Proteasome inhibitors stimulate activator protein-1 pathway via reactive oxygen species production

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Abstract In this report we explored the effects of proteasome inhibitors (MG132, aLLN, lactacystin and MG262) on interleukin-8 (IL-8) induction. In HEK293 cells, proteasome inhibitors could concentration-dependently increase IL-8 promoter and activator protein-1 (AP-1) activities, but inhibited nuclear factor (NF)-κB activation induced by cytokines. The stimulating effects on IL-8 promoter and AP-1 were reduced by *N*-acetylcysteine, glutathione, diphenyleneiodonium, rotenone and antimycin A. Fluorescent analysis using 2′,7′-dichlorodihydrofluorescin diacetate further confirmed the abilities of proteasome inhibitors to induce reactive oxygen species (ROS) production. These results suggest that ROS production by proteasome inhibitors leads to AP-1 activation, which in the absence of NF-κB activation still transactivates IL-8 gene expression.

Key words: Proteasome; Reactive oxygen species; Interleukin-8; Activator protein-1; Nuclear factor-κB; MG132

1. Introduction

Proteasomes are large multi-subunit protease complexes, which are localized in the nucleus and cytosol, and selectively degrade intracellular proteins. Accumulating evidence has strengthened the crucial roles of proteasomes in the degradation of many proteins involved in cell cycling, proliferation, and apoptosis [1]. A vast majority of short-lived, normal proteins and misfolded, abnormal proteins are degraded by this system. A protein marked for degradation is first covalently attached to multiple molecules of ubiquitin, and is then escorted for rapid hydrolysis by proteasomes. Proteins which undergo regulation by this mechanism include cyclins, Bcl-2 [2], p53 [3], c-Fos [4], c-Jun, nuclear factor (NF)-κB precursor, JAKs [5], STATs, HIF-1α, retinoid X receptor [6], retinoic acid receptor [7], peroxisome proliferator-activated receptor γ [8], and estrogen receptor [9]. It is thus conceivable that a great variety of cellular regulatory mechanisms, examples ranging from the progression of the cell cycle to the pathways controlling signal transduction and metabolism, are controlled by the ubiquitin/proteasome system.

NF-κB and activator protein-1 (AP-1) are two major transcriptional systems distinctly regulated by proteasomes. In the NF-κB signaling pathway, the inhibitory protein of NF-κB, IκB, can be degraded by the proteasome. This results in the up-regulation of the expression of many NF-κB-dependent genes. Viewing the crucial roles of NF-κB in inflammation and promoting cell survival, the inhibition of IκB degradation and NF-κB activation by proteasome inhibitors has set up a novel strategy for anti-inflammatory and anti-cancer drug development [10,11]. However, in the aspect of regulating the AP-1 signaling cascade, the proteasome inhibitor MG132, on the contrary, can activate c-Jun N-terminal kinase (JNK) activation and induce some downstream responses. AP-1-dependent cell apoptosis [12], neurite outgrowth [13], and transcriptional gene expression of monocyte chemoattractant protein 1, stromelysin, mitogen-activated protein kinase, phosphatase 1, and cyclooxygenase-2 all have been reported for MG132 action [14,15]. Although MG132 prevention of c-Fos and/or c-Jun degradation might account for AP-1 activation, the rapid JNK activation by MG132 is another alternative pathway [15]. However, the mechanism responsible for JNK-related AP-1 activation and the action specificity for other proteasome inhibitors are still uninvestigated.

Since proteasome inhibitors have been considered as therapeutic drugs for the treatment of inflammation, it is interesting to explore their actions on interleukin (IL)-8 expression. IL-8 is a CXC chemotactic factor for neutrophils, T cells and basophils [16], and has been implicated in several inflammatory diseases [17]. IL-8 secretion is primarily regulated at the transcription level, and its promoter region contains the DNA binding sites for NF-κB and AP-1 [18–20]. Since the involvement of both transcription factors for IL-8 induction is stimulus-specific [19–22], it is interesting to explore the mechanisms responsible for IL-8 induction by proteasome inhibitors.

2. Materials and methods

2.1. Reagents and plasmids

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). aLLN, lactacystin, MG132, MG262, *N*-acetylcysteine (NAC) and glutathione (GSH) were purchased form Calbiochem (Darmstadt, Germany). H_2O_2, diphenyleneiodonium (DPI), rotenone and antimycin A were obtained from Sigma Aldrich

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Abbreviations: NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; AP-1, activator protein-1; IL, interleukin; NAC, *N*-acetylcysteine; GSH, glutathione; DPI, diphenyleneiodonium; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NF-IL-6, nuclear factor-interleukin-6

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(St. Louis, MO, USA). All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Luciferase expression vectors containing the 5′-flanking region of the IL-8 gene (−133 to −50) and specific mutants of NF-κB, AP-1 and nuclear factor-interleukin-6 (NF-IL-6) were provided by Dr. N. Mukaida (Kanazawa University, Ishikawa, Japan). AP-1-Luc construct was provided by Dr. Guy Haegeman (University of Gent-VIB, Gent, Belgium). pGL2-ELAM-Luc (κB-Luc) under the control of one NF-κB binding site was constructed.

2.2. Cell culture

Human embryonic kidney (HEK) 293 cells, obtained from American Type Culture Collection (Manassas, VA, USA), were grown at 37°C in 5% CO_2 using DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Transfection and reporter gene assay

For transfection assays, 5×10^5 HEK293 cells were seeded into six-well plates. Cells were transfected on the following day by the calcium phosphate precipitation method. Premix DNA with 33.4 μl 0.1×TE buffer, 12.6 μl 1 M $CaCl_2$ in a tube for each well, then mix slowly with 46 μl 2×Hanks' balanced salt solution in 25 s. Incubate the mixture for 25 min at room temperature, and add into each well. After 24 h incubation, transfection was complete, and cells were incubated with the indicated concentrations of proteasome inhibitors. After another 24 h incubation, the media were removed, and the cells were washed once with cold phosphate-buffered saline. To prepare lysates, 100 μl of reporter lysis buffer (Promega) was added to each well, cells were scraped from dishes. Collect the supernatant after centrifugation at 13 000 rpm for 30 s. Aliquots of cell lysates (5 μl) containing equal amounts of protein (10–20 μg) were placed into the wells of an opaque black 96-well microplate. An equal volume of luciferase substrate (Promega) was added to all samples, and the luminescence was measured in a microplate luminometer (Meriden, CT, USA). The luciferase activity value was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector (pCR3lacZ; Pharmacia, Sweden), and is presented as the percentage of luciferase activity measured in the presence of each proteasome inhibitor relative to the activity of control cells with no stimulation.

2.4. Flow cytometry for ROS formation

2′,7′-Dichlorodihydrofluorescin diacetate (DCFH-DA) was used as an indicator for the formation of intracellular reactive oxygen species (ROS). Cells were pretreated with DCFH-DA (50 μM) for 30 min, and then indicated concentrations of proteasome inhibitors were added for different time periods. Once ROS was generated, the DCFH oxidation product, DCF fluorescence can be detected by flow cytometer (FACScan, Becton Dickinson). The fluorescence was assessed by counts of FL1-H, and the mean value would represent the ability of a chemical compound to induce ROS formation.

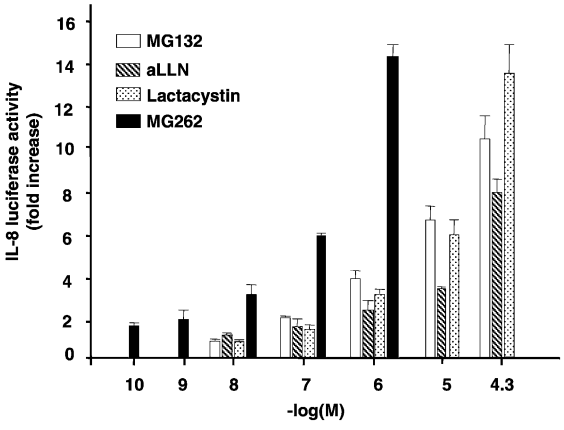

Fig. 1. Four proteasome inhibitors increased IL-8 promoter activity in HEK293 cells in a concentration-dependent manner. HEK293 cells were co-transfected with wild-type cDNA construct of IL-8 promoter (0.5 μg) and β-gal-lacZ (0.5 μg). Twenty-four hours later, 0.01–50 μM of MG132, aLLN, lactacystin, and 0.1 nM–1 μM MG262 were added for another 24 h, and then cell lysates were prepared and IL-8 promoter activity was assessed by measurement of luciferase activity. Results were normalized for transfection efficiency with β-gal-lacZ. Each data point represents the mean ± S.E.M. of at least three independent experiments, which were performed in duplicate.

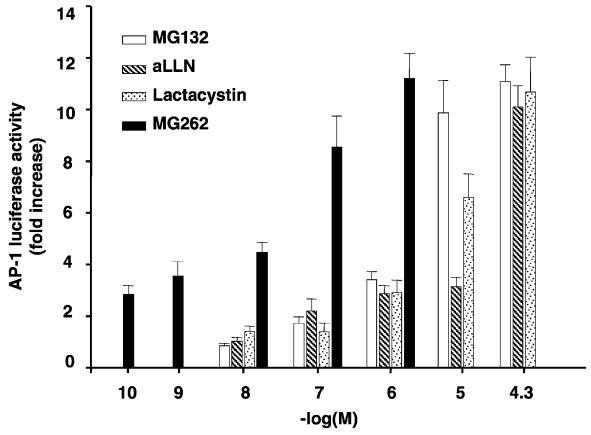

Fig. 2. Four proteasome inhibitors increased AP-1 reporter activity. HEK293 cells were co-transfected with wild-type AP-1 promoter (0.5 μg) and β-gal-lacZ (0.5 μg). Twenty-four hours later, 0.01–50 μM of MG132, aLLN, lactacystin, and 0.1 nM–1 μM of MG262 were added for 24 h, and then cell lysates were prepared for reporter activity assay. Results were normalized for transfection efficiency with β-gal-lacZ. Each data point represents the mean ± S.E.M. of at least three independent experiments, which were performed in duplicate.

2.5. Statistical evaluation

Values are expressed as the mean ± S.E.M. of at least three experiments, which were performed in duplicate. Analysis of variance was used to assess the statistical significance of the differences, and a P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Proteasome inhibitors increased IL-8 and AP-1 luciferase activity

To determine the gene regulatory effect of proteasome inhibitors on IL-8 expression, we performed reporter gene assays to test four proteasome inhibitors, MG132, aLLN, lactacystin, and MG262. As shown in Fig. 1, these four proteasome inhibitors increased IL-8 promoter activity in a concentration-dependent manner. MG262 was the most potent proteasome inhibitor to achieve IL-8 stimulation within 0.1 nM–1 μM. MG132 and lactacystin, on the other hand, exhibited a moderate and comparable potency within 0.1–50 μM. Among the proteasome inhibitors tested, aLLN was the weakest one to stimulate IL-8 response. The stimulation efficacy at the highest concentration of these inhibitors examined, 1 μM for MG262, 50 μM for MG132 and lactacystin, was around a 12–14-fold increase, while that for 50 μM of aLLN was about eight-fold. At these concentrations examined, no cytotoxicity was seen. However, increasing the concentrations of MG132, lactacystin and aLLN to 100 μM resulted in cytotoxicity within 24 h, as assessed from the MTT assay (data not shown).

Consistent with previous studies showing that MG132 can cause AP-1 activation [12–15], all four proteasome inhibitors possessed similar effects to stimulate AP-1 luciferase activity. Moreover, the relative potency and efficacy for AP-1 stimulation were correlated to IL-8 gene induction (Fig. 2).

3.2. Proteasome inhibitors inhibited cytokine-induced κB reporter activity

To verify the inhibitory action of these proteasome inhibitors on NF-κB transcription, the κB-luciferase reporter gene assay was carried out. The results revealed that while κB luciferase activity in basal condition is not altered by MG132, aLLN, lactacystin, or MG262, its stimulation by two potent NF-κB inducing cytokines, IL-1β (10 ng/ml) and tumor necrosis factor α (TNF-α) (50 ng/ml), was antagonized by the presence of proteasome inhibitors (data not shown). The IC_{50} values against cytokine-induced NF-κB activation were 0.1–0.3 μM for MG132, 1 μM for aLLN and lactacystin, and 1–3 nM for MG262.

3.3. ROS-dependent effects of proteasome inhibitors

Since several reports have indicated AP-1 transcription factor as a target of ROS [20,23,24], we next wanted to address the role of ROS in the action of proteasome inhibitors. Another reason to address this issue is the recent finding that lactacystin can lead to significant oxidative damage in NT-2

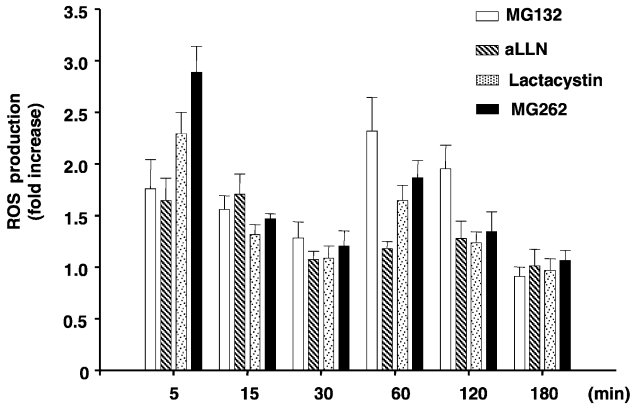

Fig. 4. Proteasome inhibitors induced ROS production. HEK293 cells loaded with 50 μM DCFH-DA for 30 min were treated with vehicle or proteasome inhibitors (10 μM MG132, 10 μM aLLN, 10 μM lactacystin, and 0.1 μM MG262) in the dark for different periods. Then fluorescent intensity was measured by flow cytometry. Each data point represents the mean ± S.E.M. of three to five independent experiments.

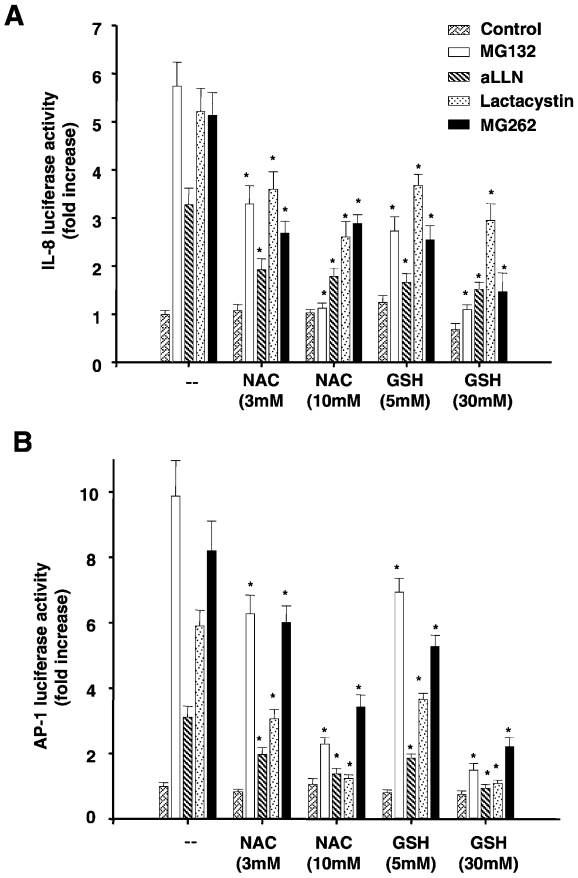

Fig. 3. Antioxidants reduced the stimulatory effects of proteasome inhibitors. HEK293 cells were co-transfected with either IL-8 promoter (0.5 μg) (A) or AP-1 promoter (0.5 μg) (B) together with β-gal-lacZ (0.5 μg). Twenty-four hours later, cells were pretreated with NAC (3 or 10 mM) or GSH (5 or 30 mM) for 30 min, and then indicated concentrations of MG132 (10 μM), aLLN (10 μM), lactacystin (10 μM) and MG262 (0.1 μM) were added for another 24 h, followed by luciferase assay. Results were normalized for transfection efficiency with β-gal-lacZ. Each data point represents the mean ± S.E.M. of at least three independent experiments, which were performed in duplicate. $^*P < 0.05$ as compared to the control response without NAC or GSH treatment.

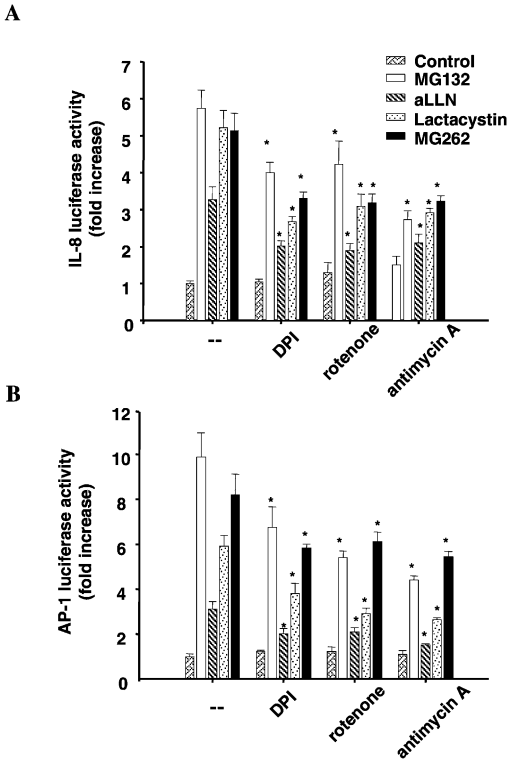

Fig. 5. NADPH oxidase and mitochondrial inhibitors reduced the stimulatory effects of proteasome inhibitors. HEK 293 cells were co-transfected with either IL-8 promoter (0.5 μg) (A) or AP-1 promoter (0.5 μg) (B) together with β-gal-lacZ (0.5 μg). Twenty-four hours later, cells were treated with DPI (10 μM), rotenone (3 μM), or antimycin A (1 μM) for 30 min, followed by MG132 (10 μM), aLLN (10 μM), lactacystin (10 μM), or MG262 (0.1 μM). After incubation for 24 h, luciferase assay as normalized by transfection efficiency with β-gal-lacZ was determined. Each data point represents the mean ± S.E.M. of at least three independent experiments, which were performed in duplicate. $^*P < 0.05$ as compared to the control response without inhibitor treatment.

and SK-N-MC cell lines [25]. Fig. 3 shows that when the antioxidant NAC (3 or 10 mM) or GSH (5 or 30 mM) was pretreated for 15 min, the increased extents of IL-8 and AP-1 reporter activities caused by proteasome inhibitors were markedly attenuated. This result suggested the involvement of ROS as intermediators in the signaling pathway induced by proteasome inhibitors. To verify this suggestion, we used the fluorescent agent DCFH-DA to measure the intracellular content of ROS. When examining the concentrations of these proteasome inhibitors that induced maximal and comparable stimulation on IL-8 and AP-1 (10 μM for MG132, aLLN and lactacystin, and 0.1 μM for MG262), the results shown in Fig. 4 indicated the time-dependent increases of intracellular ROS following exposure to proteasome inhibitors. Within 3 h incubation, the effects of MG132, lactacystin and MG262 on ROS production exhibited biphasic features, which peaked at 5 min, declined gradually and re-induced around 1 h. The efficacy in terms of the rapid stimulation at 5 min is MG262 > lactacystin > MG132, aLLN, while that occurring at 1 h is MG132 > MG262, lactacystin > aLLN. When examining MG132, aLLN and lactacystin at 0.1 and 1 μM, and MG262 at 0.01 μM, the ROS production at 5 min was also increased by 40–80% (data not shown).

To further determine the source(s) of proteasome inhibitors to generate ROS, we examined DPI, the NADPH oxidase inhibitor; rotenone, a potent inhibitor of mitochondria complex I; and antimycin A, an inhibitor of mitochondria electron transport. As shown in Fig. 5, DPI, rotenone, and antimycin A all reduced proteasome inhibitor-stimulated luciferase activities of IL-8 promoter and AP-1.

4. Discussion

Previous studies have demonstrated that IL-8 gene transcription is regulated mainly by NF-κB and AP-1 [18–20]. Although NF-κB has been reported to be essential for IL-8 induction by IL-1β and TNF-α [19,20], NF-κB-independent, but AP-1-dependent regulation has been demonstrated in H_2O_2 action [20–22]. Following observation of the increased IL-8 promoter activity by proteasome inhibitors, we therefore examined the roles of NF-κB and AP-1. Reporter gene assay clearly demonstrated that proteasome inhibitors could reduce NF-κB activation, especially in the presence of cytokines IL-1β and TNF-α, which were demonstrated to trigger NF-κB activation through IκB kinase-dependent phosphorylation, ubiquitination and degradation of IκB [26,27]. In this study, blockade of proteasome-dependent IκB degradation and NF-κB activation by proteasome inhibitors was shown. In addition, the potency order for NF-κB inhibition seen in this cell system (MG262 ≫ MG132, lactacystin, aLLN) correlates quite well with previous findings in terms of K_i values for in vitro proteasome inhibition and IC_{50} values for NF-κB inhibition [25,28–31].

In contrast to NF-κB inhibition, AP-1 is stimulated by proteasome inhibitors, and this action accounts for the gene induction of IL-8. Pharmacological approaches using antioxidants and selective inhibitors of ROS production further suggest ROS as a key player for AP-1 signaling. In this aspect, mitogen-activated protein kinases, the upstream essential regulators of AP-1 activation, are signaling targets of ROS [32–37]. Moreover, since inhibitors of NADPH oxidase and mitochondrial respiratory chain reaction can markedly diminish the stimulatory effects of proteasome inhibitors, ROS production from both NADPH oxidase and mitochondria are suggested to be involved. Consistent with this point, we for the first time demonstrate that intracellular ROS level is increased by four proteasome inhibitors at concentration ranges to induce AP-1 and IL-8 promoter activation, and support a previous study showing the ability of lactacystin (1–25 μM) to induce oxidative protein damage [25]. However, since antioxidants and inhibitors of ROS production did not abolish the increased IL-8 promoter and AP-1 activities, whether any other intermediate molecules and/or action mechanisms, besides ROS-related signaling, contribute to the actions of proteasome inhibitors needs to be investigated.

In summary, we demonstrate that ROS production possibly from NADPH oxidase and mitochondria mediates the major signaling pathway for proteasome inhibitors to stimulate IL-8 gene expression. ROS-dependent AP-1 transcriptional activation is sufficient to induce IL-8 response in the absence of NF-κB activation.

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