 °C for 1 min. The primer sequences are summarized in Table 1.

2.9. Measurement of cell viability

Cell viability was determined using a Cell Counting Kit-8 (WST-8, Dojindo Molecular Technology, Kumamoto), according to the manufacturer's protocol. Cells were incubated in DMEM containing 5% WST for 1 h, then absorbance of the cell culture medium was measured at 450 nm.

2.10. RNA interference (RNAi) of CHIP and p62

Transfection of siRNAs was conducted using Lipofectamine™ RNAiMAX, according to the manufacturer's specifications. Briefly, each siRNA solution (1 μM final concentration) was added to Lipofectamine™ RNAiMAX solution (1:25), which was incubated for 20 min. This transfection mixture was diluted in 500 μL of serum-free Opti-MEM I®, which was treated to cells for 6 h. After replacing the medium with DMEM containing 10% FBS, the cells were incubated for another 24 h.

2.11. Statistical analysis

Each experiment was performed at least 3 times and values are shown as the mean ± SD, where applicable. Statistically significant differences between groups in each assay were determined using a Tukey–Kramer test, Dunnett's test, and Student's *t*-test (two-sided).

3. Results

3.1. ZER insolubilized cellular proteins in vitro

To evaluate the effect of ZER on protein solubility, we determined the amounts of insoluble proteins after ZER exposure. ZER increased insoluble proteins in a concentration-dependent manner, with similar results seen following heat shock treatment (Fig. 1). It should be noted that use of α-humulene, an inactive analog of ZER lacking a carbonyl group, and *N*-ethylmaleimide, a hydrophilic compound known as a thiol modifier, led to very slight insolubilization. These findings indicate the potential of ZER for altering the solubility of cellular proteins, which might be important for protein denaturation.

Table 1
Primer sequences for qRT-PCR.

Gene	Primer	Sequence (5'–3')
p62	Sense	gCTgCCCTATACCCACATCT
	Antisense	CgCCTTCATCCgAgAAAC
Cdkn2a	Sense	TgATgATgATgggCAACg
	Antisense	ACgggAACgCAAATATCg
Gabarapl1	Sense	CATCgTggAgAAggCTCCTA
	Antisense	ATACAgCTggCCCATggTAg
Hsp90aa1	Sense	AAAggCAggCTgACAAgA
	Antisense	AggggAggCATTTCTTCAgT
Hspa8	Sense	gCTggATAAgAACCAg
	Antisense	ACTgCTgCACTCTggTACAgCTTgg
HPRT	Sense	gTAATgATCAgTCAACggggAC
	Antisense	CCAgCAAgCTTgCAACCTTAACCA

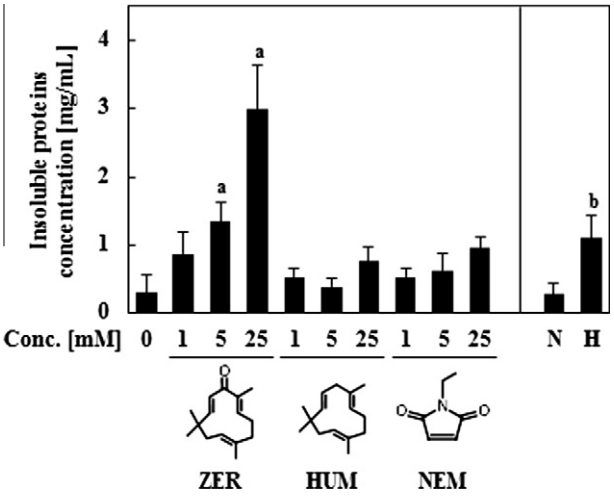

Fig. 1. ZER insolubilized cellular proteins *in vitro*. Cell extracts lysed with lysis buffer from a ReadyPrep™ Protein Extraction Kit (soluble/insoluble) were incubated with ZER, α-humulene (HUM), or *N*-ethylmaleimide (NEM) (0, 1, 5, 25 mM) for 6 h, then the pellets were lysed with lysis buffer for insoluble proteins. Cell lysates exposed to heat shock (incubation at 45 °C in a water bath) for 6 h were used as a positive control (H), whereas non-treated lysates served as a negative control (N). Protein concentrations in each sample were determined using the Bradford method. a, vs. DMSO (0) by Dunnett's test; b, vs. N by Student's *t* test ($P < 0.05$).

3.2. ZER induced ubiquitination and aggregation of cellular proteins

Abnormal proteins generated in the cytoplasm are known to be ubiquitinated by CHIP, a chaperone dependent E3 ligase, for their degradation [8]. ZER dramatically induced protein ubiquitination in Hepa1c1c7 cells, which was suppressed by CHIP silencing by siRNA (Fig. 2A). p62 has been reported to aggregate with abnormal proteins by binding to ubiquitinated proteins [14] and its band shift to a higher molecular weight range is suggested to reflect conjugation [15]. As shown in Fig. 2B, a remarkable p62 band shift was seen following ZER treatment, which was time- and concentration-dependent. Additionally, the p62 monomer was up-regulated by ZER at 10 μM, which was not seen with severe conditions (50 μM for 24 h). To confirm the formation of aggresome, cells treated with ZER were stained with molecular rotor dye, which increased the level of cellular aggresome. Treatment with lactacystin, a proteasome inhibitor used as a positive control, had a similar result (Fig. 2C). Consistent with these observations, ubiqutinated proteins and p62 in the insoluble fraction were markedly increased by ZER in a time-dependent manner (Fig. 2D).

3.3. ZER activated UPS and autophagy

We anticipated that proteolysis machineries might be activated for adapting to proteo-stress induced by ZER. Expression of β5, the 20S proteasome subunit essential for chymotrypsin-like activity, was up-regulated by ZER (Fig. 3A), while ZER also increased cellular chymotrypsin-like activity (Fig. 3B). Subsequently, we evaluated autophagy activation by western blot analysis of LC3-II, whose increment is a hallmark of this phenomenon [16], resulted in up-regulation of LC3-II by ZER (Fig. 3C). To confirm this autophagic response to ZER, cellular acidic vesicular organelles including autolysosomes and lysosomes were stained by acridine orange, which resulted in an increase of acidic vesicles by ZER (Fig. 3D).

3.4. ZER induced p62 expression and conferred resistance to protein modification

We also examined the activity of ZER for regulating the expressions of a total of 84 key genes involved in autophagy using a PCR

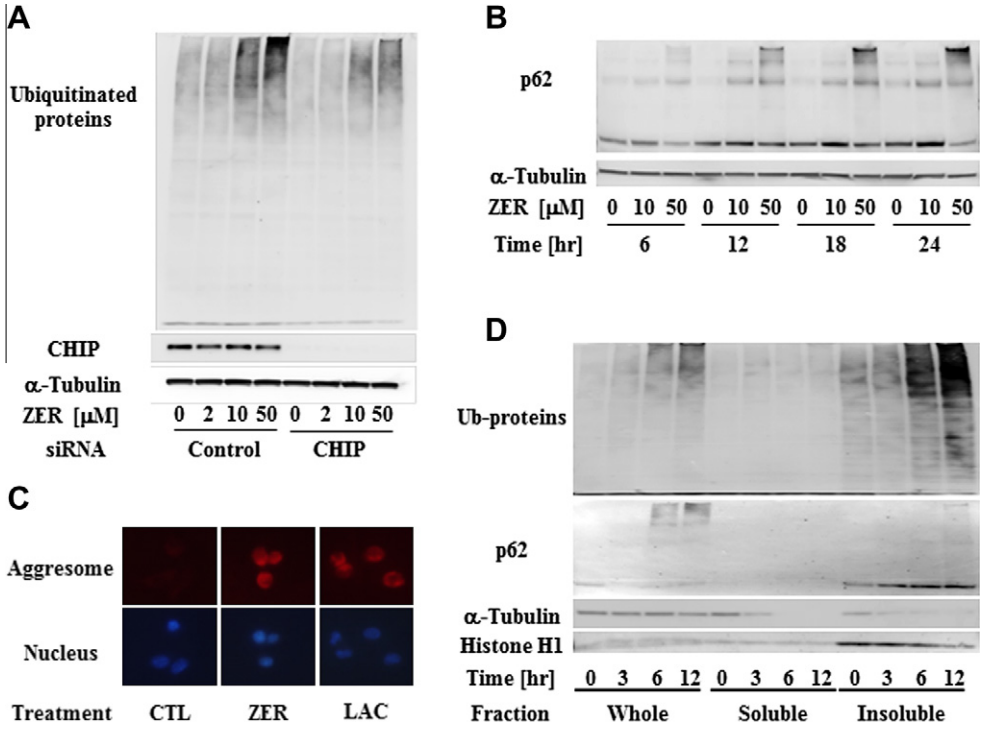

Fig. 2. ZER induced ubiquitination and aggregation of cellular proteins. (A) Hepa1c1c7 cells were treated with Lipofectamine™ RNAiMAX and an siRNA solution (control or CHIP, 5 nM) for 6 h. After recovery with DMEM containing 10% FBS for another 24 h, cells were treated with ZER (0, 2, 10, 50 μM) for 6 h, then lysed for western blot analysis. (B) Cells were treated with ZER (0, 10, 50 μM) for 6–24 h, then lysed for western blot analysis. (C) Cells were treated with ZER or lactacystin (LAC) (0, 50 μM) for 6 h, then cellular aggresome was stained using a ProteoStat Aggresome Detection Kit (red: aggresome, blue: nucleus). (D) Cells treated with ZER (50 μM) for 0–12 h were lysed, then fractionated into soluble and insoluble proteins using a ReadyPrep™ Protein Extraction Kit for western blot analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

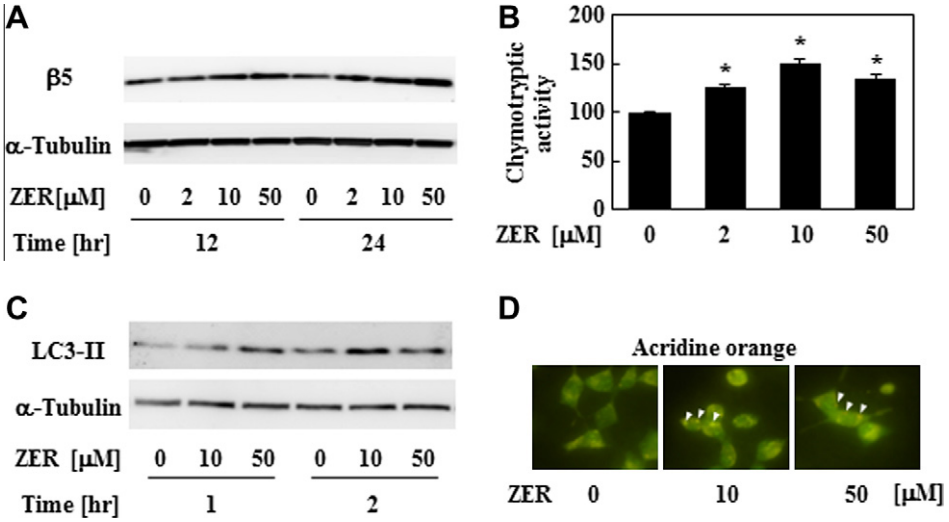

Fig. 3. ZER activated UPS and autophagy. (A) Hepa1c1c7 cells treated with ZER (0, 2, 10, 50 μM) for 12 or 24 h were lysed for western blot analysis. (B) Cells treated with ZER (0, 2, 10, 50 μM) for 6 h then cultured in DMEM with 10% FBS for another 18 h. Then cells were lysed for measurement of chymotrypsin activity using a 20S Proteasome Assay Kit. Values for proteasome activities were corrected by the protein concentrations of the samples. $^{*}P < 0.05$ vs. DMSO (0) by Dunnett's test. (C) Cells were treated with ZER (0, 10, 50 μM) for 1 or 2 h, then lysed for western blot analysis. (D) Cells were treated with ZER (0, 10, 50 μM) for 24 h, then stained with 1 μg/mL acridine orange (green: neutral area, yellow or red: acidic area, arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

array (data not shown), which revealed that 5 genes were markedly regulated by ZER. Expressions of these genes were confirmed by real time RT-PCR analysis, which showed significant induction of 5 pro-autophagic genes by ZER; *p62*, *Cdkn2a*, *Gabarapl1*, *HSP90aa1*, and *Hspa8* (Fig. 4A). These results imply the possibility that ZER not only induces acute autophagy, but also amplifies autophagic capacity. Considering that p62 monomer up-regulation by ZER (50 μM) at 12 and 18 h showed a decline at 24 h (Fig. 2B), cells were washed by PBS after ZER treatment, followed by incubation in ZER-free medium to mitigate chemical stress. Interestingly, the p62 band shift induced by ZER was attenuated after washing the cells (Fig. 4B), which might have been degraded through autophagy. We also noted that expression of the p62 monomer was dramatically up-regulated after clearance of its conjugates. To

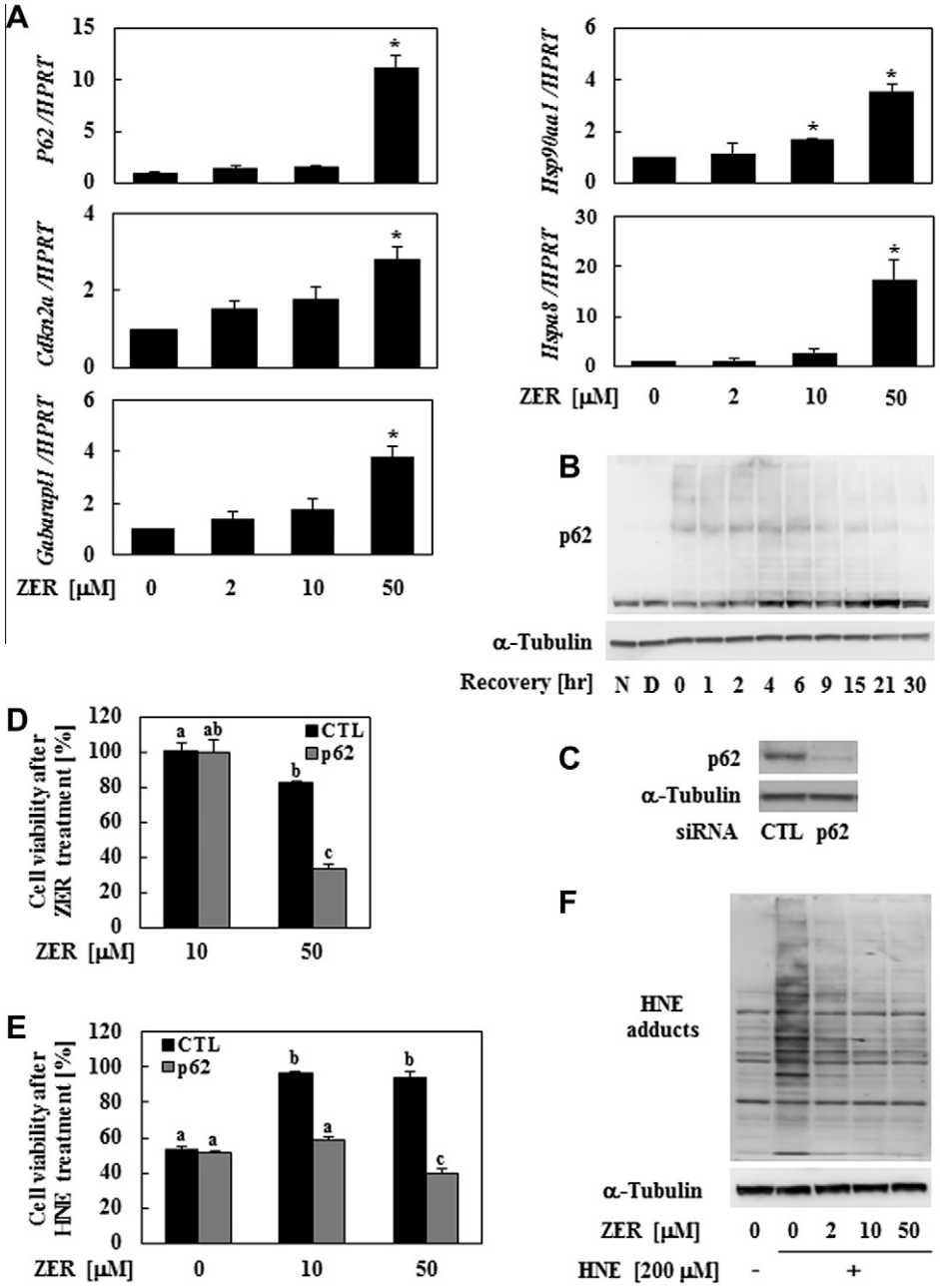

Fig. 4. ZER induced p62 expression and conferred resistance to protein modification. (A) Hepa1c1c7 cells were treated with ZER (0, 2, 10, 50 μM) for 12 h, the expressions of *p62*, *cdkn2a*, *gabarapl1*, *hsp90aa1*, and *hspa8* were semi-quantified by qRT-PCR. *HPRT* expression was also measured as an internal standard. $^{*}P < 0.05$ vs. DMSO (0) by Dunnett's test. (B) Cells were treated with ZER (0, 50 μM) for 6 h, then cultured in DMEM containing 10% FBS for another 1–30 h and lysed for western blot analysis. (C–E) Cells were treated with Lipofectamine™ RNAiMAX and an siRNA solution [control (CTL) or p62, 5 nM] for 6 h. After recovery with DMEM containing 10% FBS for another 24 h, cells were treated with ZER (0, 10, 50 μM) for 6 h, followed by incubation with DMEM containing 10% FBS for another 18 h. Cells were treated with HNE (0, 100 μM) for 1 h, then viability was determined using a WST-8 test. The various characters were significantly different, as shown by Tukey–Kramer test results ($P < 0.05$). (F) Cells were treated with ZER (0, 2, 10, 50 μM) for 6 h, then cultured in DMEM with 10% FBS for another 18 h. After HNE treatment (0, 200 μM) for 2 h, cells were lysed for western blot analysis.

validate the significance of p62 for protein quality control systems, we down-regulated the expression of p62 by siRNA transfection (Fig. 4C) and found that ZER cytotoxicity was enhanced in p62-silenced cells (Fig. 4D), which supports our findings showing its substantial proteo-toxicity (Figs. 1 and 2). Importantly, pretreatment of control cells with ZER conferred a phenotype resistant to cytotoxicity by HNE, a major lipid peroxidation product that is known to intensively modify cellular proteins [17]. However, this phenotype was abolished in p62-silenced cells (Fig. 4E). Pretreatment with ZER also suppressed cellular protein modification by HNE in a concentration-dependent manner (Fig. 4F). These results indicate that ZER increases p62 expression for adapting to additional proteo-stresses.

4. Discussion

In the present study, we examined the possibility that a proteotoxic compound can activate intracellular proteolysis mechanisms to maintain protein homeostasis. Incubation of cell lysates with ZER caused protein insolubilization (Fig. 1), which may be associ-

ated with their increase in molecular hydrophobicity by ZER modifications (ClogP value; 5.34). α-Humulene and *N*-ethylmaleimide are considered to be inactive because of their low levels of electrophilicity and hydrophobicity, respectively. Importantly, HSPs recognize denatured proteins by binding to their hydrophobic surface, leading to their ubiquitination [18]. In the present study, ZER treatment of Hepa1c1c7 cells induced ubiquitination of cellular proteins by CHIP, a chaperone-dependent E3 ligase (Fig. 2A), followed by formation of cellular aggresome (Fig. 2B–D). These findings indicate that ZER possesses substantial proteo-toxicity, which is considered to be caused by protein modifications. Given that other bioactive compounds such as curcumin and benzyl isothiocyanate were also reported to induce similar protein ubiquitination [19,20], such proteo-toxicity may be broadly conserved among electrophilic compounds with hydrophobicity.

The expression and activity of proteasome are known to be enhanced *via* the Keap1-Nrf2 signaling pathway [11]. For example, Kwak et al. showed that protein expressions of 20S subunit genes including β5 were up-regulated in mouse livers by oral administration of 3*H*-1,2-dithiole-3-thione (D3T) in an Nrf2-dependent manner [21]. Since ZER activates Nrf2 [3], mechanisms similar to that of D3T might have contributed to the increased proteasome activity (Fig. 3B). On the other hand, recent studies have revealed that aggrephagy is regulated by specific cargo receptors, such as p62 and NBR1. p62 recruits cellular aggresome to autophagosomes by binding to both ubiquitinated proteins and LC3 [14]. Lipopolysaccharide (LPS)-induced aggresome was reported to be degraded through p62-dependent aggrephagy in RAW264.7 mouse macrophages [22]. Given that aggresome induced by ZER was shown to be comprised of p62 (Fig. 2D), it may enhance aggrephagy by mechanisms similar to those of LPS.

It is interesting that ZER up-regulated the expressions of pro-autophagic genes (Fig. 4A). p16, the gene product of *Cdkn2a*, is known to inhibit CDK4, an anti-autophagic signaling molecule [23]. GABARAP, a homolog of Atg8, is essential for autophagosome formation in a manner similar to that of LC3 [24]. HSPs are involved in the induction of chaperone-mediated autophagy [25] and play a role in lysosome stabilization [15]. Notably, the expression of p62 is markedly enhanced at protein levels (Fig. 4B), which may be mediated by Nrf2 activation [14]. As shown in Fig. 4E, p62-induction by ZER conferred a phenotype resistant to cytotoxicity by HNE in Hepa1c1c7 cells. These results suggest that ZER amplifies autophagic capacity through p62 induction for adaptation to additional proteo-stresses, though it should be noted that xenobiotics detoxifying mechanisms [3] may partly contribute to such phenotypes. On the other hand, ZER treatment under severe conditions (50 μM, 24 h) resulted in attenuation of p62-inducing activity (Fig. 2B). Collectively, we consider the effects of ZER on proteolysis mechanisms to be hormesis [26], which provides beneficial functions by mild biological stresses.

Recently, a large number of studies have shown activation of proteolysis mechanisms, whose dysfunction is associated with ageing and various disorders [9]. However, their excess activation by modulation of this specific signaling pathway has been revealed to induce side-effects such as autophagic cell death [27]. Interestingly, MG132, a proteasome inhibitor, was reported to induce accumulation of denatured proteins, leading to p62-dependent aggrephagy [28], which may be more effective for selective clearance of abnormal proteins. Our present findings are the first to show activation of proteolysis mechanisms in response to a proteo-toxic compound.

Acknowledgments

The authors would like to thank Dr. Norio Yamamoto (House Wellness Foods Co., Ltd) for providing the Hepa1c1c7 cells. This study was partly supported by a Grant-in-aid for Scientific Research (C) (No. 23580164 to A.M.) and a Grant-in-aid for the Promotion of Science for Young Scientists (No. 22.3355 to K.O.) from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

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