Proteasome inhibitors up-regulate haem oxygenase-1 gene expression: requirement of p38 MAPK (mitogen-activated protein kinase) activation but not of NF- κ B (nuclear factor κ B) inhibition

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Regulation of intracellular protein stability by the ubiquitin-dependent proteasome system plays a crucial role in cell function. HO-1 (haem oxygenase) is a stress response protein, which confers cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis. In the present study, we found a novel action of proteasome inhibitors MG132 and MG262 on HO-1 induction, and characterized the underlying mechanisms. MG132 ($\geq 0.1 \mu\text{M}$) treatment resulted in a marked time- and concentration-dependent induction of the steady-state level of HO-1 mRNA in RAW264.7 macrophages, followed by a corresponding increase in HO-1 protein. Actinomycin D and cycloheximide inhibited MG132-responsive HO-1 protein expression, indicating a requirement for transcription and *de novo* protein synthesis. The involvement of signal pathways in MG132-induced HO-1 gene expression was examined using chemical inhibitors. Antioxidant *N*-acetylcysteine and SB203580, an antioxidant and inhibitor of p38 MAPK (mitogen-activated protein kinase), abolished MG132-inducible HO-1 expression. Further-

more, MG132 activated the p38 MAPK pathway. The half-life of HO-1 protein was prolonged by MG132, indicating that the up-regulation of HO-1 by proteasome inhibitor is partially attributable to the inhibition of protein degradation. MG132 can ablate I κ B α degradation and NF- κ B (nuclear factor κ B) activation induced by lipopolysaccharide, similar to the effect of another NF- κ B inhibitor pyrrolidine dithiocarbamate. We found HO-1 up-regulation by MG132 and pyrrolidine dithiocarbamate is unrelated to their inhibition of NF- κ B, since leptomycin B, another NF- κ B inhibitor, did not elicit similar induction of HO-1. Taken together, we found a novel effect of proteasome inhibitor on induction of HO-1 expression. This action is ascribed to the activation of the p38 MAPK pathway, but is not dependent on NF- κ B inhibition.

Key words: haem oxygenase-1, leptomycin B, p38, pyrrolidine dithiocarbamate (PDTC), proteasome inhibitor, reactive oxygen species.

INTRODUCTION

HO-1 (haem oxygenase-1) is the rate-limiting enzyme that catalyses the degradation of haem and maintains optimal haem levels necessary for preserving cellular homeostasis. Haem metabolism by HO-1 leads to the formation of biliverdin and carbon monoxide with a concurrent release of iron, which can drive the synthesis of ferritin for iron sequestration [1]. HO-1 is expressed at low levels under basal conditions, and can be highly induced in response to various agents causing oxidative stress, including hyperthermia, UV irradiation [2], hydrogen peroxide [2], heavy metals [2–5], heat shock [6], inflammatory cytokines [7,8], endotoxin [9], hypoxia [10], hyperoxia [11] and nitric oxide [12].

In general, HO-1 induction can provide cytoprotection against oxidative stress and apoptosis. This action is demonstrated not only in cultured cell systems [13–15], but also in *in vivo* studies [16,17]. Although the mediators and mechanisms by which HO-1 provides protection are not clear and depend on cell types and stimuli, accumulating lines of evidence point to the important role of CO [18,19]. A low concentration of CO can exert protection through a guanylyl cyclase and cGMP pathway [14,20].

In eukaryotic cells, the ubiquitin-dependent proteasome pathway is the central non-lysosomal pathway for intracellular protein degradation [21]. Appropriate and co-ordinated degradation of short-lived targeted proteins is essential for cellular proliferation, apoptosis and homeostasis. Owing to cellular functions of the

proteasome system, there is considerable interest in proteasome inhibitors that play a major role in cancer chemotherapy and anti-inflammation [22,23]. In the present study, we examined the effects of proteasome inhibitors on the molecular regulation of HO-1 gene transcription and expression in murine RAW264.7 macrophages.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, FBS (foetal bovine serum), penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Rabbit polyclonal antibodies specific for HO-1, I κ B α [inhibitor of NF- κ B (nuclear factor κ B)], β -actin and p38 MAPK (mitogen activated protein kinase) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibody specific for phosphorylated p38 MAPK was obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies and the ECL[®] detection reagent were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). pGL2-ELAM-Luc (κ B-Luc) under the control of one NF- κ B binding site was constructed. The AP-1 (activator protein 1)-Luc construct was provided by Dr G. Haegeman (University of Ghent-VIB, Ghent, Belgium). β -Galactosidase expression vector (pCR3lacZ) was

Abbreviations used: AP-1, activator protein 1; FBS, foetal bovine serum; HO, haem oxygenase; I κ B α , inhibitor of NF- κ B (nuclear factor κ B); JNK, c-Jun N-terminal kinase; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; RT, reverse transcription.

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purchased from Pharmacia (Uppsala, Sweden). MG132, MG262, Ro31-8220, PD98059, U0126, SB203580, GF109203X and herbimycin A were purchased from Calbiochem (La Jolla, CA, U.S.A.). SP600125 was obtained from Tocris Cookson (Ellisville, MO, U.S.A.). NAC (*N*-acetylcysteine), LPS (lipopolysaccharide) and PDTC (pyrrolidine dithiocarbamate) were obtained from Sigma (St. Louis, MO, U.S.A.). Leptomycin B was a gift from Dr M. Yoshida (Department of Biotechnology, University of Tokyo, Japan). All materials for SDS/PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Cell culture

Murine RAW264.7 macrophages obtained from A.T.C.C. (Manassas, VA, U.S.A.) were grown at 37 °C in 5 % CO₂ using Dulbecco's modified Eagle's medium containing 10 % (v/v) FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

Immunoblotting analysis

After stimulation, cells were rinsed twice with ice-cold PBS, and 100 µl of cell lysis buffer (20 mM Tris/HCl, pH 7.5/125 mM NaCl/1 % Triton X-100/1 mM MgCl₂/25 mM β-glycerophosphate/50 mM NaF/100 µM Na₃VO₄/1 mM PMSF/10 µg/ml leupeptin/10 µg/ml aprotinin) was then added to each plate. Protein was denatured in SDS, electrophoresed on SDS/polyacrylamide (10 % gel), and transferred on to nitrocellulose membrane. Non-specific binding was blocked with TBST (50 mM Tris/HCl, pH 7.5/150 mM NaCl/0.1 % Tween 20) containing 5 % (w/v) non-fat milk for 1 h at room temperature. After incubation with the appropriate first antibodies, membranes were washed three times with TBST. The secondary antibody was incubated for 1 h. After three washes with TBST, the protein bands were detected with the ECL[®] reagent.

RT (reverse transcription)-PCR

To amplify HO-1 mRNA, the specific primers for RT-PCR analysis were synthesized. Macrophages treated with the indicated agents were homogenized with 1 ml of RNazol B reagent (Gibco), and total RNA was extracted by acid guanidinium thiocyanate/phenol/chloroform extraction. RT was performed using StrataScript RT-PCR kit, and 10 µg of total RNA was reverse-transcribed to cDNA according to the manufacturer's instructions. RT-generated cDNA encoding HO-1 and β-actin genes were amplified using PCR. The oligonucleotide primers used correspond to the mouse HO-1 (5'-GAGAATGCTGAGTTCATG-3' and 5'-ATGTTGAGCAGGAAGGC-3') and mouse β-actin (5'-GACTACCTCATGAAGATCCT-3' and 5'-CCACATCTGCTGGAAGGTGG-3'). PCR was performed in a final volume of 50 µl containing Taq DNA polymerase buffer, all four dNTPs, oligonucleotide primers, Taq DNA polymerase and RT products. After an initial denaturation for 2 min at 94 °C, 35 cycles of amplification (94 °C for 45 s, 65 °C for 45 s and 72 °C for 2 min) were performed followed by a 10 min extension at 72 °C. PCR products were analysed on 2 % (w/v) agarose gel. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Nitrite measurement

Cells were cultured in 24-well plates in 500 µl of culture medium until confluence. They were treated with LPS in the absence or presence of MG132 for 24 h, and then the culture media were collected. Nitrite was measured by adding 100 µl of Griess reagent (1 % sulphanilamide and 0.1 % naphthylethylenediamide

in 5 % (v/v) phosphoric acid) to 100 µl samples of the culture medium. The absorbance at 550 nm (*A*₅₅₀) was measured using a microplate reader, and the nitrite concentration was calculated by comparing with the *A*₅₅₀ produced using standard solutions of sodium nitrite in the culture medium.

Transfection and reporter gene assay

Using electroporation (280 V, 1070 µF, 30 ms time constant), cells (2 × 10⁷ cells/cuvette) were co-transfected with 1 µg of κB promoter construct and 1 µg of β-galactosidase expression vector (pCR3lacZ; Pharmacia). After electroporation, cells were cultured in a 24-well plate at 2 × 10⁶ cells/well. After a 24 h incubation, cells were incubated with the indicated concentrations of agents. After another 24 h incubation, the media were removed, and the cells were washed once with cold PBS. To prepare lysates, 100 µl of reporter lysis buffer (Promega, Madison, WI, U.S.A.) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 12 000 *g* for 30 s. Aliquots of cell lysates (5 µl), containing equal amounts of protein (10–20 µg), were placed inside the wells of an opaque, black, 96-well microplate. An equal volume of luciferase substrate (Promega) was added to all the samples and the luminescence was measured in a microplate luminometer (Packard, Meriden, CT, U.S.A.). Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression and are presented as a percentage of luciferase activity measured with LPS stimulation alone or in the absence of the indicated agents.

Propidium iodide staining for DNA content

The propidium iodide staining assay used flow cytometry to measure the extent of cell death. Cells were initially seeded at the rate of 5 × 10⁶ cells in a 6-well plate and incubated with the indicated concentration of drugs or 0.1 % DMSO for selected time periods. After treatment, all cells were collected. Cells were then centrifuged, washed once in PBS and re-suspended in ice-cold 70 % (v/v) EtOH with vortexing and stored at 0 °C until analysis. Fixed cells were collected by centrifugation, washed once in PBS and incubated in 400 µl phosphate/citric acid buffer (0.2 M Na₂HPO₄/0.1 M citric acid, pH 7.8) for 10 min at room temperature and stained with 0.5 ml of propidium iodide staining buffer (1 % Triton X-100/1 mg/ml of RNase A/80 µg/ml of propidium iodide) for more than 30 min in the dark. Before cytometric analysis, cells were filtered on a nylon mesh filter. The samples were analysed using FACScan and Cellquest programs (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cell-cycle distribution was also analysed by flow cytometry.

Statistical evaluation

Values are expressed as means ± S.E.M. for at least three experiments, which were performed in duplicate. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a *P* < 0.05 was considered to be statistically significant.

RESULTS

Proteasome inhibitors transcriptionally induce HO-1 gene expression

Immunoblotting analysis showed that MG132 at 3 µM was able to cause HO-1 protein induction in murine RAW264.7 macrophages. At basal state, a weak immunoreactivity of HO-1 protein was

Figure 1 Time- and concentration-dependent effects of MG132 and MG262 on HO-1 induction

(A) Cells were treated with 3 μM MG132 for 18 h at different time periods. (B) Cells were treated with different concentrations of MG132 (0.1–3 μM) or MG262 (0.03–0.3 μM) for 12 h. After stimulation, cell lysates were subjected to SDS/PAGE and immunoblotted with HO-1 antibody. The β -actin protein level was considered as an internal control. The results were representative of three independent experiments.

detected. The stimulating action of 3 μM MG132 displayed time-dependence, occurring after 3 h exposure, peaking at 12 h and being maintained up to 18 h (Figure 1A). Experiments with different concentrations of MG132 showed that 0.1 μM MG132 was capable of inducing this effect for 12 h of incubation, and a striking increase was seen at 3 μM (Figure 1B). We found that another proteasome inhibitor MG262 also mimicked MG132 in HO-1 induction. The potency of MG262 was higher than that of MG132, a weak increase was observed at 0.03 μM , and a steady level achieved at 0.1–0.3 μM (Figure 1B).

With regard to cell toxicity, as observed previously for proteasome inhibitors, which are novel agents for cancer treatment [24,25], we performed cell-cycle analysis to measure subG1 appearance, an index of DNA fragmentation and a common process of cell apoptosis. Results in Figure 2 show that for 12 h of incubation with 1 and 3 μM MG132 or 0.03 and 0.1 μM MG262, only a slight population of cells (2–7%) is apoptotic. To avoid interference with cell toxicity, in the present study, we used 3 μM MG132 to treat cells for periods less than or up to a maximum of 12 h.

To understand further whether HO-1 protein induction was derived from gene transcription or not, RT-PCR analysis of mRNA was performed. MG132 (3 μM) treatment for a period of as little as 1 h is sufficient to increase HO-1 mRNA level, which was further increased to 3-fold at 2 h and slightly decreased to 1.8-fold at 5 h (Figure 3A). Verifying this action resulting from gene transcription followed by protein translation, we found MG132-induced HO-1 protein production was abrogated by the presence of actinomycin D (an inhibitor of gene transcription) and cycloheximide (an inhibitor of protein translation) (Figure 3B).

MG132 action is dependent on p38 activation and ROS (reactive oxygen species) production

To dissect the signalling pathways involved in HO-1 gene regulation, several pharmacological manipulations were tested. Figure 4(A) shows that HO-1 induction by 3 μM MG132 was markedly diminished by SB203580 (a p38 MAPK inhibitor) and NAC (*N*-acetylcysteine, an antioxidant), but not by PD98059, U0126 (two MEK (MAPK/ERK kinase) inhibitors), SP600125 [an inhibitor of JNK (c-Jun N-terminal kinase)], GF109203X, Ro318220 [two PKC (protein kinase C) inhibitors] or herbimycin

Figure 2 Effects of MG132 and MG262 on cell viability

RAW264.7 cells were incubated with indicated concentrations of proteasome inhibitors for 12 h. After incubation, cells were stained with propidium iodide and cell-cycle allocation was determined by flow cytometry. The percentage of cells with subG1 appearance is indicated. Data from a typical experiment, which was repeated three times with similar results, are presented.

A (a non-selective tyrosine kinase inhibitor). Confirming this effect, the up-regulated expression of HO-1 mRNA was blocked by SB203580 and NAC (Figure 4B). Furthermore, immunoblotting to examine p38 MAPK activation, assessed by its phosphorylation as an index, we showed a delayed but significant stimulation of p38 MAPK by MG132 (Figure 4C). Compared with the rapid onset of LPS in this event, an obvious effect seen at 15 min, MG132 induced p38 MAPK phosphorylation after 4 h of incubation.

MG132 enhances HO-1 protein stability

We speculate whether MG132, acting as a proteasome inhibitor, affects HO-1 protein degradation. To explore this issue, we determined the stability of induced HO-1 under conditions without protein translation. First, we used PDTC (50 μM) as a stimulator to induce HO-1 expression, followed by washout with

Figure 3 Transcriptional increase of HO-1 gene expression by MG132

(A) Cells were treated with 3 μ M MG132 for different time periods. Total RNA was prepared and subjected to RT-PCR analysis in order to determine the HO-1 mRNA level. The β -actin mRNA level was considered to be the internal control. The results are representative of three separate experiments. HO-1 mRNA level was quantified after normalization by β -actin mRNA expression. (B) Cells were pretreated with vehicle, 3 μ g/ml actinomycin D or 3 μ g/ml cycloheximide for 30 min before stimulation with 3 μ M MG132. The protein level of induced HO-1 was determined by immunoblotting. The results are representative of three independent experiments.

fresh medium and addition of cycloheximide to stop continuous HO-1 translation. Under these circumstances, the gradual decline in HO-1 protein in control group indicated its half-life to be 2.3 h. The presence of MG132 after cycloheximide addition slowed the degradation rate significantly, and prolonged the half-life to 6 h (Figure 5).

NF- κ B inhibition-independent effect of HO-1 induction

To address further whether HO-1 induction by MG132 is associated with NF- κ B inhibition, we compared the actions of MG132, leptomycin B and PDTC. Before determining HO-1 expression, we first verified their abilities to inhibit NF- κ B activation. For MG132, κ B α was determined as a positive control to indicate its degradation through the ubiquitination/proteasome system. In the presence of MG132 (3 μ M), LPS (1 μ g/ml)-induced rapid I κ B α degradation was inhibited and delayed (Figure 6A). Luciferase assay of NF- κ B transactivity showed that MG132, PDTC and leptomycin B are able to inhibit this event in a concentration-dependent manner (Figures 6B and 7B). Concomitant NO release induced by LPS was decreased in a concentration-dependent manner by MG132 (results not shown), PDTC and leptomycin B (Figure 7A). Even though these agents are all NF- κ B inhibitors, we found that MG132 and PDTC, but not leptomycin B, could induce HO-1 expression (Figure 7C), indicating that the HO-1 expression is not associated with NF- κ B action.

MG132 activates transactivity of AP-1

Since AP-1 was shown to be involved in HO-1 gene transcription [9,26–28], we explored the effects of MG132 and PDTC on the

Figure 4 HO-1 induction by MG132 was inhibited by p38 MAPK inhibitor and antioxidant

(A) Cells were pretreated with various pharmacological inhibitors (10 μ M SB203580, 30 μ M PD98059, 1 μ M U0126, 10 μ M SP600125, 3 μ M GF109203X, 10 μ M Ro318220, 3 μ M herbimycin A, 5 mM NAC) for 30 min, and then 3 μ M MG132 was added for another 12 h. The protein level of induced HO-1 was determined by immunoblotting. (B) Similar to Figure 3(A), HO-1 mRNA determined by RT-PCR, in the presence of SB203580 or NAC, was quantified. (C) Cells were treated with vehicle or 3 μ M MG132 for different time periods. Total cell lysates were collected and hybridized with specific antibody detecting the phosphorylated form (p-p38) or total amount of p38 MAPK. The results are representative of three independent experiments.

Figure 5 Effect of MG132 is partially dependent on the stabilization of HO-1 protein

Cells were treated with vehicle or 50 μ M PDTC for 12 h, and after washing with the medium, they were incubated in a medium containing 3 μ g/ml cycloheximide for 1 h. Subsequently, MG132 (3 μ M) was added and incubated for different time periods. The protein level of HO-1 in cell lysates was determined by immunoblotting followed by quantification using a densitometer. Values in parentheses indicate the percentage change of HO-1 protein level compared with that immediately after 1 h treatment with cycloheximide. Data represent the means $\pm$ S.E.M. from at least three independent experiments. The time-dependent degradation of an HO-1 level represents protein stability.

Figure 6 MG132 inhibits LPS-induced IκBα degradation and NF-κB activation

(A) Cells were pretreated with MG132 (3 μM) for 30 min, followed by stimulation with LPS (1 μg/ml) for different time periods. The protein levels of IκBα and β-actin in cell lysates were determined by immunoblotting. The results are representative of three independent experiments. The values in parentheses indicate the percentage of IκBα level compared with the control level before LPS stimulation. **(B)** Cells transfected with the κB reporter gene and β-gal-lacZ plasmid were treated with LPS (1 μg/ml) and/or MG132 (0.03–3 μM) for 12 h. The luciferase activity derived from κB activation was normalized to the transfection efficiency with β-gal-lacZ. Data represent the means ± S.E.M. for at least three independent experiments. **P* < 0.05 indicates the capability of MG132 to inhibit LPS-induced κB activation.

transactivity of AP-1. As shown in Figure 8, both agents as well as LPS were capable of stimulating AP-1 transactivity in a concentration-dependent manner.

DISCUSSION

Modulation of gene transcription is the principal mechanism by which HO-1 is regulated. In the present study, we determined the ability of proteasome inhibitors to activate the transcription of HO-1 gene in murine RAW264.7 macrophages, and examined the signalling pathway. The inhibitory effects of actinomycin D, which inhibits transcription, and cycloheximide, a potent inhibitor of translation, indicate mRNA and protein synthesis *de novo* for HO-1 induction by proteasome inhibitors. Furthermore, we suggest that production of ROS, p38 MAPK and AP-1 activation are required for this event.

The proteasome-dependent pathway for protein degradation has been implicated in the regulation of several signalling pathways, such as activation of NF-κB [29], down-regulation of STAT (signal transduction and activators of transcription) activation [30] as well as the production of ROS [31,32]. Since HO-1 can be induced in response to various treatments, which change the intracellular redox state, such as hydrogen peroxide, glutathione depletors, UV radiation, hyperoxia, ischaemia-reperfusion injury, NO, LPS, 15dPGJ₂ and dopamine [2,3,10,33–38], it has been suggested that ROS may mediate the induction of HO-1, and HO-1 may consequently function against oxidative stress. Our current results indicate that ROS indeed participates in the inductive effect of MG132 on HO-1 expression.

Figure 7 HO-1 induction is dissociated from NF-κB inhibition

According to the methods described above, cells treated with PDTC and leptomycin B at different concentrations with LPS (1 μg/ml) were assayed by NO production **(A)** and NF-κB reporter activity **(B)**. HO-1 protein expression was also assayed by immunoblotting after MG132, PDTC or leptomycin B treatment **(C)**. Data in **(A, B)** represent the means ± S.E.M. for at least three independent experiments. **P* < 0.05 indicates significant inhibition of LPS-induced NO production and κB activation.

Figure 8 AP-1 activation by MG132 and PDTC

Cells transfected with the AP-1 reporter gene and β-gal-lacZ plasmid were treated with MG132, PDTC or leptomycin B at the concentrations indicated, or with 1 μg/ml LPS for 24 h. The luciferase activity derived from AP-1 activation was normalized to the transfection efficiency with β-gal-lacZ. Data represent the means ± S.E.M. for at least three independent experiments.

The MAPKs are a family of structurally related signal-transducing enzymes participating in the regulation of many essential cellular processes [39]. Three major subgroups of the MAPK family identified to date include ERK (extracellular-signal-regulated kinase), JNK and p38 MAPK. Recent mechanistic studies on HO-1 induction have pointed out the critical intermediate nature of the p38 MAPK pathway in the regulation of HO-1 expression by TGF- β [8], NO [40], arsenite [41], cadmium [42], hypoxia [43], ischaemia reperfusion [44], IL (interleukin)-10 [45] and 15dPGJ₂ [46]. Although the involvement of ERK in some stressful stimuli-induced HO-1 expression was identified [41], the JNK pathway appeared not to be involved [47]. In the present study, we found that the effect of MG132 depends only on p38 MAPK.

Transcription factor AP-1 behaves as a redox-sensitive transcription factor that can be activated by either oxidant or anti-oxidant stimuli [27,48,49]. The mouse HO-1 gene contains two inducible enhancers E1 and E2. E1 contains three stress-response elements, which encompass the consensus motifs for AP-1 proteins. Deletion and mutational analyses of the regulatory element of the HO-1 gene indicate that these AP-1-binding sites in stress-response elements play an important role in mediating HO-1 gene regulation by different stimuli [9–11,26–28,43,50,51]. This suggests a commonality in response to multiple agents in the activation mechanism of HO-1. Our results demonstrated that proteasome inhibitors induce AP-1 activation, which, as indicated previously with strong evidence, leads to the induction of HO-1 gene transcription. When previous studies explored the transcription factors responsible for HO-1 transcription, different proteins were identified, according to the variety of HO-1 inducers and cell types. For example, Fos and c-Jun were activated for binding to an AP-1 regulatory element by IL-1 β [51]. Nrf2, one of the four CNC-bZIP proteins, which functions as a transcription activator, was activated by cadmium, cobalt, haem, arsenite and 15dPGJ₂ [4,5,36,42,52,53]. Nrf2 was suggested to form a heterodimer with Maf, and bind to the stress-response element for HO-1 transcription [42,52]. In addition to AP-1 activation by up-regulating the gene expression and increasing the protein stability of Fos and c-Jun [54,55], a p38 MAPK signalling pathway can also mediate the transactivity caused by Nrf2 through an unknown mechanism [42]. The identity of members of the AP-1 protein and CNC-bZIP protein families activated after proteasome inhibitor stimulation in RAW264.7 macrophages, including Fos, c-Jun, JunD, Nrf1, Nrf2, ATFs and Maf, needs further characterization.

Moreover, our results are consistent with previous studies [27] showing that PDTC has a similar effect on AP-1 activation and HO-1 induction. These results confirm the notion that AP-1 is a redox-sensitive transcription factor that can be activated not only by oxidant stimuli but also by antioxidant stimuli and hypoxia. This action explains that HO-1 gene is up-regulated by hypoxia [56] and antioxidants, such as PDTC [27], curcumin [43,57] and diphenylene iodonium [43]. In the present study, we provide further evidence to exclude the correlative action of MG132 on HO-1 induction and NF- κ B inhibition. Although leptomycin B is a potent NF- κ B inhibitor, owing to its binding to nuclear protein CRM1 and blockade of the nuclear export of I κ Bs [58–60], it cannot evoke HO-1 gene induction.

In conclusion, we provide evidence to show the ability of proteasome inhibitors in inducing HO-1 gene expression in murine macrophages. This effect is mediated by the production of ROS and activation of p38 MAPK and AP-1.

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