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Adiponectin suppression of late inflammatory mediator, HMGB1-induced cytokine expression in RAW 264 macrophage cells

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Running title: Adiponectin inhibition of HMGB1-induced response

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Summary

High mobility group protein B1 (HMGB1) is a late inflammatory mediator released from inflammatory cells when stimulated, resulting in exaggerating septic symptoms. We recently demonstrated that full-length adiponectin, a potent anti-inflammatory adipokine, inhibits lipopolysaccharide-induced HMGB1 release. However, the effects of adiponectin on HMGB1-induced exaggerating signals currently remain unknown. This study aimed to investigate the effects of adiponectin on the pro-inflammatory function of HMGB1 in RAW264 macrophage cells. The treatment of RAW264 cells with HMGB1 significantly up-regulated the mRNA expression of tumor necrosis factor-α, interleukin-1β, and C-X-C motif chemokine 10. HMGB1-induced cytokine expression was markedly suppressed by a toll-like receptor 4 antagonist, and slightly suppressed by an antagonist of the receptor for advanced glycation end products. A prior treatment with full-length or globular adiponectin dose-dependently suppressed all types of HMGB1-induced cytokine expression, and this suppression was abolished by compound C, an AMP kinase inhibitor, but not by the heme oxygenase (HO)-1 inhibitor, zinc protoporphyrin IX. Both forms of adiponectin also reduced the mRNA expression of TLR4. These results suggest that full-length and globular adiponectin suppress HMGB1-induced cytokine expression through an AMP kinase-mediated HO-1-independent pathway.

Key words: Adiponectin, High mobility group protein B1, Inflammation, Macrophages, Toll-like receptor 4.

High mobility group protein B1 (HMGB1) is a highly conserved, ubiquitous non-histone nuclear protein that is abundantly expressed in most kinds of mammalian cells (*1, 2*). The major structural features of HMGB1 are two positively charged DNA-binding domains, termed the A and B boxes, and a negatively charged C-terminal acidic region. Full-length HMGB1 and its B box act like cytokine and activate macrophage through a cell surface receptor as described below, whereas the A box acts like an antagonist to compete with HMGB1 for the receptor binding (*3, 4*). HMGB1 contains two non-classical nuclear localization sequences, resulting in the predominantly nuclear localization of HMGB1 under physiological conditions (*5*). Nuclear HMGB1 stabilizes the structure of chromatin and participates in DNA replication, transcription, and repair. In response to infection or injury, HMGB1 is actively secreted by innate immune cells and/or passively released by injured or damaged cells.

Once in the extracellular space, HMGB1 acts as a damage-associated molecular pattern molecule and triggers inflammation in a manner that depends on its redox state. HMGB1 with all cysteine residues reduced stimulates immune cell infiltration and initiates chemotaxis via the chemokine receptor CXCR4 and the receptor for advanced glycation end products (RAGE). Partially oxidized HMGB1 with a Cys23-Cys45 disulfide bond and reduced Cys106, the main isoform that accumulates in the extracellular space and serum during acute and chronic inflammation, is a pro-inflammatory cytokine-like molecule that activates macrophages/monocytes via multiple cell surface receptors including RAGE (*6, 7*), Toll-like receptor (TLR) 2, TLR4 (*8, 9*), TLR9 (*10, 11*), cluster of differentiation 24 (CD24)/Siglec-10 (*12*), and Mac-1 (*13*), as well as single transmembrane domain proteins (e.g., syndecans) (*14*) to produce proinflammatory cytokines, chemokines, and adhesion molecules. Fully oxidized HMGB1 (sulfonyl HMGB1) is devoid of chemotactic and cytokine activities (*15-17*).

Numerous studies have focused on the biological role of extracellular HMGB1 since it was first identified as a late mediator of endotoxemia (*18*). Moreover, HMGB1 has been established as a pathogenic mediator of infection- and injury-elicited inflammatory diseases including

sepsis (*19, 20*), arthritis (*21-23*), ischemia-reperfusion injury (*24-27*), pancreatitis (*28, 29*), obesity (*30*), and cancer (*31, 32*). The administration of neutralizing antibodies and other selective HMGB1 antagonists reverses inflammation and prevents organ damage and lethality in the above-described inflammatory diseases.

Adiponectin is an adipokine secreted by adipose tissue that regulates glucose and lipid metabolism as well as immuno-responses (*33-37*). Full-length adiponectin is a 30-kDa protein that circulates in plasma as trimer, hexamer, and multimeric complexes that may differ in their signaling effects and biological activities (*38-41*). The cleavage of full-length adiponectin by proteases secreted from activated monocytes and/or neutrophils generates globular adiponectin, which is reportedly more biologically active than full-length adiponectin (*42*). Plasma adiponectin levels were previously shown to be reduced in obese humans, particularly those with visceral obesity, and inversely correlated with insulin resistance (*43, 44*).

The anti-inflammatory effects of adiponectin are mainly exerted on monocytes/macrophages via the adiponectin receptors, AdipoR1 and AdipoR2, both of which are expressed on macrophages. The anti-inflammatory potential of adiponectin is driven by multiple signaling mechanisms. It inhibits the up-regulation of endothelial adhesion molecules in response to inflammatory signals and suppresses the phagocytic activity of and cytokine production by lipopolysaccharide (LPS)-stimulated macrophages (*33, 34*). Adiponectin has also been shown to desensitize macrophages to TLR4-dependent signaling (*34, 45-47*). We previously demonstrated that the exposure of RAW264 macrophage cells to full-length adiponectin inhibits LPS-induced HMGB1 release via AMP kinase and the HO-1-dependent pathway (*48*). Furthermore, supplementation with adiponectin has been reported to decrease serum HMGB1 and HMGB1 mRNA expression in lung tissues in polymicrobial sepsis mouse models (*49*). Collectively, these findings prompted us to speculate that adiponectin desensitizes macrophages to HMGB1 and subsequently attenuates its pro-inflammatory activity. Therefore, this study aimed to investigate whether adiponectin attenuates the proinflammatory function of

HMGB1 in macrophages and, if so, elucidate the underlying mechanisms responsible for this effect.

Materials and Methods

Materials

Recombinant mouse full-length adiponectin expressed in HEK293 cells was purchased from Biovendor (Asheville, NC, USA). Recombinant mouse globular adiponectin was purchased from Antibody and Immunoassay Services, the University of Hong Kong (Hong Kong). Recombinant murine IL-10 and anti-mouse IL-10 antibody were purchased from PeproTech (Rocky Hill, NJ, USA) and Biolegend (San Diego, CA, USA), respectively. OPTI-MEM I was purchased from Invitrogen (Carlsbad, CA, USA). The following antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA): a rabbit anti-HMGB1 antibody, anti-His Tag antibody, anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase, an anti-phospho-NF-κB p65 (Ser536) antibody, anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, and anti-phospho-p44/42 MAPK (ERK1/2) antibody. An anti-β-actin antibody was purchased from BD Bioscience (San Jose, CA, USA). RIPA buffer (Cell Lysis Solution) and phosphatase inhibitor cocktail were obtained from Nacalai Tesque (Kyoto, Japan). Zinc protoporphyrin IX (ZnPP) was purchased from Frontier Scientific (Logan, UT, USA), while dorsomorphin (compound C) was purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Hexyl-3,4,6-trihydroxy-2-methoxy-5-oxo-5H-benzo[7]annulene-8-carboxylate(CU-CPT22) and N-benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1) were purchased from Calbiochem (San Diego, CA, USA), and resatorvid (TAK-242) was purchased from ChemScene (Monmouth Junction, NJ, USA).

Preparation of recombinant mouse full-length HMGB1 and the A box.

The coding regions of the mouse HMGB1 and A box proteins were amplified by PCR from mouse spleen cDNA using specific primers with attB sites (Supplementary Table 1) and their sequences were confirmed by DNA sequencing. The PCR product was inserted into

pDEST17 (Invitrogen) according to the manufacturer's protocol, and the plasmids were transformed into *Escherichia coli*, *KRX strain* (Promega, Madison, USA). The recombinant proteins were expressed by adding rhamnose (Wako Pure Chemicals, Osaka, Japan) and isopropylthio-β-galactoside (Invitrogen) at final concentrations of 0.1% and 1 mM, respectively. Cells were harvested by centrifugation at 12,000 rpm at 4 °C for 15 min. The pellet was treated with Bugbuster® protein extraction reagent (Millipore, Billerica, MA, USA) containing deoxyribonuclease I (Wako), lysozyme (Wako), and phenylmethylsulfonyl fluoride (Sigma–Aldrich). After centrifugation at 12,000 rpm at 4 °C for 15 min, the supernatant was collected as a soluble fraction.

Recombinant HMGB1 and A box proteins were purified using the Nickel His Trap HP column (GE Healthcare, Buckinghamshire, UK). Briefly, the soluble fraction was subjected to overnight dialysis against phosphate-buffered saline (PBS) with 20 mM imidazole and then applied to the Nickel His Trap column, which was pre-equilibrated with binding buffer containing 20 mM imidazole and 500 mM NaCl. The column was washed and the recombinant protein was eluted with elution buffer containing 500 mM imidazole and 500 mM NaCl. The eluted protein was dialyzed against PBS and the purity of the recombinant protein was verified by Coomassie Brilliant Blue (CBB) staining and Western blotting with an anti-His-tag antibody. Contaminating LPS from protein preparations was removed by the Pierce™ High capacity endotoxin removal spin column (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. LPS concentration in the HMGB1 preparation was less than 0.2 endotoxin unit/ μg protein.

Cell culture and treatments

The murine macrophage-like cell line, RAW264 (RCB0535; RIKEN Cell Bank, Japan) was maintained in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemicals) supplemented with 10% fetal bovine serum (FBS, Trace Scientific Ltd., Melbourne, Australia),

100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of humidified 5% CO_2 at 37 °C. When RAW264 cells reached 80-90% confluence, they were washed twice with and subsequently cultured in serum-free OPTI-MEM I for 12 h before all treatments. When included, the cells were pretreated with compound C, ZnPP, TAK-242, CU-CPT22, FPS-ZM1, or anti-IL-10 antibody for 1 h and then stimulated with adiponectin and/or HMGB1. In stimulation experiments, polymyxin B (PMB; a LPS inhibitor; Sigma-Aldrich) was routinely added to the cell culture medium at a concentration of 10 μg/ml in order to suppress any contaminating endotoxin in protein preparations.

Western blot analysis

RAW264 cells were rinsed twice with ice-cold PBS, collected by scraping and centrifuged at 3,000 × *g* for 1 min. Whole cell extracts were prepared by the resuspension of cells in RIPA buffer supplemented with phosphatase inhibitor cocktail and incubated at 4 ℃ for 20 min under gentle shaking. Insoluble material was removed by centrifugation (12,000 × *g*, 4 °C, 30 min) and the supernatant was used in Western blot experiments. Equal amounts of protein (20 μg) were mixed with SDS loading buffer (500 mM Tris-HCl (pH 7.4), 10% SDS, 0.5% bromophenol blue, 5% 2-mercaptoethanol), boiled at 100 °C for 5 min, separated on 15% SDS-polyacrylamide gels, and transferred onto polyvinylidene fluoride membranes (Immobilon; Millipore). The membranes were blocked at room temperature for 1 h in blocking buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1%Tween 20 (TBS-T), and 5% skimmed milk) and then incubated at 4 °C overnight with a specific primary antibody. Membranes were then washed with TBS-T for 15 min and incubated with the appropriate horseradish peroxidase-linked goat anti-rabbit or anti-mouse secondary antibody at room temperature for 1 h. Signals were visualized using chemiluminescent HRP Substrate (Millipore) according to the manufacturer's instructions and detected using the ImageQuant LAS 500

system (GE Healthcare). The intensity of the chemiluminescence of the corresponding bands was quantified using ImageJ software (v. 1.48, http://imagej.nih.gov/ij).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RAW264 cells using the RNAiso reagent (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed using a 15-mer oligo (dT) adaptor primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed on a fluorescence thermal cycler (Light Cycler System; Roche Diagnostics, Mannheim, Germany) using FastStart Essential DNA Green Master PCR kits (Roche). Expression levels were measured using the standard curve method with respective cDNA fragments as standards. Expression levels are expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression as an internal control. The primer sequences used in this study and the lengths of each PCR product are listed in Supplementary Table 2.

Statistical Analysis

IBM SPSS Statistics version 22.0 software (SPSS, Chicago, IL, USA) was used for statistical analyses. Data are presented as means ± standard error (SE). Statistical comparisons between multiple groups were performed with a one-way analysis of variance followed by Dunnett's post hoc test. A p value of <0.05 was considered to be significant.

Results

The recombinant full-length HMGB1 and A box proteins were successfully produced from *E. coli*. The purity of the recombinant HMGB1 produced was higher than 95%, as judged by CBB staining (Fig. 1A). In order to examine the biological activity of the HMGB1 protein, RAW264 macrophage cells were treated with HMGB1 (5 μg/ml) in the presence or absence of polymyxin B (PMB), an inhibitor of LPS. The HMGB1 treatment increased tumor necrosis factor (TNF)-α mRNA expression, even in the presence of PMB, while the boiled HMGB1 protein (100 °C for 30 min) failed to do (Fig. 1B). In contrast, LPS treatment with or without boiling increased TNF-α mRNA expression, and inclusion of PMB clearly inhibited the LPS effect (Fig. 1C). Moreover, LPS in the HMGB1 preparation was less than the detection limit, these results suggest that the recombinant HMGB1 protein, but not contaminated LPS was responsible for its biological activity.

The treatment of cells with increasing concentrations of HMGB1 dose-dependently induced TNF-α mRNA expression, and this was accompanied by the time- and dose-dependent activation of the NF-κB, p38MAPK, and ERK pathways (Figs. 1D and 1E). Furthermore, a prior treatment with the HMGB1 A box protein, which was inactive in the induction of TNF-α (Fig. 1B), antagonized HMGB1-induced responses (Fig. 2A), indicating HMGB1 influences RAW264 cells through a receptor-mediated pathway. To date, ten separate receptors and proteins have been implicated in mediating biological responses to HMGB1: RAGE, TLR2, TLR4, TLR9, Mac-1, syndecan-1, phosphacan, protein-tyrosine phosphatase-ζ/β, and CD24 (*6-14, 50, 51*). In addition, previous studies reported that TLR2, TLR4, and RAGE are required to mediate HMGB1-induced inflammatory responses in macrophages (*7, 9, 51*). In order to identify the receptors that mediate the inflammatory reaction induced by HMGB1 in RAW264 cells, cells were treated with pharmacological inhibitors for TLR2, TLR4, or RAGE for 1 h before the addition of HMGB1. TAK-242, a TLR4 antagonist, significantly decreased

HMGB1-induced TNF-α mRNA expression, while CU-CPT22 and FPS-ZM1, TLR2 and RAGE antagonists, respectively, did not (Fig. 2B). However, when the expression of interleukin (IL)-1β was analyzed, antagonists for TLR4 and RAGE significantly suppressed HMGB1-induced IL-1β mRNA expression (Fig. 2B), suggesting that TLR4 and RAGE are, at least in part, involved in HMGB1 signaling in RAW264 cells. The activation of TLR4 transduces its signals through myeloid differentiation primary response protein 88 (MyD88) adaptor protein-dependent and -independent pathways, and the expression of TNF-α and IL-1β are controlled by a MyD88-dependent pathway (*52, 53*). Preliminary experiments revealed that HMGB1 induced the mRNA expression of C-X-C motif ligand 10 (CXCL10) and interferon β, which are mediated by a MyD88-independent pathway (*52*) (data not shown).

In order to investigate the effects of adiponectin on HMGB1-induced inflammatory responses in RAW264 macrophage cells, cells were treated with increasing concentrations of the globular and full-length forms of adiponectin for 18 h prior to the stimulation with HMGB1. Globular and full-length adiponectin dose-dependently suppressed the HMGB1-induced mRNA expression of TNF-α and IL-1β as well as that of CXCL10 (Fig. 3).

We previously reported that full-length adiponectin inhibits LPS-induced HMGB1 release through an AMP kinase- and heme oxygenase (HO)-1-dependent pathway in RAW264 macrophage cells (*48*). In order to investigate whether the same pathway is involved in the effects of both forms of adiponectin on cytokine expression, changes in HO-1 mRNA expression by globular adiponectin were examined. As shown in Figure 4, globular adiponectin doubled the expression of HO-1 mRNA, whereas full-length adiponectin increased HO-1 mRNA expression 4-fold (*48*). The treatment of RAW264 cells with compound C, an AMP kinase inhibitor, 1 h before the addition of adiponectin completely abolished the suppressive effects of both forms of adiponectin on HMGB1-induced TNF-α, IL-1β, and CXCL10 mRNA expression (Fig. 5). However, the treatment with zinc protoporphyrin IX (ZnPP), a HO-1

inhibitor, failed to abolish this suppression (Fig. 5), suggesting that suppression by both forms of adiponectin is mediated through a HO-1-independent mechanism.

The quantification of mRNA expression revealed that the treatment of RAW264 cells with globular adiponectin increased IL-10 expression, but decreased TLR4 expression without affecting TLR2 or RAGE expression (Fig. 4). Interestingly, the prior treatment with IL-10 effectively inhibited HMGB1-induced cytokine expression (Fig. 6). Moreover, neutralization antibody to IL-10 prevented globular adiponecttin-induced suppression of HMGB1 effect (Fig.7). Thus, IL-10 does appear to be involved in the suppressive effects elicited by globular adiponectin.

Discussion

In the present study, purified recombinant HMGB1 produced in *E. Coli* was passed through LPS removal resin, and contaminated LPS concentration in this HMGB1 preparation was less than 0.2 endotoxin unit/μg protein. As LPS induces TNF-α mRNA *via* TLR4 (*54*), we included PMB, anti-LPS, in the RAW264 culture to minimize LPS effect, if present. Actually, PMB suppressed LPS (10 ng/ml) effect, but not HMGB1-induced expression of TNF-α mRNA, suggesting our HMGB1 preparation was biological active, but free from LPS. Furthermore, difference in heat sensitivity between LPS and HMGB1 supported the above suggestion. However, as LPS in pg/ml concentrations, that is sufficient for causing its biological activity, is heat-inactivated (*55*), it should be noticed that only assessment of boiled recombinant protein cannot exclude the possibility of LPS contamination.

We here demonstrated that recombinant HMGB1 induced the expression of cytokines through TLR4 and RAGE, but not TLR2 in RAW264 cells. These results are partly supported by previous findings showing that TLR2, TLR4, and RAGE are required to mediate HMGB1-induced inflammatory responses in macrophages; however, other miscellaneous proteins have been suggested to play roles (*6-14, 50, 51*). Furthermore, since the TLR4 antagonist was the most effective among the antagonists used and the mRNA expression levels of RAGE were 1/100 those of TLRs, TLR4 appears to predominantly mediate HMGB1 signaling in cells.

We also demonstrated that the prior treatment of RAW264 cells with full-length and globular adiponectin suppressed HMGB1-induced cytokine expression. Since we recently reported that the exposure of RAW264 macrophage cells to full-length adiponectin inhibits the LPS-induced release of HMGB1 via AMPK and the HO-1-dependent pathway (*48*), full-length and globular adiponectin were assumed to both activate AMPK through adipoR2 and adipoR1, respectively, resulting in the induction of HO-1 and subsequent HO-1-dependent inhibition of

cytokine expression. As expected, the effects of both forms of adiponectin were blocked in the presence of an AMPK inhibitor. However, although both forms of adiponectin significantly up-regulated HO-1 expression, the HO-1 inhibitor failed to block these effects. Thus, AMPK appears to be involved in the suppression of HMGB1-induced cytokine expression by adiponectin.

In the present study, we mainly analyzed the mRNA expression of three cytokines: TNF-α, IL-1β, and CXCL10. The two former cytokines have been classified as MyD88-dependent cytokines, while the latter is MyD88-independent. MyD88 is directly associated with TLR4 and transduces the receptor signal as an adaptor protein, leading to the production of pro-inflammatory cytokines (*52*). The TLR4 signal is also transduced in a MyD88-independent pathway that elicits the expression of IFNβ and IFN-inducible genes (*52*). Since both forms of adiponectin suppressed HMGB1-induced cytokine expression irrespective of dependence on MyD88, an upstream molecule of the Myd88-dependent and -independent pathways may be responsible for this suppression. Both forms of adiponectin decreased TLR4 mRNA expression (Fig. 4) (*48*), and reductions in cell surface TLR4 were previously reported in macrophages treated with globular adiponectin (*46*). Therefore, the reductions induced in TLR4 by adiponectin may be attributed, at least in part, to the suppression of HMGB1-induced cytokine activity by adiponectin.

Previous studies reported that the anti-inflammatory effects of adiponectin on LPS-stimulated TNF-α, IL-1β, and CXCL10 mRNA expression were mediated by a HO-1-dependent pathway (*45, 46, 56*). Since LPS is a potential activator of TLR4, the discrepancy between the present results and previous findings on LPS indicates that macrophage responses to HMGB1/TLR4 signaling differ from those to LPS/TLR4 signaling. In our preliminary results, TNF-α and IL-1β expression levels induced by HMGB1 were markedly lower than those by LPS, whereas the expression level of CXCL10 induced by

HMGB1 was similar to that by LPS (data not shown). Thus, the modes of signaling through TLR4 appear to differ between stimuli such as LPS and HMGB1.

It has been reported that IL-10 induction contributes to the anti-inflammatory effect of globular adiponectin (*56*). In the present study, we have found that IL-10 shows anti-inflammatory effect, and that inclusion of neutralizing antibody against IL-10 prevented the globular adiponectin suppression of HMGB1-induced cytokine expression. Thus, it is confirmed that IL-10 is one of anti-inflammatory mediator of globular adiponectin. In contrast, IL-10 may not play a role in the suppression of HMGB1-induced cytokine expression by full-length adiponectin, because it is reported that full-length adiponectin fails to induce IL-10 in RAW264 cells (*48*) and that its anti-inflammatory effects are independent on IL-10 (*45*).

Several studies showed that obese individuals with low adiponectin levels exhibit higher insulin resistance associated with stronger inflammatory reactions (57, 58), and that the increased accumulation of adipose tissue macrophages caused by obesity is associated with the development of chronic inflammation and metabolic dysfunction (*59-61*). In addition, obesity leads to M1 macrophage polarization in rodent adipose tissue, which contributes to inflammatory responses and tissue destruction (*62, 63*). HMGB1 was recently reported to be elevated during obesity (*64*) and has been identified as a new biomarker of metabolic syndrome in obese children (*65*). The neutralization of HMGB1 has been suggested to reduce weight gain and liver inflammation in mice fed an obesogenic diet (*30*). Therefore, additional research is needed in order to clarify whether adiponectin affects obesity-associated HMGB1 release and functions *in vivo*.

In summary, we herein demonstrated that the prior treatment of cells with full-length and globular adiponectin suppressed HMGB1-induced TLR4-mediated TNF-α, IL-1β, and CXCL10 mRNA expression, possibly through AMPK-dependent, HO-1-independent reductions in TLR4 in the RAW264 mouse macrophage cell line.

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Figure legends

Figure 1. Recombinant HMGB1 dose-dependently induced TNF-α expression in RAW264 macrophage cells. **A**. Recombinant HMGB1 and its A-box fragment were purified from an *E. coli* extract by an Ni^{+} column and subjected to SDS-PAGE with CBB staining (left) and a Western blot analysis using anti-HMGB1 (middle) or His tag (right) antibodies. **B.** RAW264 cells were cultured in DMEM supplemented with 10% FBS and then in serum-free OPTI-MEM I medium for 12 h. Cells were treated with HMGB1 (5 μg/ml) and A-box (5 μg/ml), which were or were not boiled for 30 min, in the presence or absence of the anti-lipopolysaccharide (LPS), polymyxin B (PMB, 10 μg/ml) for 3 h. RNA was extracted and the expression of TNF-α and Gapdh mRNA was measured by qRT-PCR. The results obtained are expressed as means ± SE of three independent experiments (** $p < 0.01$ significantly different from the control). **C.** Cells were cultured as in **B** and stimulated with LPS (10 ng/ml) in the presence or absence of PMB (10 μg/ml) or boiled LPS (30 min, 100°C) for 3 h. Expression of TNF-α and Gapdh mRNA was measured by qRT-PCR. Data are representative of two independent experiments. **D**. Cells were cultured as in B and stimulated with increasing concentrations of HMGB1 (1 - 5 μg/ml) for 3 h in the presence of PMB. The expression of TNF-α mRNA was measured and expressed as means ± SE of three independent experiments (*** $p < 0.001$, *$p < 0.05$ significantly different from the control). **E**. Cells were cultured as in B and stimulated with HMGB1 (6 μg/ml) for 0-60 min and increasing concentrations of HMGB1 (1- 10 μg/ml) for 30 min. Representative Western blots showed the time- and dose-dependent activation of the NF-κB, p38MAPK, and ERK pathways by HMGB1 (left and right panel, respectively). β-actin served as a loading control.

Figure 2. HMGB1-induced inflammatory cytokine expression was mediated by the receptor for advanced glycation end products (RAGE) and Toll-like receptor (TLR) 4, but not by TLR1/2 in RAW264 cells. Cells were cultured as described in Figure 1B and treated with HMGB1 A-box in (A), and antagonists of RAGE (FPS-ZM1), TLR1/2 (CU-CPT22), and TLR4 (TAK-242) in (B) 1 h before the addition of HMGB1. The expression of TNF-α, IL-1β, and Gapdh mRNA 2.5 h after the addition of HMGB1 was measured by qRT-PCR. The results obtained are expressed as means ± SE of three independent experiments (**** $p < 0.0001$, ***

$p < 0.001$ significantly different from the control, $^{++++}p < 0.0001$, $^{+++}p < 0.001$, $^{++}p < 0.01$, $^{+}p < 0.05$ significantly different from the HMGB1 treatment).

Figure 3. Full-length and globular adiponectin suppressed HMGB1-induced inflammatory responses in RAW264 cells. Cells were cultured in DMEM supplemented with 10% FBS and cultured in serum-free OPTI-MEM I medium for 12 h. Cells were treated with increasing concentrations of globular (gAPN, A) or full-length (flAPN, B) adiponectin for 18 h, and then stimulated with HMGB1 (6 μg/ml) for another 2.5 h in the presence of PMB (10 μg/ml) in order to suppress any contaminating LPS in the protein preparations. The expression of TNF-α, IL-1β, CXCL10, and Gapdh mRNA was measured by qRT-PCR. The results obtained are expressed as means ± SE of three independent experiments (*** $p < 0.001$ significantly different from the control, $^{+}p < 0.05$, $^{++}p < 0.01$ significantly different from HMGB1-treated cells).

Figure 4. Effects of globular adiponectin on mRNA expression in RAW 264 cells. RAW264 cells were cultured as described in Figure 3 and treated with gAPN (3 μg/ml) for 18 h. RNA was extracted and the expression of TLR4 (A), TLR2 (B), RAGE (C), IL-10 (D), HO-1 (E), and Gapdh (control) mRNAs was measured by qRT-PCR. The results obtained are expressed as means ± SE of three independent experiments (** $p < 0.01$, *$p < 0.05$ significantly different from the control (Cont)).

Figure 5. Effects of AMPK and HO-1 inhibitors on the suppression of HMGB1-induced inflammatory responses by full-length and globular adiponectin in RAW264 cells. Cells were cultured as in Figure 3 and treated with an AMPK inhibitor (compound C, 10 μM) or heme oxygenase-1 inhibitor (ZnPP, 1 μM) 1 h before the treatment with gAPN (A) or flAPN (B) for 18 h, and were subsequently stimulated with HMGB1 (6 μg/ml) for another 3 h. The expression of TNF-α, IL-1β, CXCL10, and Gapdh mRNA was measured by qRT-PCR. The

results obtained are expressed as means ± SE of three independent experiments ($^{+}p < 0.05$, $^{++}p < 0.01$ significantly different from HMGB1-treated cells, $^{\#}p < 0.05$ significantly different from HMGB1 plus ZnPP-treated cells).

Figure 6. Effects of recombinant IL-10 on HMGB1-induced inflammatory responses in RAW264 cells. Cells were cultured as in Figure 3, pretreated with increasing concentrations of recombinant IL-10 for 18 h, and then stimulated with HMGB1 (6 μg/ml) for another 2.5 h. The expression of TNF-α, IL-1β, CXCL10, and Gapdh mRNA was measured by qRT-PCR. The results obtained are expressed as means ± SE of three independent experiments (*** $p < 0.001$ significantly different from the control, $^{+}p < 0.05$, $^{++}p < 0.01$ significantly different from HMGB1-treated cells).

Figure 7. Effects of anti-IL-10 antibody on globular adiponectin suppression of HMGB1-induced cytokine expression in RAW264 cells. Cells were cultured as in Figure 3 and treated with anti-IL-10 antibody 1 h before the treatment with gAPN for 18 h, and were subsequently stimulated with HMGB1 (6 μg/ml) for another 2.5 h. The expression of TNF-α, IL-1β, and Gapdh mRNA was measured by qRT-PCR. The results obtained are expressed as means ± SE of three independent experiments ($^{+}p < 0.05$, $^{++}p < 0.01$ significantly different from HMGB1-treated cells).

Supplementary Table 1. Primer sequences for the construction of full-length HMGB1 and the A box expression vector.

Mouse gene	Forward primer	Reverse primer	Product size (bp)
HMGB1	GGGGACAAGTTTGTACAAAAAA GCAGGCTTCGAAGAAGATAGAA CCATGGGCAAAGGAGATCCTAA	GGGGACCACTTTGTACAAGAAA GCTGGGTTTATTCATCATCATCAT CTT	648
A box	GGGGACAAGTTTGTACAAAAAA GCAGGCTTCGAAGAAGATAGAA CCATGGGCAAAGGAGATCCTAA	GGGGACCACTTTGTACAAGAAA GCTGGGTGAACTTCTTTTTGGTC TCCCTTA	267

Supplementary Table 2. Primer sequences for quantitative real-time PCR and the length of each PCR product.

Mouse gene	Gene product	Forward primer	Reverse primer	Product Size (bp)
Ager	RAGE	AATTGTGGATCCTGCCTCTG	TCTCAGGGTGTCTCCTGGTC	173
Cxcl10	CXCL10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA	157
Gapdh	GAPDH	GAAGGTCGGTGTGAACGGATT	GAAGACACCAGTAGACTCCAC	294
Hmox1	HO-1	TTCAGAAGGGTCAGGTGTCC	CAGTGAGGCCCATACCAGAA	193
IFNβ	IFNβ	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACTCTTCTGCAT	138
Il-10	IL-10	GCCAAGCCTTATCGGAAATG	TTTTCACAGGGGAGAAATCG	163
Il-1β	IL-1B	TGCCACCTTTTGACAGTGATG	GAGTGATACTGCCTGCCTGA	167
Tlr2	TLR2	CGGAGGTAGAGTTCGACGAC	AACTGGGGGATATGCAACCT	127
Tlr4	TLR4	CAGCAAAGTCCCTGATGACA	AGAGGTGGTGTAAGCCATGC	179
Tnf-α	TNF-α	TCGAGTGACAAGCCTGTAGC	GGGAGTAGACAAGGTACAAC	162

Figure 1

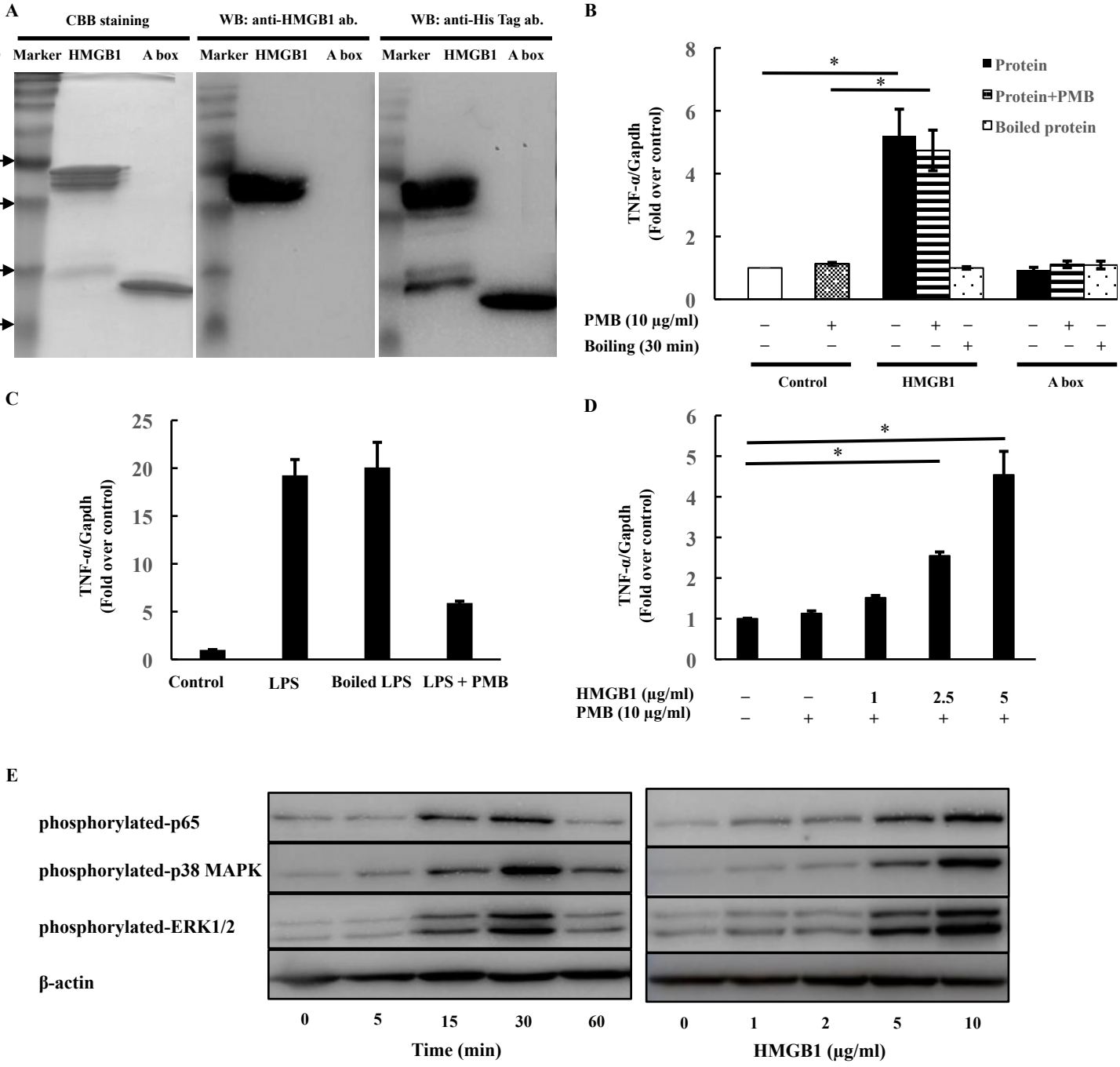

A
CBB staining
WB: anti-HMGB1 ab.
WB: anti-His Tag ab.
kD Marker HMGB1 A box
Marker HMGB1 A box
Marker HMGB1 A box
36
28
21
9
B
TNF-α/Gapdh (Fold over control)
Protein
Protein+PMB
Boiled protein
PMB (10 μg/ml)
Boiling (30 min)
Control
HMGB1
A box
C
TNF-α/Gapdh (Fold over control)
Control
LPS
Boiled LPS
LPS + PMB
D
TNF-α/Gapdh (Fold over control)
HMGB1 (μg/ml)
PMB (10 μg/ml)
E
phosphorylated-p65
phosphorylated-p38 MAPK
phosphorylated-ERK1/2
β-actin
Time (min)
HMGB1 (μg/ml)

Figure 2

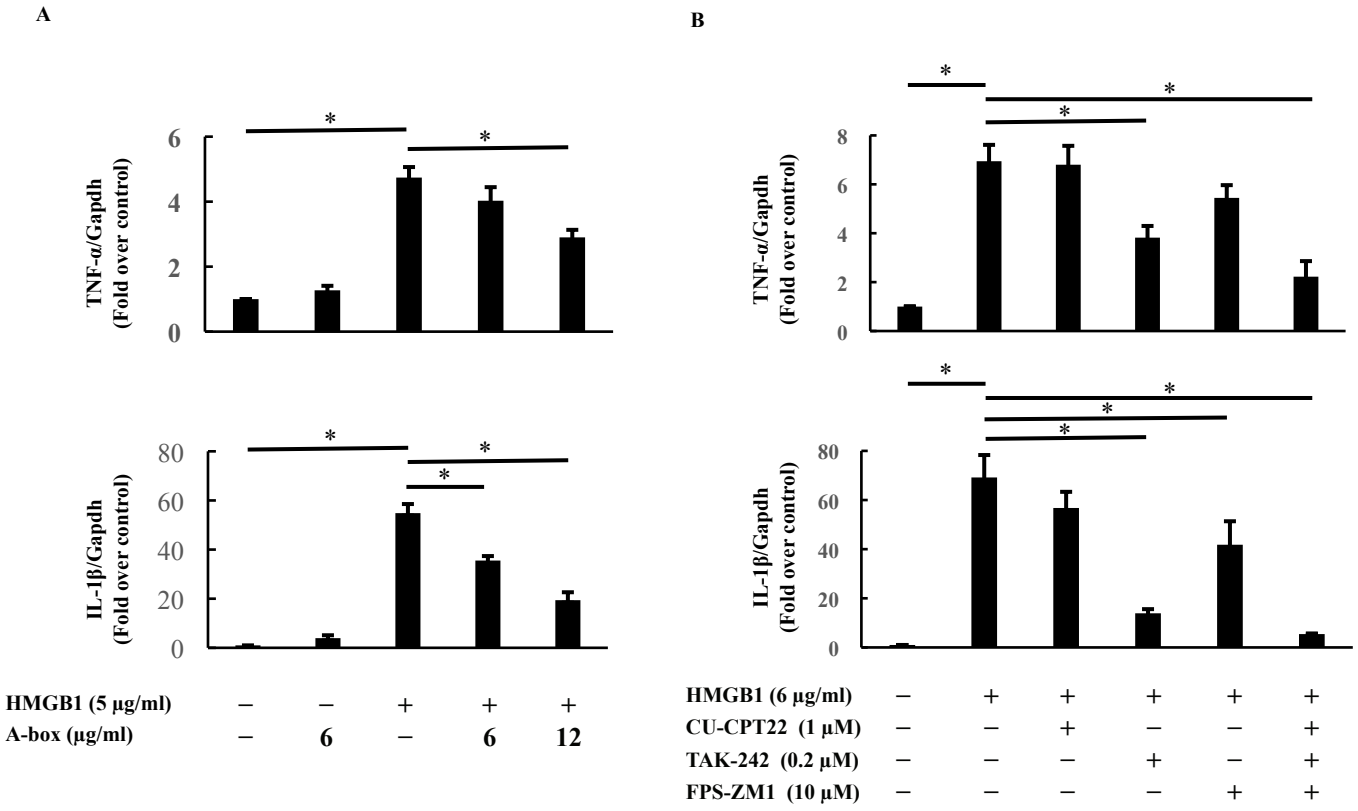

A
TNF-α/Gapdh (Fold over control)
IL-1β/Gapdh (Fold over control)
HMGB1 (5 μg/ml) – – + + +
A-box (μg/ml) – 6 – 6 12
B
TNF-α/Gapdh (Fold over control)
IL-1β/Gapdh (Fold over control)
HMGB1 (6 μg/ml) – + + + + +
CU-CPT22 (1 μM) – – + – – +
TAK-242 (0.2 μM) – – – + – +
FPS-ZM1 (10 μM) – – – – + +

Figure 3

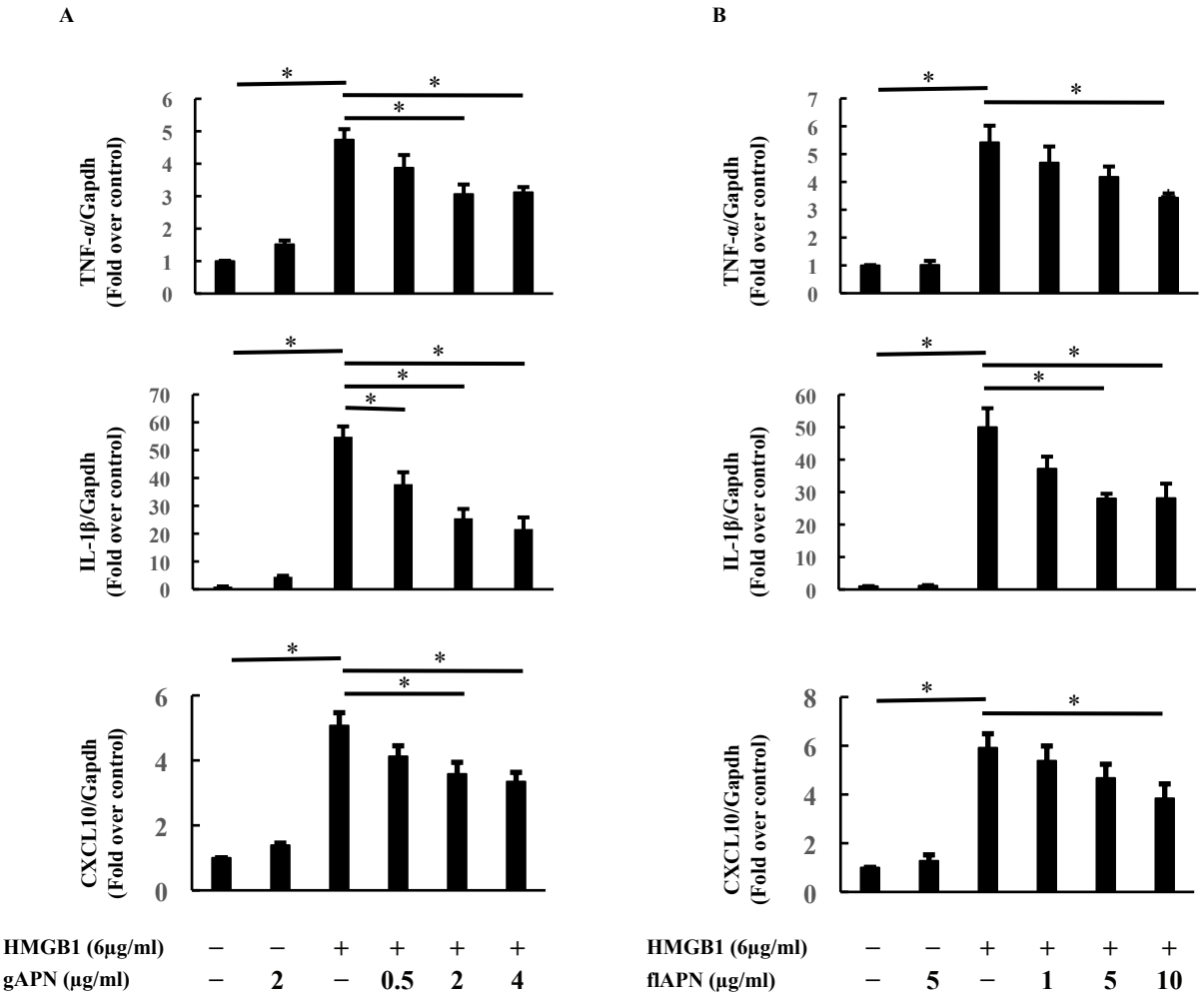

A
B
TNF-α/Gapdh (Fold over control)
IL-1β/Gapdh (Fold over control)
CXCL10/Gapdh (Fold over control)
HMGB1 (6μg/ml) − − + + + +
gAPN (μg/ml) − 2 − 0.5 2 4
HMGB1 (6μg/ml) − − + + + +
flAPN (μg/ml) − 5 − 1 5 10

Figure 4

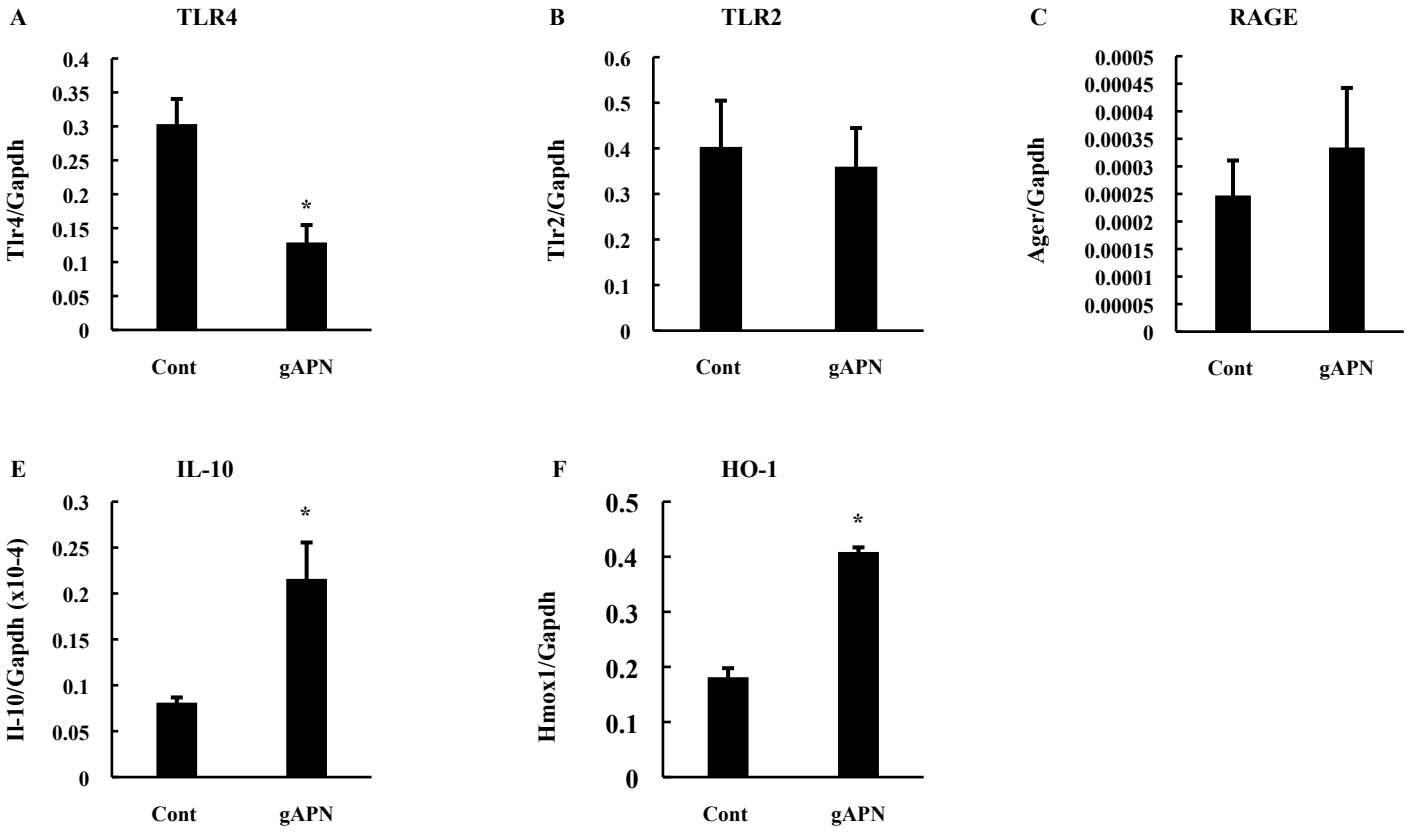

A
TLR4
Tlr4/Gapdh
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
Cont
gAPN
B
TLR2
Tlr2/Gapdh
0.6
0.5
0.4
0.3
0.2
0.1
0
Cont
gAPN
C
RAGE
Ager/Gapdh
0.0005
0.00045
0.0004
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
Cont
gAPN
E
IL-10
Il-10/Gapdh (x10-4)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
Cont
gAPN
F
HO-1
Hmox1/Gapdh
0.5
0.4
0.3
0.2
0.1
0
*
Cont
gAPN

Figure 5

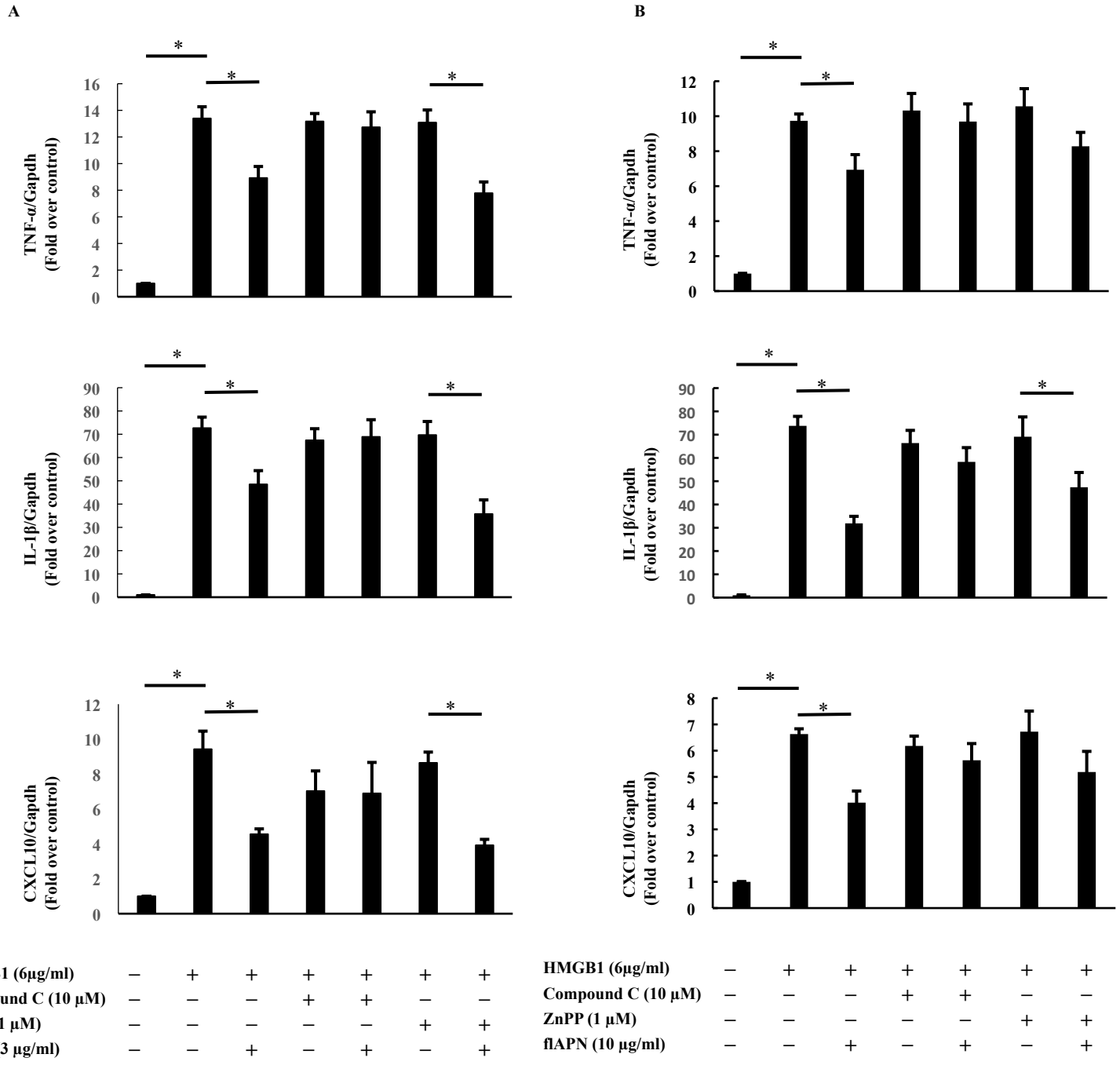

A
B
TNF-α/Gapdh (Fold over control)
IL-1β/Gapdh (Fold over control)
CXCL10/Gapdh (Fold over control)
*
HMGB1 (6μg/ml) − + + + + + +
Compound C (10 μM) − − − + + − −
ZnPP (1 μM) − − − − − + +
gAPN (3 μg/ml) − − + − + − +
HMGB1 (6μg/ml) − + + + + + +
Compound C (10 μM) − − − + + − −
ZnPP (1 μM) − − − − − + +
flAPN (10 μg/ml) − − + − + − +

Figure 6

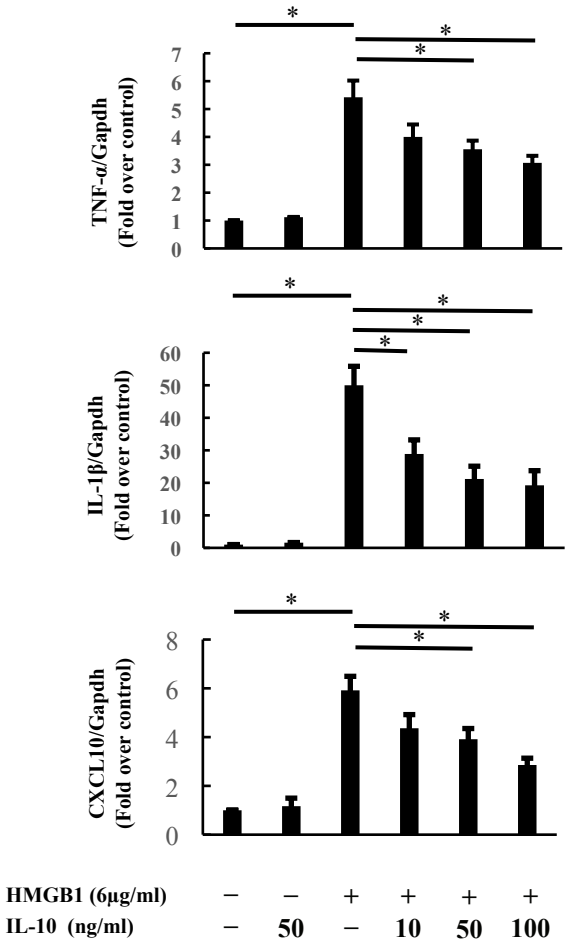
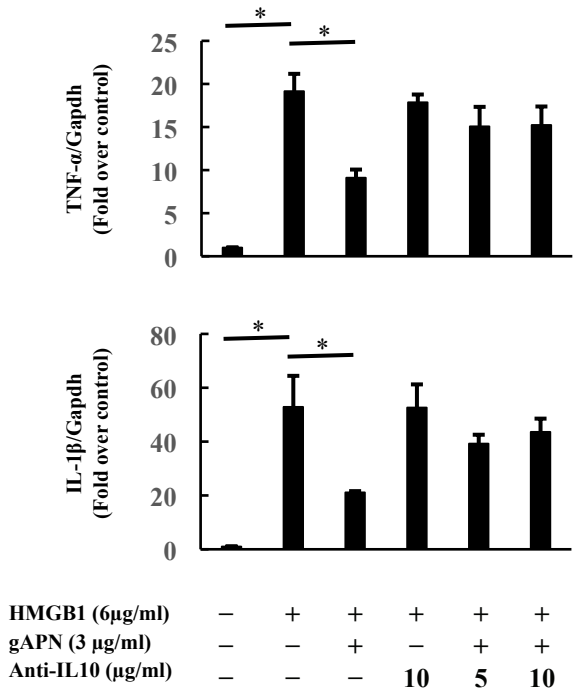

TNF-α/Gapdh
(Fold over control)
IL-1β/Gapdh
(Fold over control)
CXCL10/Gapdh
(Fold over control)
HMGB1 (6μg/ml) − − + + + +
IL-10 (ng/ml) − 50 − 10 50 100

Figure 7


TNF-α/Gapdh
(Fold over control)
25
20
15
10
5
0
*
*
IL-1β/Gapdh
(Fold over control)
80
60
40
20
0
*
*
HMGB1 (6μg/ml) – + + + + +
gAPN (3 μg/ml) – – + – + +
Anti-IL10 (μg/ml) – – – 10 5 10