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Unexpected Transcriptional Induction of Monocyte Chemoattractant Protein 1 by Proteasome Inhibition: Involvement of the c-Jun N-Terminal Kinase-Activator Protein 1 Pathway

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Proteasome inhibitors, the well-known inhibitors of NF-κB, are recently considered therapeutic agents for inflammation. However, the anti-inflammatory properties of these agents have not been fully evaluated. In this report we describe a novel effect of proteasome inhibitors on the expression of monocyte chemoattractant protein 1 (MCP-1) in mesangial cells. We found that proteasome inhibitor MG132 dose-dependently induced expression of MCP-1 at the transcriptional level. The stimulatory effect was similarly observed with other proteasome inhibitors (proteasome inhibitor 1 and lactacyclin) and in other cell types (NRK fibroblasts). The 5′-flanking region of the MCP-1 gene contains multiple AP-1 sites. To explore the mechanisms involved, we examined the effects of proteasome inhibition on the AP-1 pathway. Northern blot analysis showed that MG132 rapidly induced the expression of c-jun, but not c-fos. Immunoblot analysis showed that MG132 prevented degradation of c-Jun protein. Kinase assay revealed that c-Jun N-terminal kinase (JNK) was rapidly activated by MG132. Consistent with these results, a reporter assay showed that AP-1 activity was up-regulated after treatment with MG132. Curcumin, a pharmacological inhibitor of the JNK-AP-1 pathway, abrogated the induction of MCP-1 by MG132. Similarly, stable transfection with a dominant-negative mutant of c-Jun attenuated both MG132-induced activation of AP-1 and expression of MCP-1. The transcriptional activation by proteasome inhibitors was observed not only in MCP-1, but also in other AP-1-dependent genes, including stromelysin and mitogen-activated protein kinase phosphatase 1. These data revealed that proteasome inhibition triggered the expression of MCP-1 and other genes via the multistep induction of the JNK-c-Jun/AP-1 pathway. The Journal of Immunology, 2001, 167: 1145–1150.
N-terminal kinase (JNK), leading to activation of AP-1 and consequent induction of MCP-1 in mesangial cells.

Materials and Methods

Cells and transfectants

Rat mesangial cells (SM43) were established from isolated glomeruli of a male Sprague Dawley rat and identified as being of the mesangial cell phenotype as described previously (22). The rat fibroblast cell line NRK49F was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM/Ham’s F-12 (Life Technologies, Gaithersburg, MD) supplemented with 100 Units/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% FCS. Medium containing 0.5% FCS was generally used for experiments.

SM/JUND1 cells in which AP-1 is selectively inactivated were established by stable transfection of SM43 with a dominant-negative mutant of c-Jun, TAM-67 (23). SM/JUND1 cells exhibit attenuated activity of AP-1 under both unstimulated and stimulated conditions (23). As a control, mock-transfected SM/Ne cells that express neo alone were used (24).

Pharmacologic manipulations

Confluent cells were treated with proteasome inhibitor MG132 (1–50 μM; Peptide Institute, Osaka, Japan), proteasome inhibitor 1 (PSI-1; 50 μM; Calbiochem-Novabiochem, Nottingham, U.K.), or lactacystin (25 μM; Peptide Institute, Osaka, Japan) for 1–24 h. To examine the effects of MG132 on the inducible expression of MCP-1, cells were pretreated with or without MG132 (25 μM) for 1 h and stimulated by human recombinant IL-1β (5 ng/ml; Otsuka Pharmaceutical, Tokushima, Japan) for 8 h. To examine the effects of the c-Jun/AP-1 inhibitor curcumin and the RNA synthesis inhibitor actinomycin D, cells were pretreated with curcumin (20 μM; Sigma-Aldrich, Poole, U.K.) or actinomycin D (500 ng/ml; Serva, Heidelberg, Germany) for 1 h and stimulated with MG132. The concentrations used are sufficient to inhibit the c-Jun/AP-1 pathway and RNA synthesis in SM43 mesangial cells (25, 26). After the treatments, cells were harvested and subjected to Northern blot analysis as follows.

**Northern blot analysis**

Total RNA was extracted by the single-step method (27) and subjected to analyses as described previously (28). As probes, mouse JEM/C1 cDNA (29), human c-fos cDNA (30), human c-jun cDNA (30), rat stromelysin cDNA (31), human mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1) cDNA (32), and rat GAPDH cDNA were labeled with [32P]dCTP using the random priming method and used for hybridization.

**Western blot analysis**

Confluent mesangial cells were treated with MG132 (50 μM) for up to 24 h. After the treatment cells were lysed with sample buffer (2% SDS, 5% glycerol, 0.003% bromphenol blue, and 1% 2-ME in 125 mM Tris-HCl, pH 6.8) and subjected to Western blot analysis using an anti-MCP-1 Ab (Genzyme, Cambridge, MA) as described previously (28). To examine an effect of MG132 on the stability of c-Jun protein, cells were first treated with MG132. The concentrations used are sufficient to inhibit the c-Jun/AP-1 pathway and RNA synthesis in SM43 mesangial cells (25, 26). After the treatments, cells were harvested and subjected to Northern blot analysis as follows.

**Kinase assay**

Mesangial cells were treated with MG132 (50 μM) for 1–6 h and subjected to JNK assays using the SAPK/JNK Assay kit (New England Biolabs) and PeptidePhospho SAPK/JNK (Thr183/Tyr185) Ab kit (Cell Signaling Technology, Beverly, MA) (33, 34). JNK activity was evaluated by site-specific phosphorylation of c-Jun and JNK following protocols provided by the manufacturer.

**Reporter assay**

The activity of AP-1 was assessed by transient transfection (34). In brief, using the calcium phosphate coprecipitation method, mesangial cells cultured in 24-well plates (1.0 × 104/well) were transfected with a reporter plasmid pTRE-LacZ (a gift from Dr. A. S. Alberts, Imperial Cancer Research Fund, London, U.K.) (35) or a control plasmid pCI-βgal (a gift from Promega, Madison, WI) at 0.33 μg/well, respectively. pTRE-LacZ introduces a β-galactosidase gene (lacZ) under control of 12-O-tetradecanoyl-phorbol-13-acetate response elements (TREs). pCI-βgal introduces lacZ under control of the immediate-early enhancer/promoter of human CMV. After transfection, cells were incubated for 48 h in 0.5% FCS, stimulated with MG132 (10–25 μM) for 24 h, and subjected to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) assay (36). Activity of AP-1 was evaluated by counting X-gal-positive cells in each well. The number of X-gal-positive cells transfected with pTRE-LacZ was normalized by the number of positive cells transfected with the control plasmid pCI-βgal, and relative percentages were calculated. Assays were performed in quadruplicate.

**Statistical analysis**

Data were expressed as the mean ± SD. Statistical analysis was performed using ANOVA to compare data in different groups. A p < 0.05 was used to indicate a statistically significant difference. All experiments were repeated twice or three times, and representative data were demonstrated.

**Results**

**Induction of MCP-1 expression by proteasome inhibition**

In mesangial cells expression of MCP-1 in response to various stimuli is regulated by NF-κB (17–21). We examined the effects of proteasome inhibitors, the well-known NF-κB inhibitors, on the constitutive and IL-1β-inducible expression of MCP-1. Rat mesangial cells were pretreated with or without MG132 and incubated in the presence or the absence of IL-1β. Northern blot analysis showed that, as expected, MG132 dramatically suppressed the induction of MCP-1 by IL-1β. However, unexpectedly, MG132 increased the steady-state level of MCP-1 mRNA under the unstimulated condition (Fig. 1A). This effect was observed at concentrations >25 μM (Fig. 1B) and with incubation for >8–12 h.
h (Fig. 1C). Consistent with the increase in the mRNA level, MCP-1 protein was also induced in MG132-treated cells (Fig. 1D). A similar stimulatory effect was observed with different types of proteasome inhibitors, PSI-1 and lactacystin (Fig. 1E), and in other cell type NRK49F fibroblasts (Fig. 1F).

Up-regulation of the MCP-1 mRNA level by proteasome inhibitors may be caused by transcriptional induction or increased stability of the transcript. To test the latter, mesangial cells were pretreated with the RNA synthesis inhibitor actinomycin D and then exposed to MG132. As shown in Fig. 2, pretreatment with actinomycin D completely abolished the MG132-induced increase in MCP-1 mRNA.

**Induction and activation of the JNK-c-Jun/AP-1 pathway by proteasome inhibition**

The 5′-flanking region of the MCP-1 gene contains multiple NF-κB sites and AP-1 sites (37, 38). It suggests potential roles of NF-κB and AP-1 in the regulation of MCP-1 expression. Because proteasome inhibitors are well-known NF-κB inhibitors, we speculated that proteasome inhibition may induce MCP-1 expression via activation of AP-1. To examine this possibility, mesangial cells were treated with MG132 for up to 12 h, and expression of c-Jun and c-fos was examined. Northern blot analysis showed that the expression of c-Jun was induced within 2 h and peaked to maximum at 4 h (Fig. 3A). The increased expression of c-Jun was sustained for at least 12 h. In contrast, induction of c-fos was not detectable in MG132-treated cells.

Various signaling molecules are degraded via the ubiquitin/proteasome system. We next examined whether proteasome inhibition affects degradation of c-Jun protein. Mesangial cells were incubated with or without MG132 for 8 h in the presence or absence of the protein synthesis inhibitor cycloheximide. After the treatment, cells were subjected to Western blot analysis. As shown in Fig. 3B, c-Jun protein detected in control cells was decreased dramatically after the incubation with cycloheximide (mean ± SD relative level, 5.4 ± 2.1% vs untreated control (100%)). Treatment with MG132 in combination with cycloheximide suppressed the degradation of c-Jun (relative level, 68.1 ± 11.7%).

JNK is known to be the crucial, upstream activator of c-Jun/AP-1. The effect of MG132 on the JNK activity was also tested using c-Jun protein as a substrate. Kinase assay showed that the JNK-mediated phosphorylation of c-Jun at Ser63, a site important for JNK-dependent transcriptional activity, was induced within 1 h and peaked to a maximum after 3 h (Fig. 3C). Sustained activation of JNK was observed for at least 6 h. Consistently, phosphorylation of JNK at Thr182/Tyr185 was also observed after the treatment with MG132 (data not shown).

To further confirm that the JNK-c-Jun/AP-1 pathway is indeed activated by proteasome inhibition, a reporter assay was performed. Mesangial cells were transiently transfected with a control plasmid or an AP-1 reporter plasmid, treated with or without MG132, and subjected to X-gal assay. In the control transfection, the number of β-gal-positive cells was significantly increased by MG132 in the cells transfected with the AP-1 reporter plasmid (53 ± 8 cells/well in untreated, 79 ± 5 cells/well in 10 μM, and 624 ± 20 cells/well in 25 μM). In contrast, the number of β-gal-positive cells was significantly increased by MG132 in the cells transfected with the AP-1 reporter plasmid (53 ± 8 cells/well in untreated, 79 ± 5 cells/well in 10 μM, and 91 ± 7 cells/well in 25 μM). The normalized, relative activity of AP-1 was significantly increased by the treatment with MG132 to 169 ± 11% in 10 μM-treated cells and to 228 ± 18% in 25 μM-treated cells (p < 0.05; Fig. 3D).

**Role of the c-Jun/AP-1 pathway in proteasome inhibitor-triggered MCP-1 expression**

To examine whether the activation of AP-1 is responsible for the proteasome inhibitor-triggered MCP-1 expression, mesangial cells were pretreated with c-Jun/AP-1 inhibitor curcumin and stimulated by MG132. Northern blot analysis showed that induction of MCP-1 by MG132 was abolished by treatment with curcumin (Fig. 4A).

Curcumin is a semiselective inhibitor of c-Jun/AP-1 that may suppress other signaling molecules (39). The role of c-Jun/AP-1 was further confirmed using stably transfected mesangial cells that express a dominant-negative mutant of c-Jun. These cells exhibit...
stably expressing a dominant-negative mutant of c-jun (SM/JUNDN1) were transfected with pTRE-LacZ or pCI-βgal. After the transfection, cells were treated with (+) or without (−) MG132 (25 μM) for 24 h and subjected to X-gal assay. Assays were performed in quadruplicate. Data are shown as the mean ± SD. *, p < 0.05. C, Expression of MCP-1 in control cells and AP-1-inactive cells treated with proteasome inhibitor. SM/Neo cells and SM/JUNDN1 cells were treated with or without MG132 (50 μM) for 8 h, and Northern blot analysis was performed.

Effect of proteasome inhibition on the expression of other AP-1-dependent genes

As demonstrated above, proteasome inhibitors activate the c-Jun/AP-1 pathway. It is reasonable to speculate that proteasome inhibition induces not only MCP-1, but also other AP-1-dependent genes. Indeed, the AP-1-dependent gene, c-jun, was induced after treatment with MG132 (Fig. 3A). To further examine this possibility, we tested the expression of other AP-1-dependent genes, stromelysin and MKP-1.

Stromelysin is a member of the family of matrix metalloproteases. The 5′-flanking region of the stromelysin gene contains TRE, the binding site of AP-1 (40). We previously showed that induction of stromelysin in response to IL-1β and pyrrolidine dithiocarbamate is regulated by AP-1 in mesangial cells (23, 25). MKP-1 is a prototypic member of the family of inducible dual specificity phosphatases. The regulatory region of the MKP-1 gene contains TRE (41), and activation of JNK induces expression of MKP-1 mRNA (42), suggesting a role for AP-1 in the regulation of MKP-1.

Mesangial cells were treated with three different proteasome inhibitors, MG132, PSI-1, and lactacystin, and expression of stromelysin and MKP-1 was examined by Northern blot analysis. Consistent with the results with MCP-1, expression of stromelysin was substantially induced after treatment with all three proteasome inhibitors (Fig. 5A). Similar stimulatory effects were observed in the expression of MKP-1 (Fig. 5B).

Discussion

The ubiquitin/proteasome system plays crucial roles in the degradation of various cellular proteins, including signal transduction molecules (2). Although proteasome inhibitors have been widely used as NF-κB inhibitors, it is not surprising that these agents may affect other signaling processes. In this study, we investigated the effects of proteasome inhibitors on the c-Jun/AP-1 pathway, the crucial machinery involved in a wide range of cellular function. We showed that proteasome inhibition induced expression of c-jun, inhibited degradation of c-Jun protein, and triggered phosphorylation of JNK, leading to activation of c-Jun/AP-1 and consequent induction of MCP-1 in mesangial cells.

In this report, we demonstrated that after treatment with MG132, the stability of the c-Jun protein was increased. This result is consistent with a previous report showing that degradation of c-Jun was proteasome dependent (43). However, our results also showed that expression of c-jun mRNA was rapidly and selectively induced after proteasome inhibition. Although the proteasome system may affect the stability of certain mRNAs (44), our data indicated that the increased c-jun level was due to de novo transcription (our unpublished observation). It suggested that proteasome inhibition induced c-Jun protein via both transcriptional

attenuated activity of AP-1 under both unstimulated and stimulated conditions (23). Control cells (SM/Neo) and c-Jun/AP-1-inactive cells (SM/JUNDN1) were transfected with the AP-1 reporter plasmid and treated with or without MG132. As summarized in Fig. 4B, reporter assay showed that activation of AP-1 by MG132 was significantly lower in SM/JUNDN1 cells than that in SM/Neo cells. The relative AP-1 activity was 255 ± 26% in MG132-treated SM/Neo cells and 154.3 ± 22% in MG132-treated SM/JUNDN1 cells compared with the value of untreated SM/Neo cells (100 ± 17%). Under this experimental condition, expression of MCP-1 in response to MG132 was evaluated in both cell types. Northern blot analysis showed that induction of MCP-1 by MG132 was significantly less in SM/JUNDN1 cells than that in SM/Neo cells (Fig. 4C).
and posttranslational mechanisms. In contrast to the dramatic induction of c-jun, the expression of c-fos was not induced by proteasome inhibitors. This may be due to the fact that the expression of c-jun, but not c-fos, is regulated predominantly by AP-1 (45).

Another important finding is that activation of JNK, the selective upstream activator of c-Jun/AP-1, was rapidly induced after proteasome inhibition. Currently, it is not known how proteasome inhibition activates JNK. One possibility may be that the proteasome system participates in constitutive degradation of short-lived, upstream molecules that are crucial for activation of JNK. Another mechanism might be that inhibitors of JNK are down-regulated by proteasome inhibition. Some reports showed that other members of the MAP kinase family may also be activated by proteasome inhibitors (46, 47), and suppression of common inhibitors of MAP kinases, e.g., MAP kinase phosphatases, might be involved in the activation of JNK. However, our current results showed that MKP-1, the prototypic MAP kinase phosphatase, was, rather, up-regulated by proteasome inhibition.

Previous reports showed that the 5′-flanking region of the MCP-1 gene contains NF-κB sites and AP-1 sites (37, 38). The majority of previous data emphasized the importance of NF-κB, but not AP-1, in the induction of MCP-1. For example, in mesangial cells, induction of MCP-1 by various stimuli is NF-κB dependent (17–21) and AP-1 independent (48). In contrast, the present data showed the critical role of AP-1 in the regulation of MCP-1, i.e., MCP-1 can be induced via the AP-1-dependent, NF-κB-independent mechanism. It is consistent with our recent finding that retinoic acid suppressed basal expression of MCP-1 via intervention in the AP-1-dependent, NF-κB-independent pathway (26).

In this investigation, we focused on the importance of AP-1 in the induction of MCP-1 and other genes by proteasome inhibition. However, proteasome regulates the degradation of various other proteins. The effect of proteasome inhibitors observed here might be enhanced by stabilization of other signaling molecules. For example, it has been reported that Sp-1 is degraded by the proteasome system (49) and that its binding sites are present in the promoter regions of MCP-1 and MKP-1 genes (41, 50). We cannot exclude the possibility that the induction of these genes by proteasome inhibition could be mediated in part by stabilization of other transcription factors, including Sp-1.

Proteasome inhibitors have been considered therapeutic drugs for the treatment of malignant diseases. This is mainly due to the fact that these agents induce apoptosis of rapidly dividing tumor cells but inhibit apoptosis of normal, nondividing cells (2). Another potential, therapeutic target of proteasome inhibitors is inflammation. At inflammatory sites, various molecules, including cytokines/chemokines and leukocyte adhesion molecules, are expressed via NF-κB-dependent mechanisms (51). Because proteasome inhibitors suppress the activation of NF-κB, it is not surprising that these agents have anti-inflammatory properties. However, it should be noted that proteasome inhibitors also have the potential for activating AP-1, the crucial component involved in cell proliferation, apoptosis, and expression of inflammatory mediators (28, 34, 52). This pharmacologic property could limit the utility of these agents as therapeutic drugs in some situations. Of note, a recent report showed that proteasome inhibition caused a proinflammatory response in neural cells (53).

In summary, the present data elucidated the novel potential of the proteasome system in the suppression of MCP-1 in unstimulated cells.


