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Novel suppressive action of adiponectin on LPS- and HMGB1-induced inflammatory responses

(脂肪細胞分泌因子アディポネクチンの細菌内毒素LPSおよび核内タンパク質HMGB1に因る炎症に対する新規抑制作用)

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AUTHOR'S DECLARATION

This study is my original work and has not been presented at any other University for the award of a degree. A part of this thesis has been published in advance as follow:

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ABBREVIATIONS

AMPK	AMP-activated kinase
APN	Adiponectin
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CD	Cluster of differentiation
CXCL10	C-X-C motif chemokine 10
Cys	Cysteine
DAMPs	Damage-associated molecular patterns
DMSO	Dimethyl sulfoxide
ERK1/2	Extracellular signal-related kinase 1/2
FBS	Fetal bovine serum
flAPN	Full length adiponectin
gAPN	Globular adiponectin
HMGB1	High mobility group protein B1
HO-1	Heme oxygenase 1
IL	Interleukin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MD2	Myeloid differentiation factor 2
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor-kappa B
Nrf2	Nuclear factor erythoid-derived 2 related factor 2
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PI3K	Phosphatidylinositol-3-kinase
PMB	Polymyxin B
RAGE	Receptor for advanced glycation end products
SDS	Sodium dodecyl sulphate
SIRT	Sirtuin
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
ZnPP	Zinc protoporphyrin

PREFACE

Sepsis is a systemic, inflammatory response to infection and is associated with multiple organ failure, and death. The mortality rate from severe sepsis remains over 30%, despite advances in intensive care therapy (1). The pathogenesis of sepsis is rather complex, but partly attributable to dysregulated systemic inflammatory responses propagated by innate immune cells including macrophages and monocytes. Monocytes immediately infiltrate into the infected/injured tissues upon detecting microbial products (termed pathogen-associated molecular patterns, PAMPs) or damage-associated molecular patterns (DAMPs) (2). Upon reaching extravascular tissues, these monocytes are differentiated into tissue-specific resident macrophages, which are responsible for ingesting and eliminating invading pathogens in alliance with other phagocytes (e.g., neutrophils) (3). Additionally, macrophages/monocytes are equipped with receptors [such as the Toll-like receptors (TLRs) TLR2, TLR3, TLR4, and TLR9] specific for various PAMPs (e.g., bacterial peptidoglycan, and lipopolysaccharides (LPS)) (4, 5). The engagement of PAMPs with respective receptors triggers the sequential release of early (e.g., tumor necrosis factor (TNF)-α or interleukin (IL)-1, and interferon (IFN)-γ) and late (e.g., HMGB1) proinflammatory mediators.

High mobility group box 1 (HMGB1) protein was first described about 40 years ago as a non-histone chromosomal protein with high electrophoretic mobility (6) . It contains two internal repeats of positively charged domains (“HMG boxes” known as “A box” and “B box”) in the N-terminus, and a continuous stretch of negatively charged (aspartic and glutamic acid) residues in the C-terminus. These HMG boxes enable HMGB1 to bind chromosomal DNA, and fulfill its nuclear functions such as maintaining the nucleosomal structure and stability, and regulating gene expression (7). In response to PAMPs, DAMPs, or cytokines, macrophages/monocytes actively release HMGB1 (8-10). In addition, HMGB1 can be passively released from damaged cells following sterile tissue injury (11). Passive release happens rapidly, in contrast to the active process that requires more time. This is reflected in HMGB1 being a late mediator in diseases where it is actively secreted but an early mediator in diseases with major necrosis or cell damage.

Intriguingly, HMGB1 does not contain a secretory signal peptide and therefore not secreted

in a classical way through the endoplasmic reticulum or the golgi apparatus. Instead it is secreted through a non-classical pathway similar to the leaderless cytokine IL-1β, although the process might differ between cell types. Under normal conditions HMGB1 is continuously shuttled between the nucleus and the cytoplasm with the balance strongly tilted towards nuclear accumulation (12). Stimulation of monocytes or macrophages leads to both acetylation and phosphorylation of HMGB1, blocking it from re-entering the nucleus and leading to its accumulation in the cytoplasm (13). Via an unknown mechanism, HMGB1 is then packaged into secretory lysosomes that transport it to the extracellular milieu through exocytosis (14). The exocytosis of HMGB1-containing secretory lysosomes is induced by lysophosphatidylcholine (14, 15), a bioactive lipid that is produced several hours following monocyte activation.

Once released, extracellular HMGB1 functions as an alarmin signal to alert, recruit, and activate innate immune cells. For instance, HMGB1 is capable of stimulating the migration of monocytes, dendritic cells and neutrophils, functioning as a chemokine to facilitate the recruitment of innate immune cells to the sites of infection or injury (16-18). Furthermore, HMGB1 binds to various PAMPs (e.g., LPS), thereby facilitating their recognition by respective receptors, and consequently augmenting the PAMPs-induced inflammatory responses (19, 20). In addition, HMGB1 can bind to multiple cell surface receptors including the receptor for advanced glycation end products (RAGE) , TLR2, TLR4 , TLR9 , cluster of differentiation 24 (CD24)/Siglec-10 , Mac-1 , thrombomodulin , as well as single transmembrane domain proteins (e.g., syndecans). Consequently, it can activate macrophages and endothelial cells to produce proinflammatory cytokines, chemokines, and adhesion molecules (21, 22).

Unlike other proinflammatory cytokines, HMGB-1 is a ‘‘late’’ appearing inflammatory mediator, and therefore, it provides a wider time frame for clinical intervention against progressive inflammatory disorders. Other cytokines such as TNF-α are produced within minutes after stimulation, and their circulating levels revert to near-baseline levels within the first few hours during the progression of the disease (23, 24). Targeting of early cytokine responses is difficult, since they are released early in the course of inflammatory response, and thus leaves a

narrow therapeutic window for administration of antagonists e.g. clinical trials inhibiting the early cytokine mediators (e.g., TNF-α or IL-1) have failed to improve survival in septic patients (25, 26). In contrast to this ‘‘early’’ response, HMGB1 is secreted from macrophages approximately 20 h post-stimulation (27, 28). In endotoxemia and other experimental models of systemic inflammation, elevated serum HMGB-1 levels are detected in a ‘‘late’’ plateau beginning only 20–72 h after the onset of the disease (29, 30). In recent years, various evidences show that HMGB1 is an important and essential late inflammatory mediator of sepsis and lethal endotoxaemia and targeting it with inhibitor or antagonist provide a protection against sepsis-induced mortality in animals and humans even when the treatment is started after the onset of the disease (30-34).

Adiponectin is an insulin-sensitizing, vascular-protective, anti-inflammatory protein produced and secreted almost exclusively by adipocytes (35-38). Adiponectin circulates in high concentrations in healthy adults and mice, accounting for 0.01% of total plasma protein and its plasma levels range between 2 and 30 μg/ml in humans (39, 40). In serum, adiponectin exists as three main forms: trimers, hexamers and high-molecular weight (HMW) multimers. Low plasma adiponectin levels, known as hypoadiponectinemia, are closely associated with obesity, insulin resistance and type 2 diabetes (36-38), as well as atherosclerosis, hypertension and coronary artery disease (41).

Three adiponectin receptors, AdipoR1, AdipoR2 and T-cadherin, have been identified. AdipoR1 is ubiquitous, but is most abundantly expressed in skeletal muscle and is linked to activation of AMP-activated kinase (AMPK) pathways. AdipoR2 is most abundantly expressed in the liver and is associated with activation of peroxisome proliferator-activated receptor (PPAR)-α pathways (42, 43). A third receptor for adiponectin, T-cadherin, expressed on vascular endothelial cells and smooth muscle, is a receptor for hexameric and HMW adiponectin (44). The activation of AdipoR1 and R2 results in increased hepatic and skeletal muscle fatty acid oxidation, increased skeletal muscle lactate production, reduced hepatic gluconeogenesis, increased cellular glucose uptake and inhibition of inflammation and oxidative stress (45). Activation of T-cadherin

is protective in vascular endothelial cells against oxidative stress-induced apoptosis and is strongly expressed in regions of atherosclerosis (46, 47).

Adiponectin exerts an anti-inflammatory effect through activation of all three receptors. Adiponectin inhibits the growth of myelomonocytic progenitors and the functioning of mature macrophages (48), suppresses macrophage conversion into foam cell transformation (49), stimulates macrophage production of the anti-inflammatory cytokine IL-10 (50), inhibits both TNF-α-induced monocyte adhesion and adhesion molecule (vascular cell adhesion molecule (VCAM)-1, E-selectin and intercellular adhesion molecule (ICAM)-1) surface expression on endothelial cells (51) and inhibits TLR-mediated NF-κB activation in macrophages (52). Adiponectin also inhibits the production of reactive oxygen species in human neutrophils (53). Furthermore, recent reports indicate that adiponectin acts as a modulator of macrophage phenotypes. It was shown that adiponectin switches the phenotype from the proinflammatory classically activated macrophage (M1) to an anti-inflammatory alternatively activated macrophage (M2) (54). Moreover, adiponectin enhances the ability of macrophage to remove early apoptotic bodies, which is crucial in preventing inflammation and immune system dysfunction (55).

The specific aims of the thesis were to:

- Investigate if adiponectin could attenuate endotoxin-induced HMGB1 release.
- Explore the cytokine-inducing ability of highly purified HMGB1 and which receptor it signals through in RAW 264 macrophage cells.
- Examine whether adiponectin could desensitize macrophage to HMGB1 proinflammatory activity.

Chapter 1

Adiponectin inhibits LPS-induced HMGB1 release through an AMP kinase- and heme oxygenase-1-dependent pathway in RAW 264 macrophage cells

1.1. INTRODUCTION

Sepsis, an almost universally fatal clinical syndrome that is caused by microbial infection, results from excess stimulation of the host immune system by pathogen components to produce various pro-inflammatory cytokines (56). Overproduction of these cytokines causes systemic inflammation that can lead to tissue damage, multiple organ failure, and death (57, 58). For example, bacterial LPS, a cell wall component of gram-negative bacteria, induces an acute inflammatory response initiated by its interaction with TLR4 resulting in sequential release of "early" (e.g., TNF-α, IL-1, IL-6) and "late" (e.g., HMGB1) proinflammatory cytokines (30, 59, 60). However, therapies designed to block early released cytokines such as TNF-α or IL-1β have shown limited efficacy due to the early and transient kinetics of the production of these inflammatory cytokines (61, 62).

HMGB1 is a highly conserved, ubiquitous non-histone nuclear protein that exhibits diverse functions according to its cellular location. Nuclear HMGB1 participates in DNA replication, recombination, 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 released, HMGB1 binds with cell-surface receptors, such as RAGE and/or TLRs including TLR2 and TLR4, and mediates various cellular responses, infiltration of innate immune cells, and subsequent release of various pro-inflammatory cytokines (32, 63-65). Administration of recombinant HMGB1 to mice is lethal, while administration of anti-HMGB1 antibodies or inhibitors provides protection against LPS-induced acute tissue damage and lethal endotoxaemia (28, 30, 64, 66). Therefore, targeting HMGB1 release provides a wide window for clinical intervention against systemic inflammatory diseases.

Adiponectin, which is also known as adipocyte complement-related protein (Acrp30), is one of the most abundant of the bioactive molecules called adipokines that are secreted from adipose tissue (38). Adiponectin plays an important role in various physiological processes including in lipid metabolism, insulin sensitization, and anti-inflammatory responses (67-69). Evidence indicates that adiponectin suppresses the "early" phase of macrophage inflammatory responses.

For example, adiponectin reduces macrophage differentiation and migration (48) and promotes macrophage polarization toward an anti-inflammatory M2 phenotype both *in vivo* and in cultured macrophages (54, 70). Adiponectin also inhibits the up-regulation of the expression of adhesion molecules and the enhancement of phagocytic activity and cytokine production in LPS-stimulated macrophages (48, 71), whereas it increases the release of anti-inflammatory mediators such as IL-10 and IL-1 receptor antagonist from macrophages (72).

A number of animal studies show that adiponectin has a protective effect against the development of inflammation-related disorders. For example, treatment with adiponectin improves atherosclerosis through inhibition of macrophage aggregation (48) and improves nonalcoholic steato-hepatitis via inhibition of lipogenic factors and TNF-α (73). Moreover, adiponectin protects from endotoxin-induced disorders of organs including the liver (74), the lung (75) and the heart (76), although its deficiency is associated with severe polymicrobial sepsis with high mortality (77). However, there has been no published report regarding the effects of adiponectin on the regulation of endotoxin-mediated release of "late" pro-inflammatory mediators such as HMGB1. Therefore, in this study, we investigated the effect of adiponectin on LPS-induced HMGB1 release in murine RAW 264 macrophage cells.

1.2. MATERIALS AND METHODS

1.2.1. Materials

Rabbit anti-HMGB1 antibody was purchased from Cell Signaling Technology (CST)(Beverly, MA, USA). Recombinant mouse full length adiponectin expressed in HEK293 cells was purchased from Biovendor (Asheville, NC, USA). Recombinant murine IL-10 was purchased from PeproTech (Rocky Hill, NJ, USA). Zinc protoporphyrin IX (ZnPP) was purchased from Frontier Scientific (Logan, UT, USA). SB203580, compound C (dorsomorphin), wortmannin and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). OPTI-MEM I was purchased from Invitrogen (Carlsbad, CA, USA).

1.2.2. Cell Culture

Cells of the murine macrophage-like cell line RAW 264 (RCB0535; RIKEN Cell Bank, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemicals, Osaka, Japan) and 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 the 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. The cells were treated with or without increasing concentrations of adiponectin or IL-10 for 18 h and were then stimulated with LPS (*Escherichia coli* O55:B5, Sigma-Aldrich) dissolved in phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at a concentration of 200 ng/mL for another 24 h. When included, the cells were treated with or without ZnPP, SB203580, compound C, or wortmannin 1 h before adiponectin (10 µg/ml) or IL-10 (100 ng/ml) addition.

1.2.3. Western blotting analysis

The level of HMGB1 in the culture medium was determined by western blotting analysis as previously reported (78-81). Briefly, culture medium samples were centrifuged to remove cellular

debris, then concentrated 60-fold with the Amicon Ultra-4-10000 NMWL (Millipore, Billerica, MA, USA). The concentrated samples were mixed with SDS loading buffer (500 mM Tris-HCl, 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 a polyvinylidine fluoride membrane (Immobilon; Millipore). The membrane was incubated in a blocking buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1%Tween 20 (TBS-T), and 5% skimmed milk) and then with rabbit anti-HMGB1 polyclonal antibody (1:2,000 dilution in the blocking buffer) overnight at 4 °C. Subsequently, the membrane was washed with TBS-T for 15 min and incubated with horseradish peroxidase-linked goat anti-rabbit immunoglobulin (CST) (1:5,000 dilution in the blocking buffer) for 1 h at room temperature. The signals were visualized using chemiluminescent HRP Substrate (Millipore) according to the manufacturer's instructions and were detected using the ImageQuant LAS 500 system (GE Healthcare, Buckinghamshire, UK). The intensity of chemiluminescence of the corresponding bands was quantified using Image J software (v. 1.48, http://imagej.nih.gov/ij).

1.2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RAW 264 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 (M-MLV) 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 Diagnostics). Expression levels were determined using the standard curve method with respective cDNA fragments as standards. The levels are reported relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression as an internal control. The primer sequences used in this study and the length of each PCR product are listed in Table 1.

1.2.5. Immunofluorescence

The cellular localization of HMGB1 was investigated using an immunofluorescence staining assay. RAW 264 cells (5×10^4 cells/well) were cultured on glass coverslips in 6-well plates. The cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were permeabilized with 10% Triton X-100 in PBS supplemented with 0.5% BSA and 0.15% glycine for 10 min, following which they were blocked in PBS containing 5% BSA and 0.3% Triton X-100 for 60 min. The glass coverslips were then incubated with rabbit anti-HMGB1 antibody (1:100 dilution in PBS containing 1% BSA and 0.3% Triton X-100) overnight at 4 °C, followed by goat anti-rabbit secondary antibody conjugated with Alexa fluor 488 (1:400 dilution) (Invitrogen) in the dark for 1 h at room temperature. Cells were washed with PBS containing 0.1% Triton X-100 between all incubations steps, followed by a final wash in PBS. Nuclei were labeled by incubation with 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) for 10 min. The cells were washed three times for 5 min with PBS. The coverslips were mounted on slides using Prolong® Antifade Reagents (Invitrogen). Images were captured using a fluorescence microscope (Biorevo BZ-9000; Keyence Japan, Osaka, Japan) with a ×100 oil-immersion lens. No fluorescence was detected in control cells processed without the primary antibody. The fluorescence intensities of cytosolic and nuclear HMGB1 were quantified using Image J software.

1.2.6. Statistical Analysis

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

1.3. RESULTS

RAW264 cells released a small amount of HMGB1 into the medium under the culture conditions without any stimulation. The amount of HMGB1 that was released increased 6-fold upon stimulation of the cells with 200 ng/ml of LPS (Fig. 1-1A). Cell viability was almost 100% even after treatment with 1 μg/ml of LPS (data not shown). The increased release of HMGB1 with LPS treatment was accompanied by HMGB1 translocation from the nucleus to the cytosol (Figs. 1-1B and 1-1C). These results suggested that HMGB1 release was under the control of LPS signaling rather than being passive release due to LPS cytotoxicity. Prior treatment of the cells with full length adiponectin failed to affect basal HMGB1 release, but dose-dependently suppressed LPS-induced HMGB1 release, and was accompanied by nuclear localization of most of the HMGB1 (Figs. 1-1A-1-1C).

As it has been reported that globular adiponectin exerts its anti-inflammatory actions through induction of IL-10 (70), I examined the effect of IL-10 on LPS induction of HMGB1 release. Prior treatment of the cells with IL-10 also failed to enhance basal HMGB1 release. However, IL-10 at doses of 50 and 100 ng/ml greatly decreased the HMGB1 release into the medium that was induced by LPS (Fig. 1-2).

To further examine the mechanism behind the suppressive effect of adiponectin on LPS-induced HMGB1 release, the mRNA expression in cells treated with either full length adiponectin or IL-10 were measured. Among the genes quantified, RAW264 cells constitutively expressed TLR4 mRNA, TLR2 mRNA, and myeloid differentiation factor 2 (MD2) mRNA (Figs. 1-3A-C), all of which are plasma membrane components responsible for LPS binding and signaling. Treatment of the cells with adiponectin selectively decreased expression of TLR4 mRNA, while IL-10 treatment reduced only TLR2 mRNA expression. Distinct differences between full length adiponectin and IL-10 treatments were also observed in the expression of HMGB1 and IL-10 genes. The cells constitutively expressed HMGB1 mRNA, which was suppressed only by IL-10 treatment and not by adiponectin treatment (Fig. 1-3D). On the other hand, the cells expressed very low levels of IL-10 mRNA, which was enhanced only by IL-10 treatment, but not by full

length adiponectin treatment (Fig. 1-3E). Thus, it was unlikely that full length adiponectin exerted its suppressive effect on LPS-induced HMGB1 release through induction of IL-10.

Interestingly, both IL-10 and full length adiponectin treatments enhanced the mRNA expression of HO-1, a downstream anti-inflammatory effector of IL-10 signaling (Fig. 1-3F), while neither treatment affected the mRNA expression of nuclear factor erythoid-derived 2 related factor 2 (Nrf2), a transcription factor related with HO-1 gene expression (Fig. 1-3G). In addition, neither IL-10 nor full length adiponectin treatment increased the mRNA expression of Sirt1 or Sirt6, which are histone deacetylases that function as a chromatin silencer to regulate recombination and genomic stability (Figs. 1-3H and 1-3I). Therefore, the involvement of HO-1 in the suppressive effect of full length adiponectin on LPS-induced HMGB1 release were examined. Treatment of the cells with zinc protoporphyrin (ZnPP), a HO-1 inhibitor, did not have any effect on HMGB1 release from either LPS-stimulated or control cells (Fig. 1-4). However, treatment with ZnPP for 1 h before full length adiponectin treatment almost completely abolished adiponectin suppression of LPS-induced HMGB1 release, although it only slightly inhibited the suppression by IL-10. These results indicate that increased expression of HO-1 in response to full length adiponectin is necessary for adiponectin mediated prevention of LPS-induced HMGB1 release.

Next, i examined whether increased expression of HO-1 mRNA by full length adiponectin was mediated through AMP-activated kinase (AMPK), a main signaling pathway of adiponectin action (82). Treatment of the cells with compound C, an AMPK inhibitor, abolished the increase in expression of HO-1 mRNA by full length adiponectin, whereas neither treatment of the cells with wortmannin, a phosphatidylinositol-3-kinase (PI3K) inhibitor, nor with SB203580, a p38 mitogen-activated protein kinase (p38MAPK) inhibitor, affected this increase (Fig. 1-5). Consistent with these results, treatment of the cells with compound C, but not with SB203580, abolished adiponectin-mediated suppression of LPS-induced HMGB1 release (Fig. 1-6).

1.4. DISCUSSION

The present study demonstrated for the first time that full length adiponectin prevents LPS-induced HMGB1 translocation from the nucleus to the cytosol and its subsequent release from Raw 264 mouse macrophage cells. This process is most probably mediated by AMPK-dependent HO-1 induction, as evidenced by the following results. Both AMPK and HO-1 inhibitors prevented the suppression of LPS-induced HMGB1 release by full length adiponectin and the AMPK inhibitor also prevented induction of HO-1 mRNA by full length adiponectin. Furthermore, the mechanism of the full length adiponectin effect is supported by previous reports that showed that full length adiponectin activates AMPK activity (43, 83), that activation of AMPK by metformin or dehydrodiconiferyl alcohol enhances HO-1 expression and its activity (84, 85), and that HO-1 is indispensable for the prevention of HMGB1 release (86, 87). Of course, other events such as selective reduction in TLR4 mRNA expression by full length adiponectin might contribute, at least in part, to the suppression of LPS-induced HMGB1 release, since TLR4 is the predominant receptor for LPS (88, 89) and a similar decrease in cell surface TLR4 expression is seen in macrophage cells treated with globular adiponectin (90). However, other intracellular signaling pathways related to p38MAPK, PI3K, and the nuclear histone deacetylase sirtuin are unlikely to be involved in the mechanism, although they have been reported to be involved in some adiponectin functions (91-94) or in the processes of HO-1 induction and LPS-induced HMGB1 release (95-97).

We have also demonstrated that IL-10 is a potent inhibitor of LPS-induced HMGB1 release. However, the fact that full length adiponectin failed to induce IL-10 mRNA suggested that the suppression by full length adiponectin might not be attributed to IL-10 production. This hypothesis is supported by previous findings that the effects of full length adiponectin on macrophage function are independent of IL-10 (90, 92), although anti-inflammatory effects of globular adiponectin are mediated by IL-10 (50, 70, 72, 98, 99). The discrepancy between the role of IL-10 in the effects of full length and globular adiponectin has not been explored, but is possibly due to different signals mediated through adipoR2 and adipoR1, respectively (90).

Accumulating evidence indicates that HO-1 plays a pivotal role in the anti-inflammatory cyto-protective effects of a wide variety of compounds including statins, phytochemicals such as resveratrol, and aspirin (100). HO-1 is a microsomal enzyme that catalyzes the degradation of proinflammatory free heme and produces equimolar amounts of carbon monoxide, bilirubin, and iron (101). The mechanisms that mediate the anti-inflammatory effects of HO-1 are not fully understood, but the potent antioxidant activity of bilirubin and the signaling gas activity of carbon monoxide are reported to suppress apoptosis, necrosis, inflammation, and oxidative stress. Interestingly, HO-1 is induced by pathophysiological stimuli including LPS and hemodynamic changes, but in most cases pathophysiological activation of HO-1 results in only a transient or marginal increase in HO-1 that falls below the threshold necessary to activate downstream components such as carbon monoxide (100). In the present study, a four-fold increase in HO-1 mRNA expression compared to its basal expression was induced by full length adiponectin, whereas only a two-fold increase was induced by IL-10. Combined with the result that the HO-1 inhibitor only partially abrogated the suppression by IL-10 of LPS-induced HMGB1 release, these findings suggested that IL-10 induces only a marginal increase in HO-1 mRNA and mainly utilizes an HO-1-independent pathway for the suppression of HMGB1 release.

In summary, this report provides a novel finding that full length adiponectin suppresses HMGB1 release by LPS through an AMPK-HO-1-dependent pathway. Therefore, adiponectin plays an important role as a regulator of inflammation through inhibition of both early and late pro-inflammatory mediators under pathological conditions such as sepsis. Thus, it is possible that adiponectin might be a target for the development of therapeutic agents against sepsis and other systemic inflammatory disorders.

1.5. SUMMARY

High mobility group protein B1 (HMGB1) is a late inflammatory mediator that exaggerates septic symptoms. Adiponectin, an adipokine, has potent anti-inflammatory properties. However, possible effects of adiponectin on lipopolysaccharide (LPS)-induced HMGB1 release are unknown. The aim of this study was to investigate effects of full length adiponectin on HMGB1 release in LPS-stimulated RAW264 macrophage cells. Treatment of the cells with LPS alone significantly induced HMGB1 release associated with HMGB1 translocation from the nucleus to the cytosol. However, prior treatment with adiponectin suppressed LPS-induced HMGB1 release and translocation. Similarly the anti-inflammatory cytokine interleukin (IL)-10 suppressed LPS-induced HMGB1 release. Adiponectin treatment decreased Toll-like receptor (TLR) 4 mRNA expression and increased heme oxygenase (HO)-1 mRNA expression without inducing IL-10 mRNA, while IL-10 treatment decreased TLR2 and HMGB1 mRNA expression and increased the expression of IL-10 and HO-1 mRNA. Treatment with the HO-1 inhibitor ZnPP completely prevented the suppression of HMGB1 release by adiponectin, but only partially inhibited that induced by IL-10. Treatment with compound C, an AMP kinase (AMPK) inhibitor, abolished the increase in HO-1 expression and the suppression of HMGB1 release mediated by adiponectin. In conclusion, these results indicate that adiponectin suppresses HMGB1 release by LPS through an AMPK-mediated and HO-1-dependent IL-10-independent pathway.

Table 1-1. Primer sequences for quantitative real time PCR and the length of each PCR product

Mouse gene	Gene product	Foreword primer	Reverse primer	Product size (bp)
Gapdh	GAPDH	GAAGGTCGGTGTGAACGGATT	GAAGACACCAGTAGACTCCAC	294
Hmgb1	HMGB1	GGGAGACCAAAAAGAAGTTC	GGCAGCTTTCTTCTCATAGG	200
Hmox1	HO-1	TTCAGAAGGGTCAGGTGTCC	CAGTGAGGCCCATACCAGAA	193
Il-10	IL-10	GCCAAGCCTTATCGGAAATG	TTTTCACAGGGGAGAAATCG	163
Ly96	MD2	ACGCTGCTTTCTCCCATATT	CATTGGTTCCCCTCAGTCTT	150
Nfe2l2	Nrf2	ACATGGAGCAAGTTTGGCAG	TGGAGAGGATGCTGCTGAAA	235
Sirtuin1	SIRT1	AGGGAACCTTTGCCTCATCT	GAGGTGTTGGTGGCAACTCT	159
Sirtuin6	SIRT6	ACCTGCAACCCACAAAACAT	GGCTCAGCCTTGAGTGCTAC	178
Tlr2	TLR2	CGGAGGTAGAGTTCGACGAC	AACTGGGGGATATGCAACCT	127
Tlr4	TLR4	CAGCAAAGTCCCTGATGACA	AGAGGTGGTGTAAGCCATGC	179

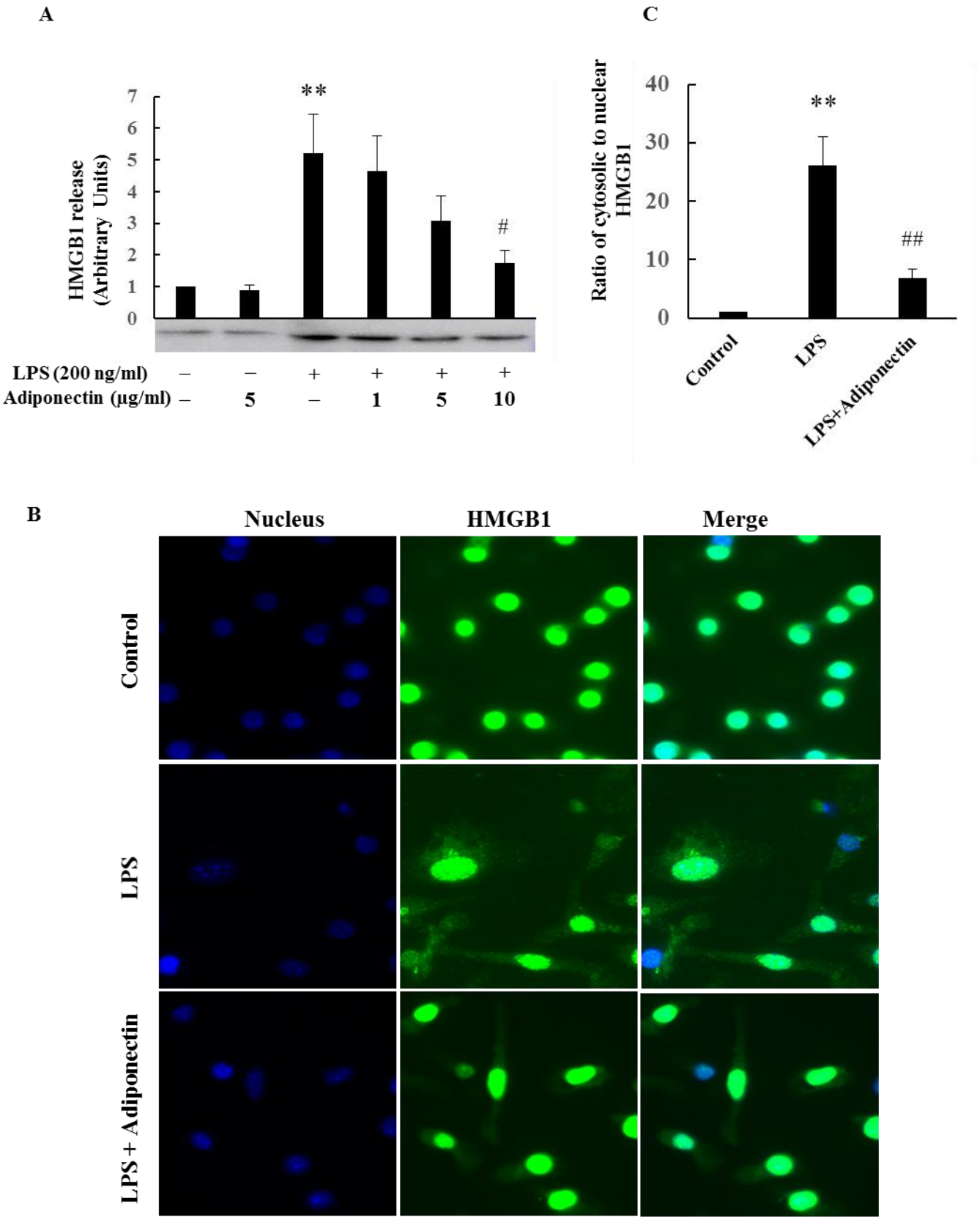

A
HMGB1 release (Arbitrary Units)
0
1
2
3
4
5
6
7
**
#
LPS (200 ng/ml) – – + + + +
Adiponectin (μg/ml) – 5 – 1 5 10
C
Ratio of cytosolic to nuclear HMGB1
0
10
20
30
40
**
##
Control
LPS
LPS+Adiponectin
B
Nucleus
HMGB1
Merge
Control
LPS
LPS + Adiponectin

Figure 1-1. Effect of recombinant adiponectin on LPS-induced HMGB1 release and HMGB1 cellular translocation. Raw 264 cells were cultured in DMEM supplemented with 10% FBS and were cultured in serum-free OPTI-MEM I medium for an additional 12 h. The cells were treated with increasing concentrations of adiponectin for 18 h, then stimulated with LPS (200 ng/ml) for another 24 h. A. Culture medium was collected and analyzed by HMGB1 western blotting, followed by quantification of the intensity of the chemiluminescent HMGB1 band. The results are expressed as means ± SE of three independent experiments (** $p < 0.01$ significance compared with control; # $p < 0.05$ significance compared with LPS-treated cells). B and C. Cellular HMGB1 was immunostained with an anti-HMGB1 rabbit primary and Alexa Fluor 488 anti-rabbit secondary antibodies. The nucleus was stained with DAPI. Merge indicates the combination of both HMGB1 (Green) and nuclear (Blue) fluorescence. The fluorescence intensities of cytosolic and nuclear HMGB1 in B were separately analyzed and the ratio of cytosolic HMGB1 to nuclear HMGB1 is shown in C (** $p < 0.01$ significance compared with control; ## $p < 0.01$ significance compared with LPS treated cells).

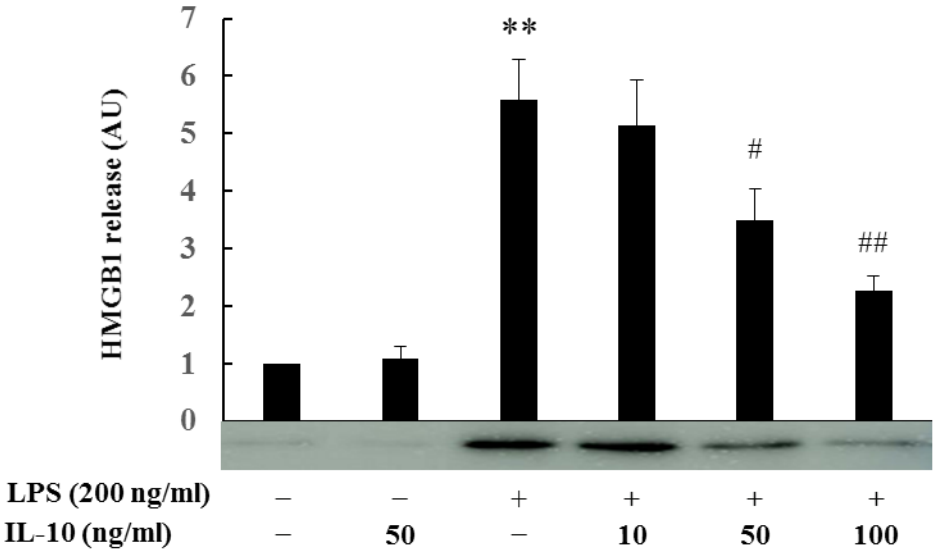


Figure 1-2. Effect of recombinant IL-10 on LPS-induced HMGB1 release. Raw 264 cells were cultured as described in the figure 1-1 legend and were treated with IL-10 for 18 h, then stimulated with LPS (200 ng/ml) for another 24 h. Culture medium was collected and analyzed by HMGB1 western blotting followed by quantification of the intensity of the chemiluminescent HMGB1 band. The results are expressed as means ± SE of three independent experiments (** $p < 0.01$ significance compared with control; $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$ significance compared with LPS-treated cells).

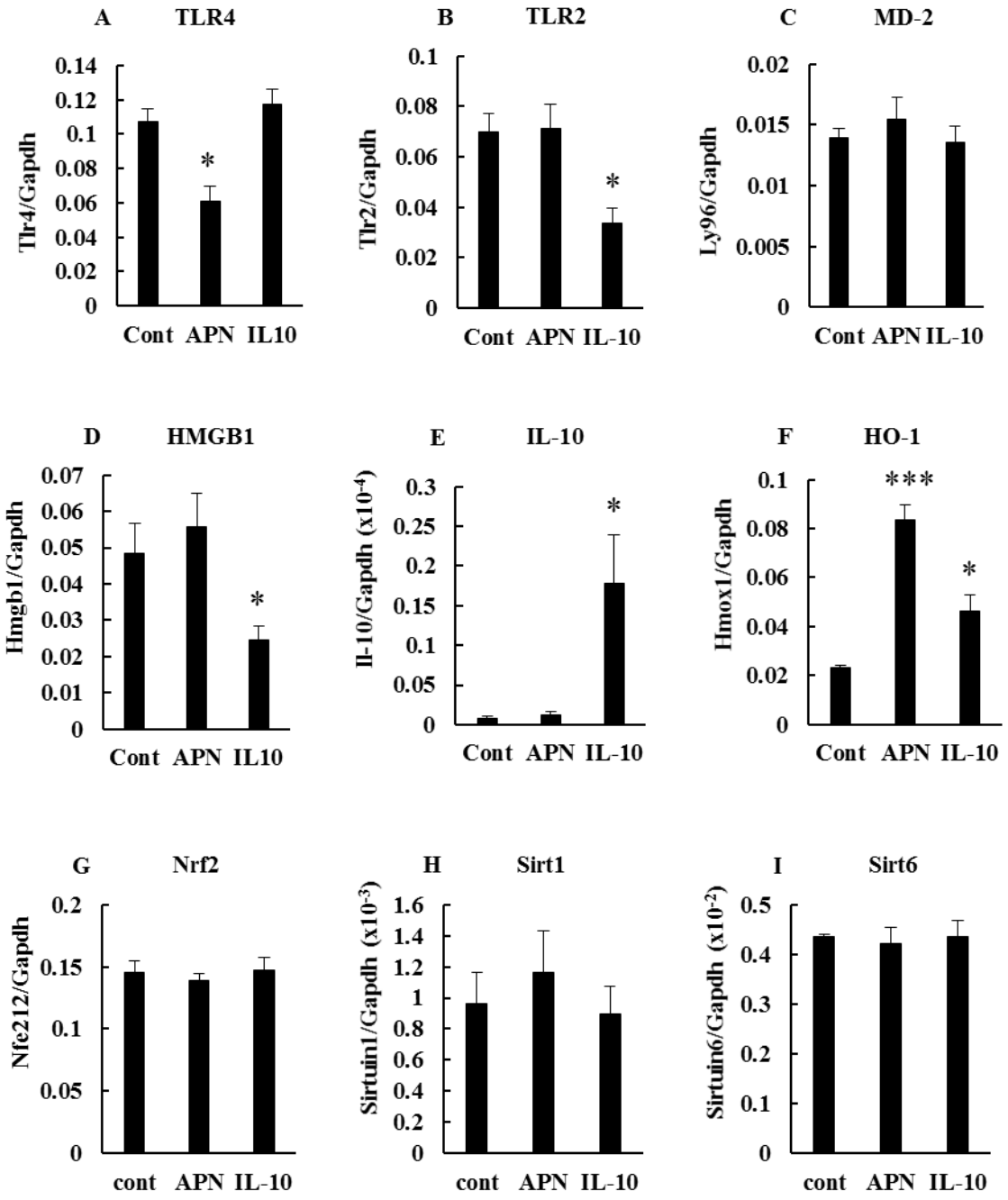


Figure 1-3. Effects of adiponectin and IL-10 on mRNA expression in RAW 264 cells. Raw 264 cells were cultured as described in the figure 1-1 legend and were treated with adiponectin (APN, 10 µg/ml) and IL-10 (100 ng/ml) for 18 h. RNA was extracted, and expression of A: TLR4, B: TLR2, C: MD2, D: HMGB1, E: IL-10, F: HO-1, G: Nrf2, H: Sirt1, I: Sirt6, and Gapdh (control) mRNAs were measured using qRT-PCR. The results are expressed as means ± SE of three independent experiments (*** $p < 0.001$, *$p < 0.05$ significance compared with control (Cont)).

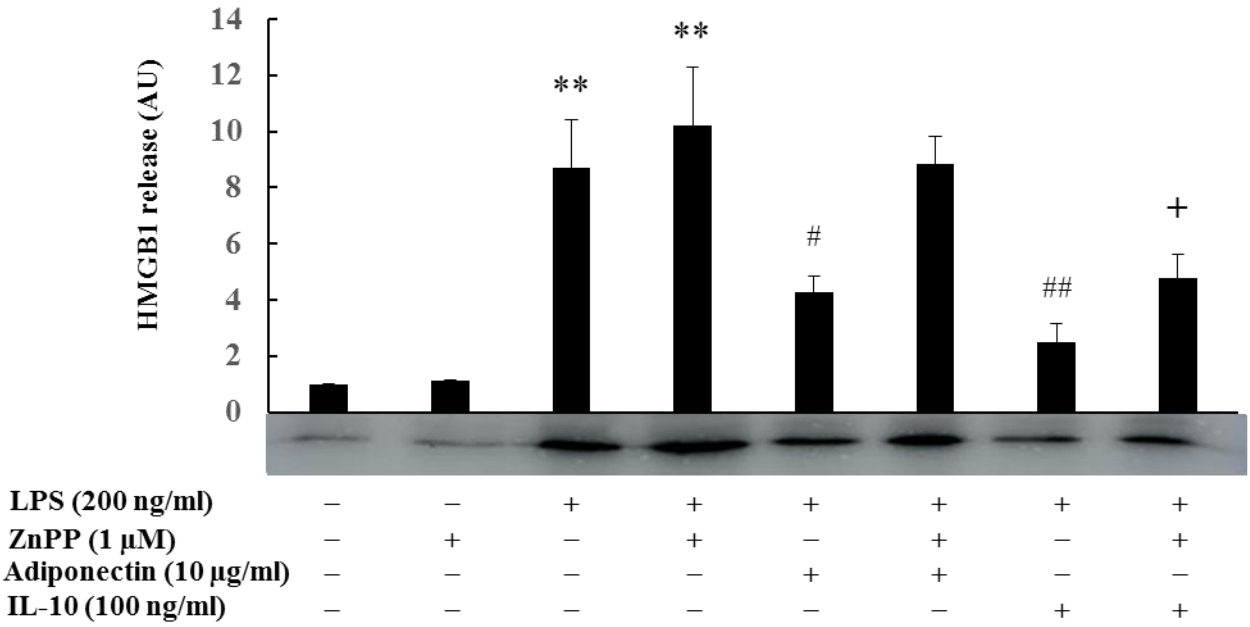


Figure 1-4. Effect of an HO-1 inhibitor on the anti-inflammatory action of adiponectin or IL-10 on LPS-induced HMGB1 release. Raw 264 cells were cultured as described in the figure 1-1 legend and were treated with dimethylsulfoxide (DMSO; control) or ZnPP (1 μM) for 1 h before treatment with adiponectin (10 μg/ml) or IL-10 (100 ng/ml) for 18 h. Cells were then stimulated with LPS (200 ng/ml) for another 24 h. Culture medium was collected and analyzed by HMGB1 western blotting followed by quantification of the intensity of the chemiluminescent HMGB1 band. The results are expressed as means ± SE of three independent experiments (** $p < 0.01$ significance compared with control; $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$ significance compared with LPS-treated cells; $^{+}$ $p < 0.05$ significance compared with LPS plus ZnPP treated cells).

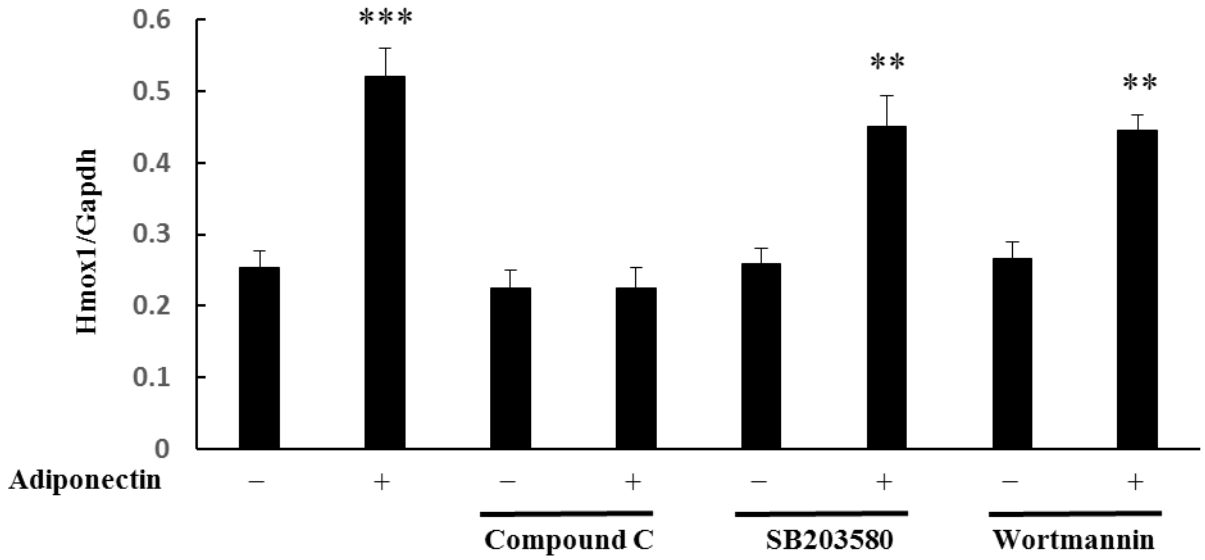


Figure 1-5. Effect of kinase inhibitors on adiponectin-induced HO-1 mRNA expression in RAW 264 cells. Raw 264 cells were cultured as described in the figure 1-1 legend and were treated with adiponectin (10 µg/ml) for 18 h in the presence of DMSO (Control), compound C (10 µM), wortmannin (1 µM), or SB203580 (10 µM). RNA was extracted, and expression of Hmox1 (HO-1) and Gapdh mRNAs were measured using qRT-PCR. The results are expressed as means ± SE of three independent experiments (*** $p < 0.001$, ** $p < 0.01$ significance compared with control).

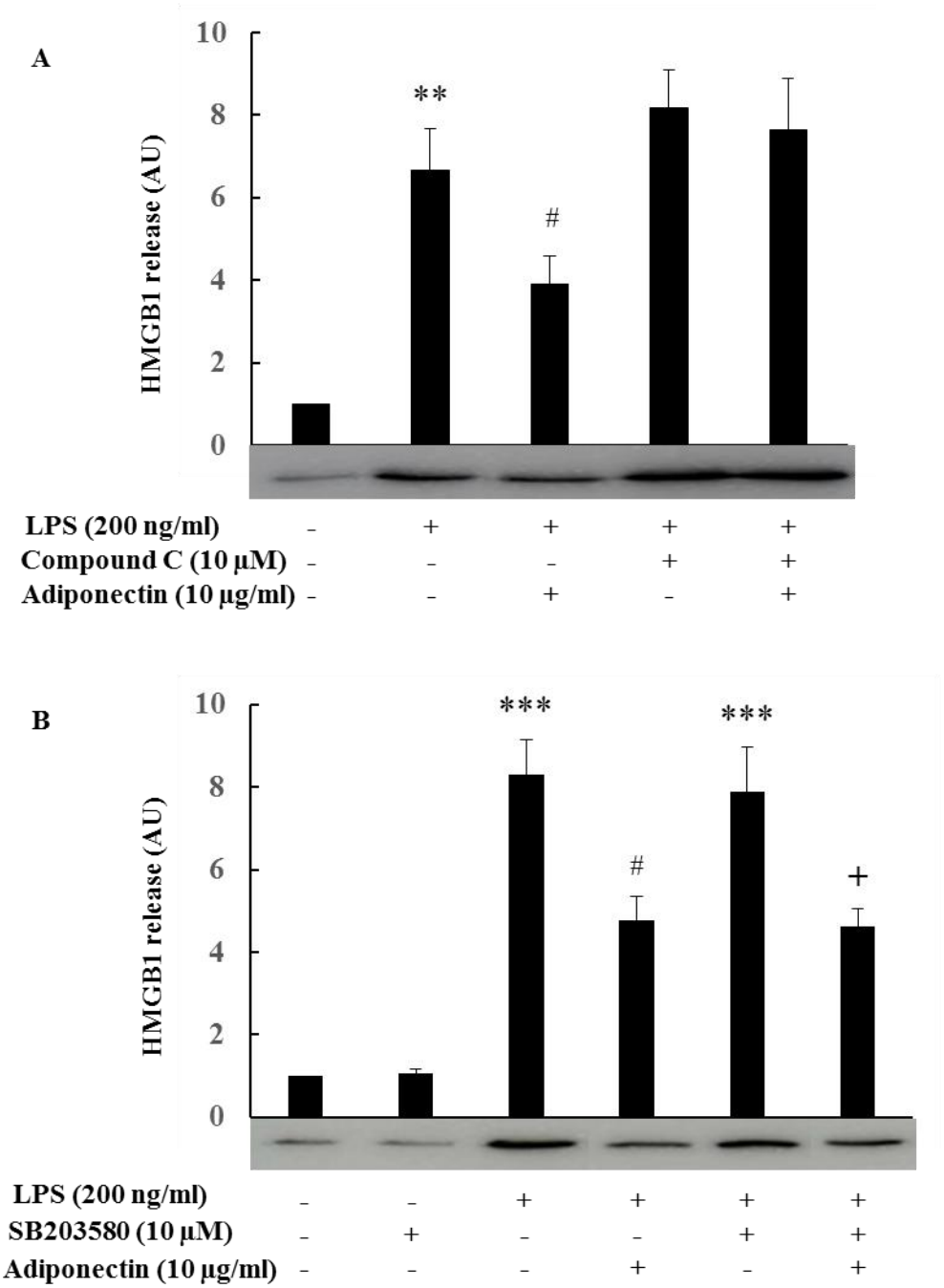


Figure 1-6. Effect of kinase inhibitors on the suppression by adiponectin of LPS-induced HMGB1 release. Raw 264 cells were cultured as described in the figure 1-1 legend and were treated with compound C (**A)** or SB203580 (**B)** for 1 h before treatment with adiponectin (10 µg/ml) for 18 h, following which they were stimulated with LPS (200 ng/ml) for another 24 h. Culture media were collected and analyzed by HMGB1 western blotting followed by quantification of the intensity of the chemiluminescent HMGB1 band. The results are expressed as means ± SE of three independent experiments (*** $p < 0.001$, ** $p < 0.01$ significance compared with control; $^{\#}$ $p < 0.05$ significance compared with LPS-treated cells; $^{+}$ $p < 0.05$ significance compared with LPS plus SB203580 treated cells).

Chapter 2

Adiponectin inhibits HMGB1-induced cytokine expression through an AMP kinase-dependent and heme oxygenase-1-independent pathway in RAW 264 macrophage cells

2.1. INTRODUCTION

HMGB1 is a highly conserved, ubiquitous non-histone nuclear protein, abundantly expressed in most kinds of mammalian cells (102, 103). The major structural features of HMGB1 are two positively charged DNA-binding domains, termed A and B boxes, and a negatively charged C-terminal acidic region. Structure–function analyses revealed that the active cytokine domain of HMGB1 is localized to the B box, whereas the A box competes with HMGB1 for binding sites on the surface of activated macrophages and attenuates the biological function of full length HMGB1 (104, 105). HMGB1 contains two non-classical nuclear localization sequences, resulting in a predominantly nuclear localization of HMGB1 under physiological conditions (12). Nuclear HMGB1 stabilizes chromatin structure, 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 act as a damage-associated molecular pattern molecule and trigger inflammation. HMGB1 is a redox-sensitive protein as it contains three conserved cysteine residues at position 23, 45, and 106. HMGB1 with all cysteine residues reduced stimulates immune cell infiltration and initiates chemotaxis via the chemokine receptor CXCR4 and RAGE. Partially oxidized HMGB1 with a Cys23-Cys45 disulfide bond and a 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 (106, 107), TLR2, TLR4 (32, 108), TLR9 (10, 20), cluster of differentiation 24 (CD24)/Siglec-10 (109), Mac-1 (110), as well as single transmembrane domain proteins (e.g., syndecans) (111) to produce proinflammatory cytokines, chemokines, and adhesion molecules. Fully oxidized HMGB1 (sulfonyl HMGB1) is devoid of both chemotactic and cytokine activities (112-114).

Numerous studies have focused on the biological role of extracellular HMGB1, since it was first identified as a late mediator of endotoxemia (115). Moreover, HMGB1 has been established as a pathogenic mediator of both infection- and injury-elicited inflammatory diseases including

sepsis (28, 116), arthritis (117-119), ischemia-reperfusion injury (120-123), pancreatitis (124, 125), obesity (126) and cancer (127, 128). Administration of neutralizing antibodies and other selective HMGB1 antagonists reverses inflammation and prevents organ damage and lethality in above-mentioned inflammatory diseases.

Adiponectin is an adipokine secreted by adipose tissue that regulates glucose and lipid metabolism as well as immuno-responses (48, 71, 129-131). Full length adiponectin is a 30-kDa protein circulates in plasma as trimer, hexamer, and multimeric complexes that are likely to differ in signaling effects and biological activity (132-135). Cleavage of full length adiponectin by proteases secreted from activated monocytes and/or neutrophils generates globular adiponectin which reported to be much more biologically active than full length adiponectin (136). Plasma adiponectin levels have also been reported to be reduced in obese humans, particularly those with visceral obesity, and to correlate inversely with insulin resistance (38, 137).

The anti-inflammatory action of adiponectin is mainly exerted on monocytes/macrophages via adiponectin receptors, AdipoR1 and AdipoR2, both are expressed on macrophages. The anti-inflammatory potential of adiponectin reported is driven by multiple signaling mechanisms. Adiponectin inhibits the up-regulation of endothelial adhesion molecules in response to inflammatory signals, suppresses phagocytic activity and cytokine production in LPS-stimulated macrophages (48, 71). Adiponectin desensitizes macrophages to TLR4-dependent signaling (71, 90, 92, 138). I have previously shown that exposure of RAW264 macrophage cells to full length adiponectin inhibits LPS-induced HMGB1 release via AMP kinase and heme oxygenase-1-dependent pathway (chapter I). In addition, adiponectin supplementation is reported to decrease serum HMGB1 and HMGB1 mRNA expression in lung tissues in polymicrobial sepsis mouse models (139). All these observations led me to speculate that adiponectin could desensitize macrophage to HMGB1 and subsequently attenuate its pro-inflammatory activity. Therefore, this study aimed to investigate whether adiponectin could attenuate proinflammatory function of HMGB1 in macrophage and, if present, to elucidate the underlying mechanisms responsible for its effects.

2.2. MATERIALS AND METHODS

2.2.1. 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, University of Hong Kong (Hong Kong). Recombinant murine IL-10 was purchased from PeproTech (Rocky Hill, NJ, USA). OPTI-MEM I was purchased from Invitrogen (Carlsbad, CA, USA). The following antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA): Rabbit anti-HMGB1 antibody, anti-His Tag antibody, anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase, anti-phospho-NF-κB p65 (Ser536) antibody, anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, anti-p38 MAPK antibody, anti-phospho-p44/42 MAPK (ERK1/2) antibody, anti-ERK1/2 Antibody. 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). Pharmacological inhibitors used in this study are listed in Table 1.

2.2.2. Preparation of recombinant mouse full length HMGB1 and A box.

2.2.2.1 Construction of full length HMGB1 and A box expression vector.

The coding region of mouse HMGB1 and A box proteins were amplified by PCR from a mouse spleen cDNA by using specific primers with attB sites (Table 2). The PCR product was inserted into pDONR221 (entry vector, Invitrogen), and transferred to pDEST17 (expression vector, Invitrogen) according to the manufacturer's protocol. The insertion in the vectors were confirmed by DNA sequencing.

2.2.2.2 Protein expression of full length HMGB1 and A box.

The recombinant plasmids were transformed into *Escherichia coli*, *KRX strain* (Promega, Madison, USA) , and then inoculated in 5 ml Luria Bertani (LB) media containing 50 µg/ml

Ampicillin and incubated at 37 °C with shaking. The next day 500 µl bacteria liquid was transfected to 50 ml fresh LB media containing 50 µg/ml Ampicillin and cultured until the density reached 0.5-0.6 at OD600. Protein expression was induced by adding rhamnose (Wako Pure Chemicals, Osaka, Japan) and isopropylthio-β-galactoside (Invitrogen) at final concentration of 0.1% and 1mM, respectively. The cells were harvested by centrifugation at 12,000 rpm for 15 min at 4 °C. The pellet was treated with Bugbuster ® protein extraction reagent (Millipore, Billerica, MA, USA) containing Deoxyribonuclease I (Wako Pure Chemicals), lysozyme (Wako Pure Chemicals) and phenylmethylsulfonyl fluoride (Sigma–Aldrich Fine Chemicals). After centrifugation at 12,000 rpm for 15 min at 4 °C, supernatant was collected as soluble fraction. Protein expression was verified by Coomassie brilliant blue (CBB) staining and western blotting with anti-His Tag antibody. For large scale production, 5 ml of overnight culture was added into 500 ml fresh LB media and cultured at the above-mentioned conditions.

2.2.2.3 Purification of recombinant mouse HMGB1 and A box.

Protein purification of recombinant HMGB1 and A box was carried out using Nickel His Trap HP column (GE Healthcare, Buckinghamshire, UK). Briefly, soluble fraction was subjected to overnight dialysis against phosphate-buffered saline (PBS) with 20 mM imidazole then applied to 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 recombinant protein was verified by CBB staining and western blotting with anti-His-tag antibody.

2.2.2.4 Removal of endotoxin from protein preparations

Contaminating LPS from protein preparations was removed by Pierce™ High capacity endotoxin removal spin column (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

2.2.3. Cell culture and treatments

Murine macrophage-like cell line, RAW264 cells (RCB0535; RIKEN Cell Bank, Japan) were 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 ^oC. When RAW 64 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 or without compound C, ZnPP, TAK-242, CU-CPT22, or FPS-ZM1 for 1 h and then stimulated with adiponectin and/or HMGB1. For stimulation experiments, polymyxin B (PMB; LPS inhibitor; Sigma-Aldrich) was routinely added to cell culture medium at concentration of 10 µg/ml to suppress any contaminating endotoxin in protein preparations.

2.2.4. Western blotting 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 resuspension of cells in a RIPA buffer supplemented with phosphatase inhibitor cocktail and incubated for 20 min under gentle shaking at 4 °C. Insoluble material was removed by centrifugation (12,000 × *g*, 30 min, 4 °C) and supernatant was used for western blotting 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 a polyvinylidine fluoride membrane (Immobilon; Millipore). The membranes were blocked for 1 h at room temperature in a 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 overnight at 4^oC with specific primary antibody. Subsequently, the membrane was washed with TBS-T for 15 min and incubated with the appropriate horseradish peroxidase-linked goat anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. The signals were visualized using chemiluminescent HRP Substrate (Millipore) according to the manufacturer's instructions and were detected using the ImageQuant LAS 500 system (GE Healthcare). The intensity of

chemiluminescence of the corresponding bands was quantified using Image J software (v. 1.48, http://imagej.nih.gov/ij).

2.2.5. 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 M-MLV 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 Diagnostics). Expression levels were determined using the standard curve method with respective cDNA fragments as standards. The expression levels are expressed relative to GAPDH expression as an internal control. The primer sequences used in this study and the length of each PCR product are listed in Table 3.

2.2.6. Statistical Analysis

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

2.3. RESULTS

The recombinant full length HMGB1 and A box proteins were successfully produced from *E.coli*. The purity of produced recombinant HMGB1 was higher than 95% as judged by CBB staining (Fig. 2-1A). To examine 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. HMGB1 treatment increased TNF-α mRNA expression even in the presence of PMB, while boiled HMGB1 protein failed to do (Fig. 2-1B). As boiling at 100 ^{o}C for 30 min is sufficient to denature the protein, but not endotoxin (140), these results suggest that recombinant HMGB1 protein, but not contaminated LPS was responsible for its biological activity.

Treatment of the cells with increasing concentration of HMGB1 induced TNF-α mRNA expression dose-dependently, accompanied with dose-dependent activation of NF-κB, p38MAPK, and ERK pathways (Figs. 2-1C and 2-1D). In addition, prior treatment with HMGB1 A box protein which was inactive in TNF-α induction (Fig. 2-1B) antagonized HMGB1-induced responses (Fig. 2-2A), indicating HMGB1 influences RAW264 cells through the receptor mediated pathway. To date ten separate receptors and proteins have been implicated in mediating the biological responses to HMGB1: RAGE, TLR2, TLR4, TLR9, Mac-1, syndecan-1, phosphacan, protein-tyrosine phosphatase-ζ/β, and CD24 (108, 109, 141). In addition, previous work has reported that TLR2, TLR4, and RAGE are required in mediating HMGB1-induced inflammatory response in macrophages (21, 32, 107). To determine the receptors that mediate the inflammatory reaction induced by HMGB1 in RAW264 cells, the cells were treated with pharmacological inhibitors for TLR2, TLR4 or RAGE for 1 h before HMGB1 addition. 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. 2-2B). However, when analyzed IL-1β expression, both antagonists for TLR4 and RAGE suppressed HMGB1-induced IL-1β mRNA expression significantly (Fig. 2-2B), suggesting that TLR4 and RAGE are, at least in part, involved in the HMGB1 signaling in RAW264 cells. It is also known

that activation of TLR4 transduces its signals through MyD88 adaptor protein-dependent and -independent pathways, and that both TNF-α and IL-1β expression are controlled by MyD88-dependent pathway (142, 143). Preliminary experiments revealed that HMGB1 induced mRNA expression of C-X-C motif ligand 10 (CXCL10) and interferon β, which are mediated by MyD88-independent pathway (142) (data not shown).

To investigate the effect of adiponectin on HMGB1-induced inflammatory response in RAW264 macrophage cells, the cells were treated with increasing concentration of both globular and full length forms of adiponectin for 18 h prior to HMGB1 stimulation. Both globular and full length adiponectin dose-dependently suppressed HMGB1-induced mRNA expression of TNF-α and IL-1β as well as CXCL10 (Fig. 2-3).

I have 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 (chapter I). To determine whether the same pathway is involved in the effects of both adiponectin on cytokine expression, first, changes in HO-1 mRNA expression by globular adiponectin was examined. As shown in Figure 2-4, globular adiponectin doubled expression of HO-1 mRNA, although full length adiponectin increased HO-1 mRNA expression 4-folds (chapter I). Treatment of RAW264 cells with compound C, an AMP kinase inhibitor, 1 h before the adiponectin addition completely abolished the suppressive effect of both forms of adiponectin on HMGB1-induced TNF-α, IL-1β and CXCL10 mRNA expression (Fig. 2-5). However, treatment with zinc protoporphyrin (ZnPP), a HO-1 inhibitor failed to abolish it (Fig. 2-5), suggesting the suppression by both forms of adiponectin is mediated through HO-1-independent mechanism.

Quantification of mRNA expression revealed that treatment of RAW264 cells with globular adiponectin increased IL-10 expression, but decreased TLR4 expression without affecting TLR2 and RAGE expression (Fig. 2-4). Full length adiponectin similarly suppressed TLR4 mRNA expression, but failed to induce IL-10 mRNA (Fig. 1-3). Thus, although prior treatment with IL-10 effectively inhibited HMGB1-induced cytokine expression (Fig. 2-6), it is unlikely that IL-10 is involved in the suppressive effects elicited by full length adiponectin while it might be one of

the pathways by which globular adiponectin exerts its anti-inflammatory effects on HMGB1-induced inflammatory cytokines.

2.4. DISCUSSION

The present study have show that recombinant HMGB1, free from LPS, induced cytokine expressions through TLR4 and RAGE, but not TLR2, in the RAW264 cells. These findings are partly supported by reports showing that TLR2, TLR4, and RAGE are required in mediating HMGB1-induced inflammatory response in macrophages, although the other miscellaneous proteins are also suggested to be involved (10, 20, 21, 32, 106-111, 141). Furthermore, as TLR4 antagonist was the most effective among the antagonists used and mRNA expression levels of RAGE was hundredth part of TLRs, TLR4 seems to mediate predominantly HMGB1 signaling in the cells.

I have also demonstrated that prior treatment of RAW264 cells with full length and globular adiponectin suppresses HMGB1-induced cytokine expression. As I have shown that exposure of RAW264 macrophage cells to full length adiponectin inhibits LPS-induced HMGB1 release via AMPK and HO-1-dependent pathway (chapter I), it was assumed that both full length and globular adiponectin activated AMPK through adipoR2 and adipoR1, respectively, resulting in induction of HO-1 and subsequent HO-1-dependent inhibition of cytokine expression. As expected, the effects by both adiponectin were blocked in the presence of AMPK inhibitor. However, although both adiponectin enhanced HO-1 expression significantly, HO-1 inhibitor failed to block the effects by both adiponectin. Thus, it is unlikely that HO-1 is involved in the suppression of HMGB1-induced cytokine expression by adiponectin. In addition, IL-10 is unlikely to be involved in the suppression, because full length adiponectin fails to induce it (chapter I).

In the present study, the mRNA expression of three main cytokines, TNF-α, IL-1β, and CXCL10 were measured. Two former cytokines are classified into a myeloid differentiation primary response protein 88 (MyD88)-dependent cytokine, while the latter is MyD88-independent. MyD88 is directly associated with TLR4 and transduce the receptor signal as an adaptor protein leading to production of pro-inflammatory cytokines (142). TLR4 signal also transduces in a MyD88-independent pathway that elicits the expression of IFNβ and IFN-

inducible genes (142). As both forms of adiponectin suppressed HMGB1-induced cytokine expression irrespective of MyD88 dependence, it is suggested that an upstream molecule of Myd88-dependent pathway is responsible for that suppression. Indeed, both adiponectin reduced TLR4 mRNA expression (Fig. 2-4, and Fig.1-3), and reduction of cell surface TLR4 is previously reported in macrophage after treatment with globular adiponectin (90). Therefore, it is likely that reduction of TLR4 by adiponectin is attributed, at least in part, to suppression of HMGB1-induced cytokine activity by adiponectin.

It is reported that the anti-inflammatory effects of adiponectin on LPS-stimulated TNFα, IL-1β, and CXCL10 mRNA expression is mediated via a HO-1–dependent pathway [12-14]. As LPS is the potential activator of TLR4, discrepancy between this study and that of LPS might indicate that macrophage response to HMGB1/TLR4 signaling is different from that of LPS/TLR4 signaling. In my preliminary results, levels of TNF-α and IL-1β expression induced by HMGB1 were markedly lower than those by LPS, whereas expression level of CXCL10 induced by HMGB1 was comparable to that of LPS (data not shown). Thus it is suggested that modes of signal through TLR4 are different between the stimuli such as LPS and HMGB1.

Several reports show that obese individuals with low adiponectin level would exhibit higher insulin resistance associated with stronger inflammatory reaction (144, 145), and that an increased accumulation of adipose tissue macrophages caused by obesity is associated with the development of chronic inflammation and metabolic dysfunction (146-148). In addition, obesity leads to the M1 macrophage polarization in rodent adipose tissue, which contributes to inflammatory response and tissue destruction (149, 150). Recently, HMGB1 was reported to be elevated during obesity (151) and identified as a new biomarker of metabolic syndrome in obese children (152). Neutralization of HMGB1 appears to reduce weight gain and liver inflammation in mice fed an obesogenic diet (126). Therefore, additional research is necessary to clarify whether adiponectin affects obesity-associated HMGB1 release and function *in vivo*.

In summary, here I have shown that 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 TLR4 reduction in RAW264 mouse macrophage cell line.

2.5. SUMMARY

High mobility group protein B1 (HMGB1) is a late inflammatory mediator released from inflammatory cells upon stimulation, resulting in exaggerating septic symptoms. In the first chapter, I have demonstrated that full length adiponectin (flAPN), a potent anti-inflammatory adipokine, inhibits LPS-induced HMGB1 release through an AMP kinase- and heme oxygenase (HO)-1-dependent pathway in RAW264 macrophage cells. However, possible effects of adiponectin on HMGB1-induced exaggerating signals are unknown. The aim of this study was to investigate effects of flAPN and globular adiponectin (gAPN) on cytokine expression in HMGB1-stimulated RAW264 macrophage cells. Treatment of the cells with recombinant HMGB1 in the presence of an anti-LPS compound, polymyxin B, induced significantly mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and C-X-C motif chemokine (CXCL) 10. The HMGB1-induced cytokine expressions were suppressed clearly by HMGB1 A-Box protein and Toll-like receptor (TLR) 4 antagonist, and marginally by receptor for advanced glycation products (RAGE) antagonist, suggesting that TLR4 predominantly mediates HMGB1 signaling in this cell line. Prior treatment with flAPN or gAPN dose-dependently suppressed all kinds of HMGB1-induced cytokine expression, and the suppression by adiponectin was abolished by compound C, an AMP kinase inhibitor. Treatments with flAPN and gAPN enhanced expression of HO-1 mRNA, however, prior treatment with the HO-1 inhibitor ZnPP failed to abolish the suppression by both adiponectin. As both adiponectin reduced expression of TLR4 mRNA to half, these results suggest that flAPN and gAPN inhibit HMGB1-induced Myd88-dependent and –independent cytokine expression through AMP kinase-mediated HO-1 independent pathway, possibly through the reduction of TLR4 expression.

Table 2-1. Pharmacological inhibitors used in this study

Inhibitor	Formal Name	Target	Company	Working Conc.
ZnPP	Zinc protoporphyrin IX	HO-1	Frontier Scientific (Logan, UT, USA)	1 μM
Compound C	dorsomorphin	AMPK	Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA)	10 μM
TAK-242	Resatorvid	TLR2	ChemScene (Monmouth Junction, NJ, USA)	1 μM
CU-CPT22	Hexyl-3,4,6-trihydroxy-2-methoxy-5-oxo-5H-benzo[7]annulene-8-carboxylate	TLR4	Calbiochem (San Diego, CA, USA)	0.2 μM
FPS-ZM1	N-Benzyl-4-chloro-N-cyclohexylbenzamide	RAGE	Calbiochem	10 μM

Table 2-2. Primer sequences for construction of full length HMGB1 and A box expression vector.

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

Table 2-3. Primer sequences for quantitative real time PCR and the length of each PCR product

Mouse gene	Gene product	Foreword 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

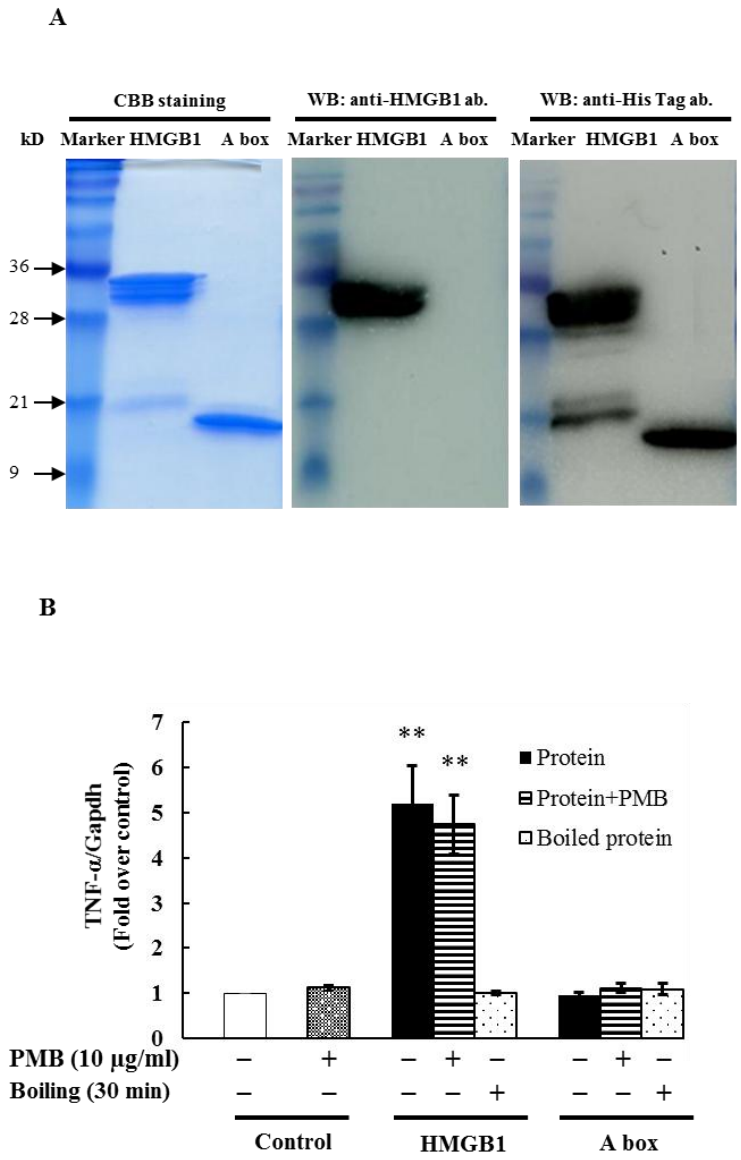

A
CBB staining
WB: anti-HMGB1 ab.
WB: anti-His Tag ab.
kD
Marker HMGB1 A box
Marker HMGB1 A box
Marker HMGB1 A box
36
28
21
9
B
TNF-α/Gapdh
(Fold over control)
Protein
Protein+PMB
Boiled protein
**
**
PMB (10 μg/ml)
Boiling (30 min)
Control
HMGB1
A box
C
TNFα/Gapdh
(Fold over control)
*
***
HMGB1 (μg/ml)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

Figure 2-1. Recombinant HMGB1 dose-dependently induced TNF-α expression in RAW264 macrophage cells. A. Recombinant HMGB1 and its A-box fragment were purified from *E. coli* extract by Ni+-column and subjected to SDS-PAGE with CBB staining (left) and 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 cultured in serum-free OPTI-MEM I medium for 12 h. The cells were treated with HMGB1 (5 μg/ml) and A-box (5 μg/ml), which were boiled or non-boiled for 30 min, in the presence or absence of anti-lipopolysaccharide, polymyxin B (PMB, 10 μg/ml) for 3 h. RNA was extracted, and expression of TNF-α and Gapdh mRNA were measured by qRT-PCR. The results are expressed as means ± SE of three independent experiments (** $p < 0.01$ significance compared with control). C. The cells were cultured as in B and stimulated with increasing concentration of HMGB1 (1 - 5 μg/ml) for 3 h in the presence of PMB. Expression of TNF-α mRNA was determined and expressed as means ± SE of three independent experiments (*** $p < 0.001$, *$p < 0.05$ significance compared with control). D. The cells were cultured as in B and stimulated with increasing concentration of HMGB1 (1- 10 μglml) for 30 min. Representative western blots showed activation of NF-κB, p38MAPK, and ERK pathways by HMGB1 dose-dependently. β-actin served as a loading control.

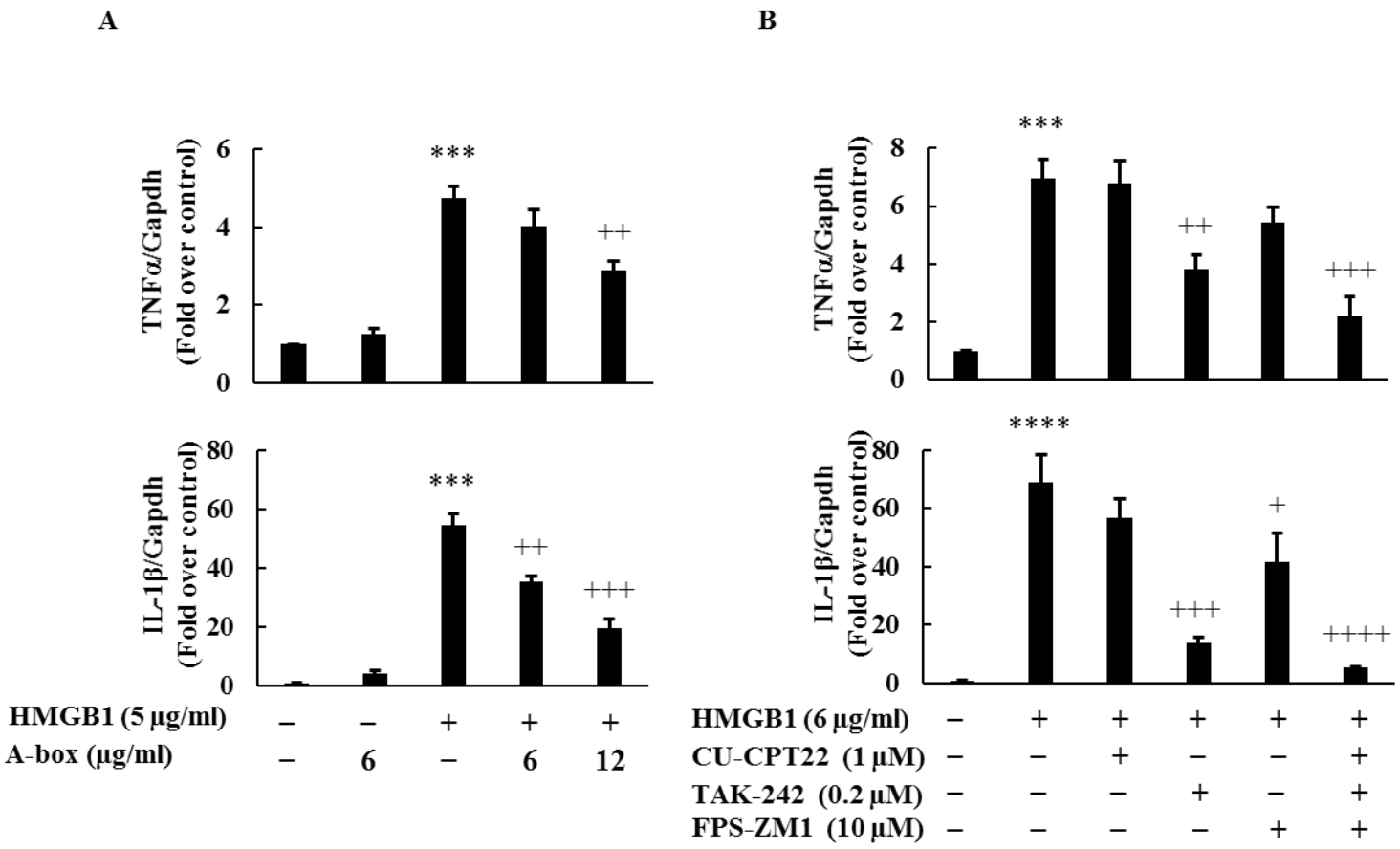


Figure 2-2. The HMGB1-induced inflammatory cytokine expression was mediated by receptor for advanced glycation end products (RAGE) and Toll-like receptor (TLR) 4, but not TLR1/2 in RAW264 cells. The cells were cultured as described in figure 2-1B and treated with HMGB1 A-box in (A), and antagonists of RAGE (FPS-ZM1), TLR1/2 (CU-CPT22) and TLR4 (TAK-242) in (B) 1 h before HMGB1 addition. Expression of TNF-α, IL-1β, and Gapdh mRNA 2.5 h after the HMGB1 addition were measured by qRT-PCR. The results are expressed as means ± SE of three independent experiments (**** $p < 0.0001$, *** $p < 0.001$ significance compared with control, $^{++++}$ $p < 0.0001$, $^{+++}$ $p < 0.001$, $^{++}$ $p < 0.01$, $^{+}$ $p < 0.05$ significance compared with HMGB1 treatment).

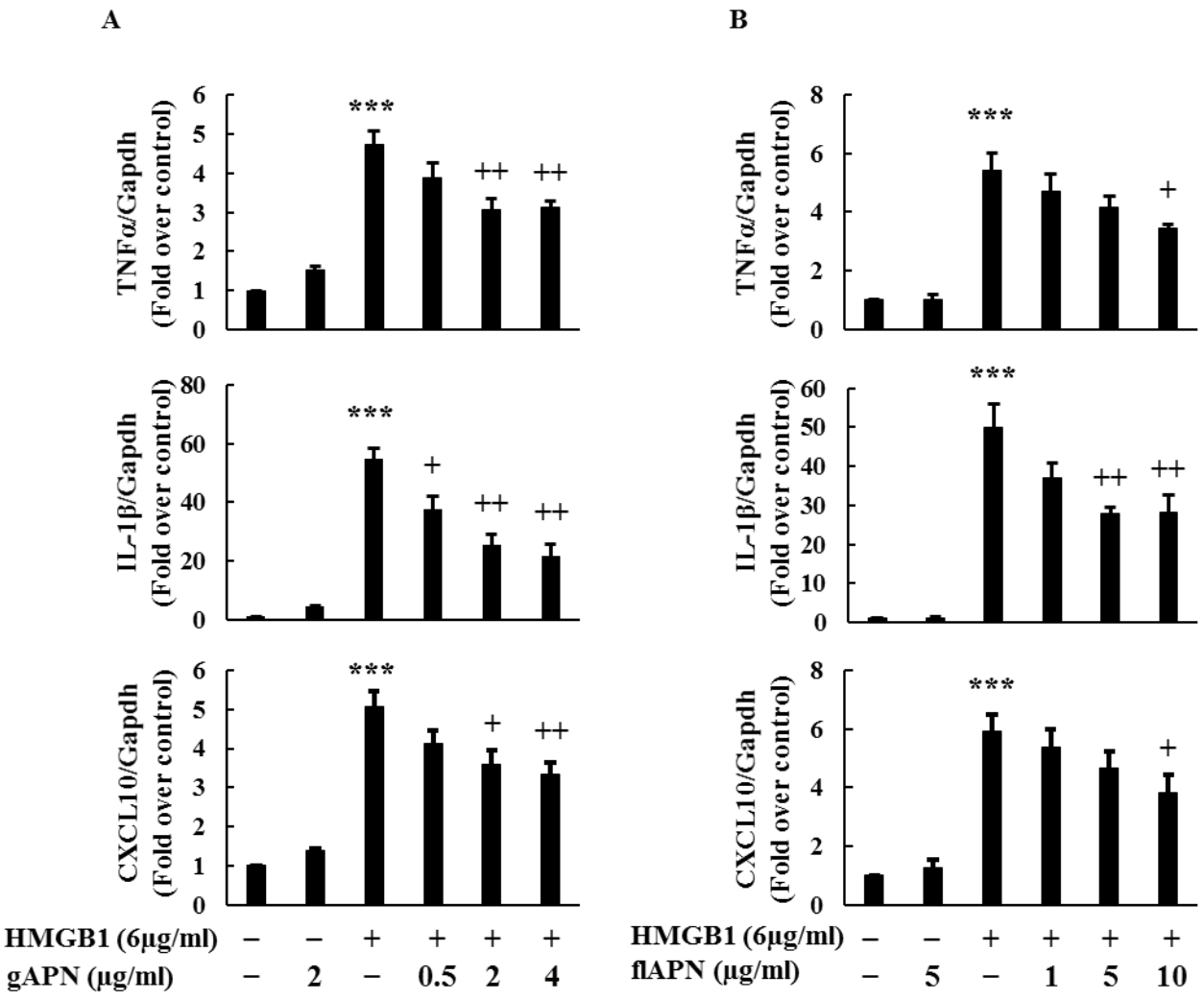


Figure 2-3. Full length- and globular adiponectin suppressed HMGB1-induced inflammatory responses in RAW264 cells. The cells were cultured in DMEM supplemented with 10% FBS and cultured in serum-free OPTI-MEM I medium for 12 h. The cells were treated with increasing concentration of globular (gAPN, A) or full length (flAPN, B) adiponectin for 18 h, then stimulated with HMGB1 (6 μg/ml) for another 2.5 h in the presence of PMB (10 μg/ml) to suppress any contaminating LPS in the protein preparations. Expression of TNF-α, IL-1β, CXCL10 and Gapdh mRNA were measured by qRT-PCR. The results are expressed as means ± SE of three independent experiments (*** $p < 0.001$ significance compared with control, $^{+}p < 0.05$, $^{++}p < 0.01$ significance compared with HMGB1-treated cells).

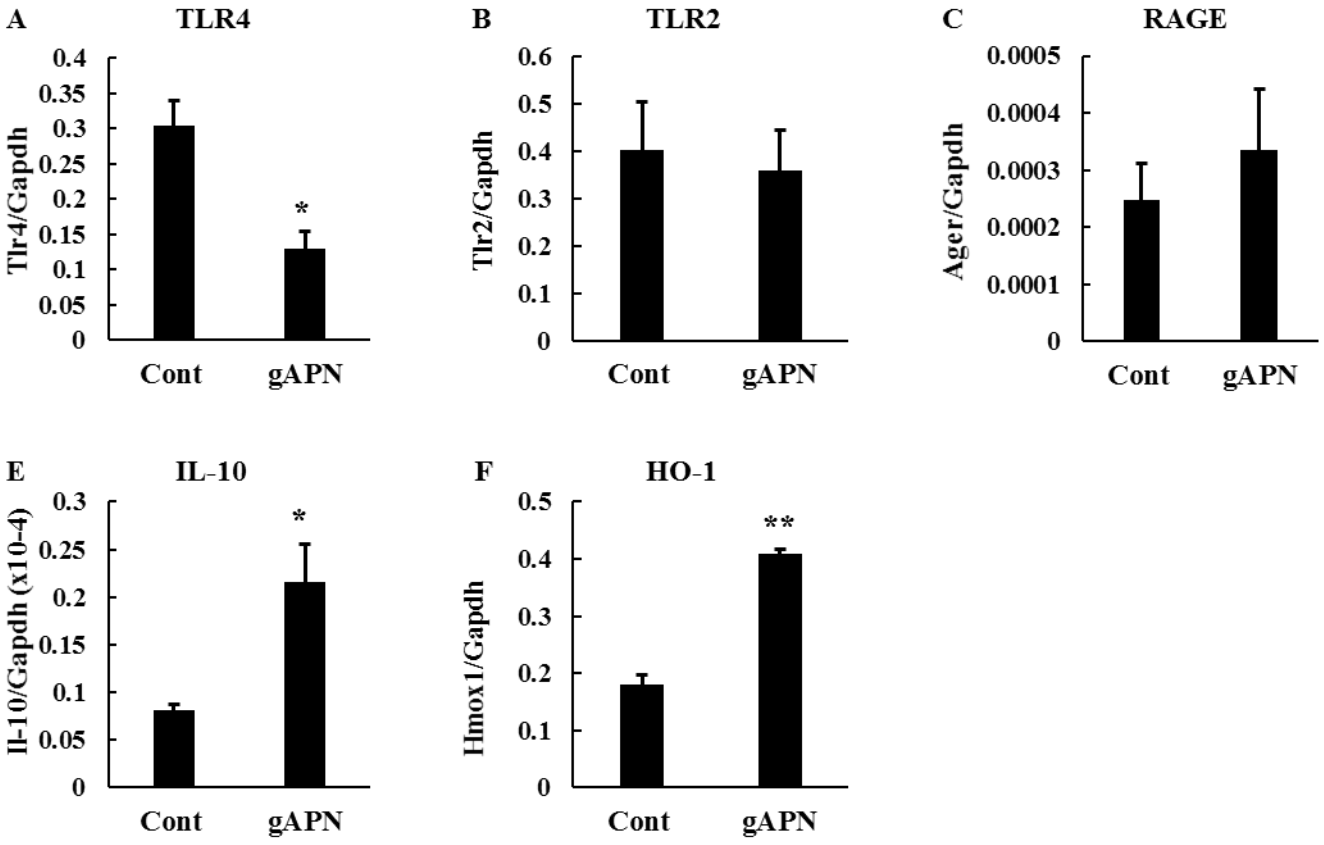
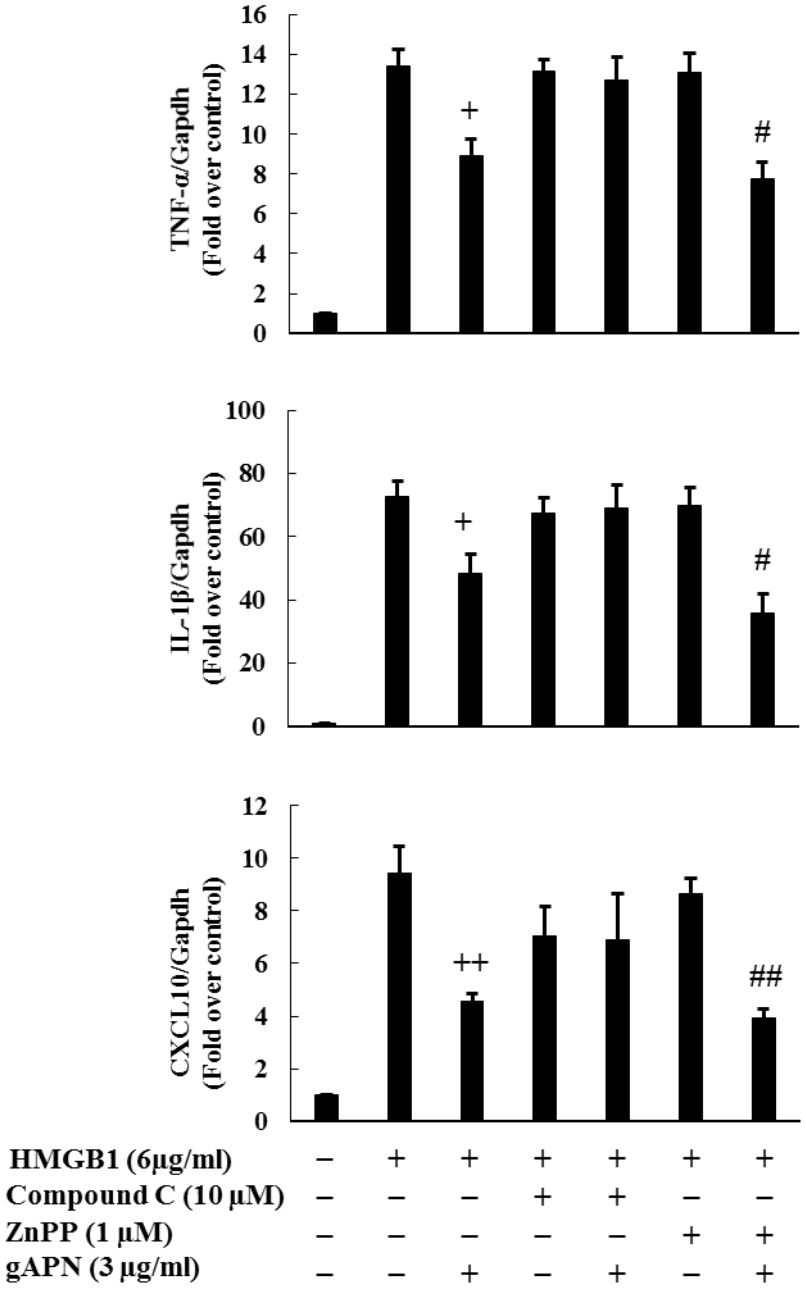


Figure 2-4. Effects of globular adiponectin on mRNA expression in RAW 264 cells. RAW264 cells were cultured as described in the figure 2-3 and were treated with gAPN (3 μg/ml) for 18 h. RNA was extracted, and expression of TLR4 (A), TLR2 (B), RAGE(C), IL-10 (D), HO-1 (E), and Gapdh (control) mRNAs were measured by qRT-PCR. The results are expressed as means ± SE of three independent experiments (** $p < 0.01$, *$p < 0.05$ significance compared with control (Cont)).

A


TNF-α/Gapdh (Fold over control)
16
14
12
10
8
6
4
2
0
+
#
IL-1β/Gapdh (Fold over control)
100
80
60
40
20
0
+
#
CXCL10/Gapdh (Fold over control)
12
10
8
6
4
2
0
++
##
HMGB1 (6μg/ml) – + + + + + +
Compound C (10 μM) – – – + + – –
ZnPP (1 μM) – – – – – + +
gAPN (3 μg/ml) – – + – + – +

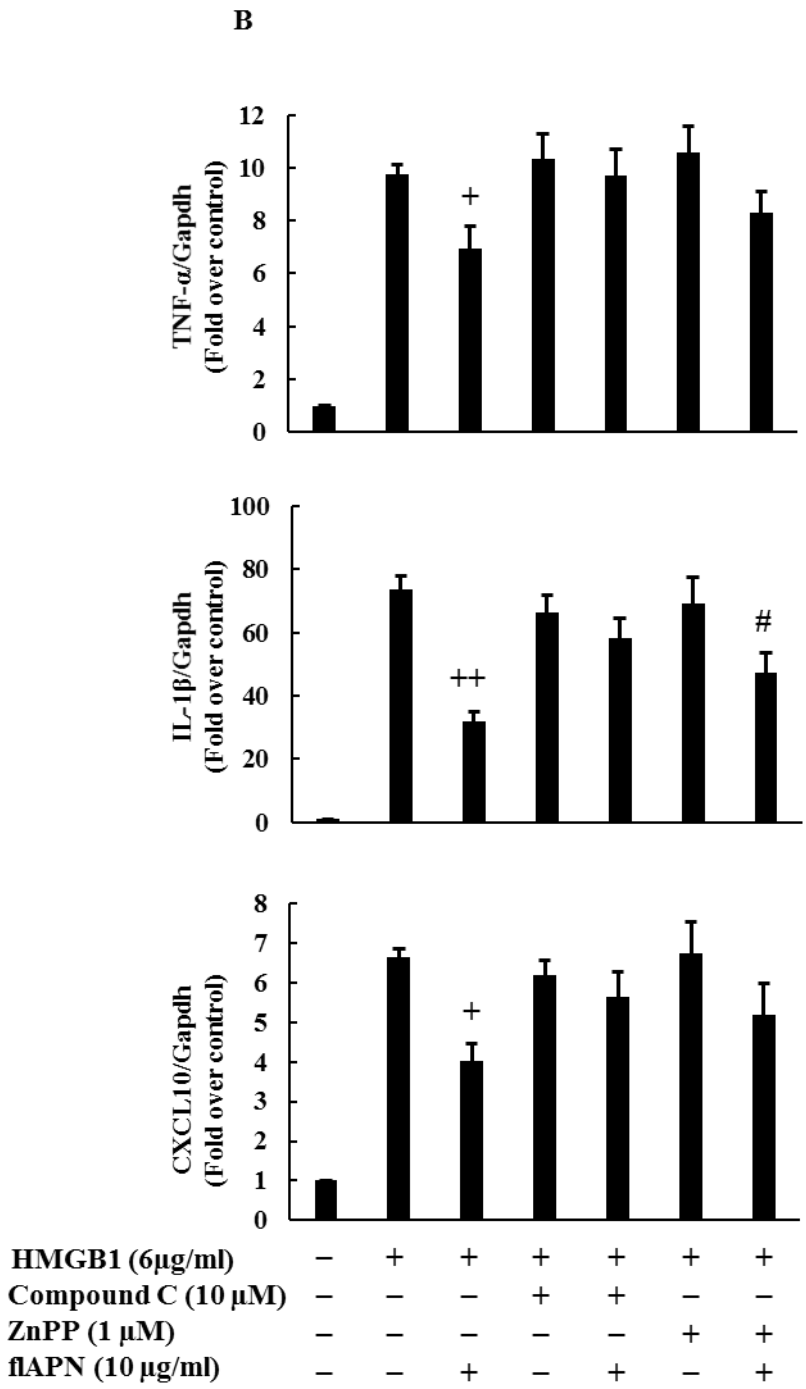


Figure 2-5. Effects of AMPK and HO-1 inhibitor on the suppression by full length and globular adiponectin of HMGB1-induced inflammatory responses in RAW264 cells. The cells were cultured as in figure 2-3 and treated with AMPK inhibitor (compound C, 10 μM) or heme oxygenase-1 inhibitor (ZnPP, 1 μM) 1 h before the treatment with gAPN (A) or flAPN (B) for 18 h, and subsequently stimulated with HMGB1 (6 μg/ml) for another 3 h. Expression of TNF-α, IL-1β, CXCL10 and Gapdh mRNA were measured by qRT-PCR. The results are expressed as means ± SE of three independent experiments ($^{+}p < 0.05$, $^{++}p < 0.01$ significance compared with HMGB1-treated cells, $^{\#}p < 0.05$ significance compared with HMGB1 plus ZnPP treated cells).

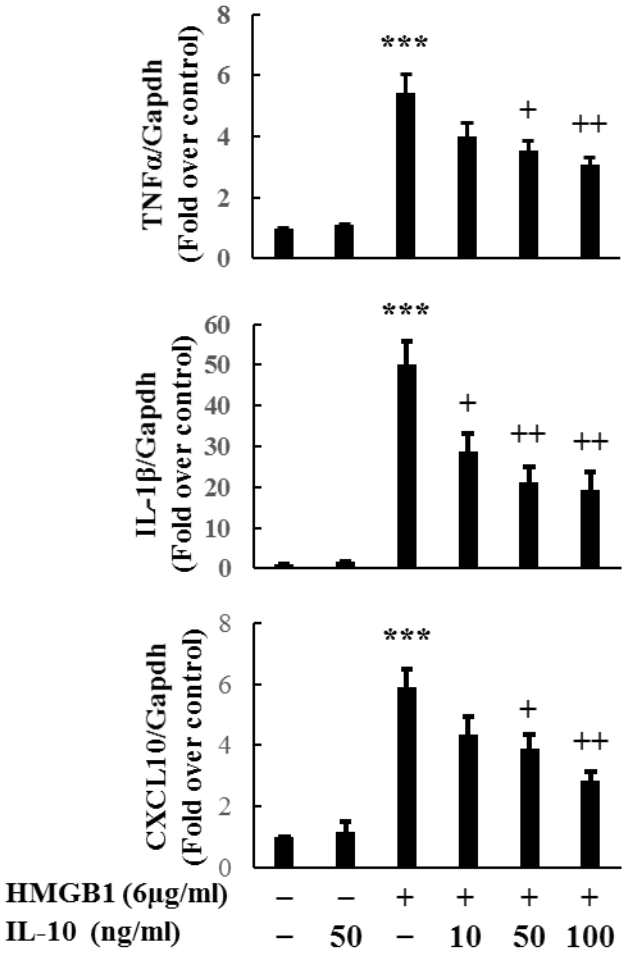


Figure 2-6. Effect of recombinant IL-10 on HMGB1-induced inflammatory responses in RAW264 cells. The cells were cultured as in figure 2-3 and pretreated with increasing concentration of recombinant IL-10 for 18 h, then stimulated with HMGB1 (6 μg/ml) for another 2.5 h. Expression of TNF-α, IL-1β, CXCL10 and Gapdh mRNA were measured by qRT-PCR. The results are expressed as means ± SE of three independent experiments (*** $p < 0.001$ significance compared with control, $^{+}p < 0.05$, $^{++}p < 0.01$ significance compared with HMGB1-treated cells).

CONCLUSION

HMGB1 is undoubtedly an important effector molecule and plays an important role in many pathological settings from acute sepsis to sterile inflammation during trauma, as well as in chronic inflammatory diseases e.g., rheumatoid arthritis. In addition, HMGB1 is implicated in other diseases characterized by cell death and damage including diabetes, atherosclerosis Alzheimer's disease, autoimmune diseases, and cancer. The major challenge in developing therapeutics for treatment of systemic inflammatory responses is that targeting of early cytokine responses is difficult and ineffective while targeting HMGB1 with inhibitor or antagonistic provides a protection against sepsis-induced mortality in animals and humans.

In chapter I, I tested the effect of flAPN on HMGB1 release from LPS-stimulated RAW 264 macrophage cells. Prior treatment of RAW 264 cells with flAPN suppressed LPS-induced HMGB1 release and translocation. Moreover, the anti-inflammatory cytokine IL-10 suppressed LPS-induced HMGB1 release. Exposure of RAW 264 cells with flAPN decreased TLR 4 mRNA expression and increased HO-1 mRNA expression without inducing IL-10 mRNA expression. Treatment with the HO-1 inhibitor ZnPP completely abolished the suppression of HMGB1 release by adiponectin. In addition, treatment with AMPK inhibitor, abolished the increase in HO-1 mRNA expression and the suppression of HMGB1 release mediated by adiponectin. These results suggest that flAPN attenuates HMGB1 release by LPS through an AMPK-mediated and HO-1-dependent IL-10-independent pathway.

In chapter II, I have proven that, TLR4 is the main receptor that mediates HMGB1-dependent activation of cytokine expression in RAW 264 macrophages. Highly purified HMGB1 is able to activate both MyD88-dependent (TNF-α, IL-1β) and –independent (CXCL10) pathway of TLR4 signaling. Prior treatment of RAW 264 macrophage cells with either flAPN or gAPN suppressed HMGB1-induced cytokine expression, and the suppression by both APN forms was abolished by compound C, an AMP kinase inhibitor. Treatment of RAW 264 with the HO-1 inhibitor ZnPP failed to abolish the suppression by both APN forms. These results suggest that the anti-inflammatory action of both APN forms against HMGB1 pro-inflammatory activity seems to be

mediated through AMPK-dependent-HO-1 independent pathways.

Taken together, this study proposes an additional mechanism for the anti-inflammatory actions of adiponectin through inhibition of both HMGB1 release and function in macrophages. In conclusion, this report sheds light on the protective therapeutic impact of adiponectin administration not only during obesity but also during other chronic inflammatory diseases such as sepsis and cancer.

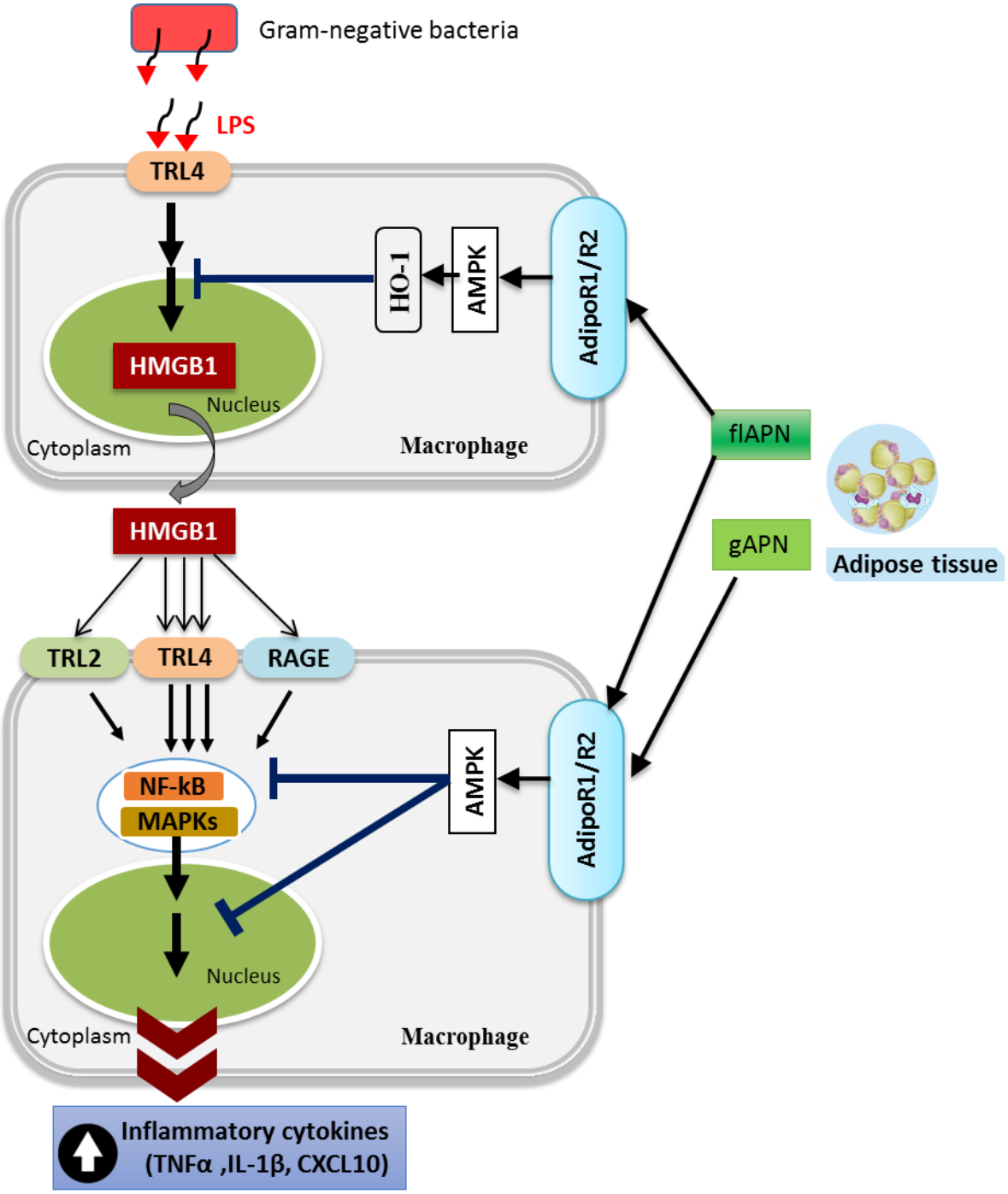


Figure 3. Schematic representation illustrating the anti-inflammatory role of adiponectin on LPS-induced HMGB1 release and HMGB1 pro-inflammatory activity in macrophage.

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JAPANESE SUMMARY

Novel suppressive action of adiponectin on LPS- and HMGB1-induced inflammatory responses

(脂肪細胞分泌因子アディポネクチンの細菌内毒素LPSおよび核内タンパク質HMGB1に因る炎症に対する新規抑制作用**)**

Mohamed Sobhy Hassan Elfeky

モハメド　ソビヒ　ハッサン　エルフェキー

非ヒストン核タンパク質HMGB1（high mobility group box protein 1）はDNAと結合し，様々な転写因子の活性を間接的に調節する。また、マウスにLPS (lipopolysaccharide)を投与して敗血症モデルを作製すると数日で死に至るが，この時、単球やマクロファージからHMGB1が能動的に、壊死組織からもHMGB1が受動的に細胞外へ放出され、炎症応答を増大させる。つまり、HMGB1は致死性炎症メディエーターとして機能する。

一方、アディポネクチンは脂肪細胞から分泌され、血中にμgオーダーで存在し、抗炎症作用を示す。脂肪蓄積が過剰となるとアディポネクチン生成は低下し、マクロファージの移入による軽度の非感染性炎症が起こる。これにより脂肪組織のみならず骨格筋などにおけるインスリン抵抗性が誘導される。この時、血中HMGB1濃度の上昇が見られ、小児肥満のマーカーとしての応用が期待されているが、その病態生理学的役割は明らかではない。

本研究ではHMGB1と関連する炎症応答とアディポネクチンの関係を明らかにすることを目的として、第一章ではアディポネクチンのLPSによるHMGB1の能動的分泌に対する作用について解析し、第二章ではアディポネクチンのHMGB1による炎症性応答に対する作用を解析した。

まずマクロファージ様細胞株RAW264細胞を用いて、全長アディポネクチンのHMGB1の能動的分泌への作用を調べた。細胞をLPSで刺激すると細胞の生存率には影響しないものの、HMGB１の細胞質への移行と細胞外液中の濃度の上昇がみられた。全長アディポネクチンの前処理はHMGB1の細胞質への移行と分泌を抑制した。また抗炎症性サイトカインであるIL-10　(Interleukin-10)も濃度依存性にHMGB1の細胞外への放出を抑制した。全長アディポネクチンの前処理はLPS受容体であるTLR （Toll-like receptor） 4 mRNAの発現を減少させ、抗酸化作用をもつHeme　oxygenase-1（HO-1）mRNAの発現を誘

導したが、IL-10　mRNAの誘導は起こさなかった。一方、IL-10の前処置はTLR2およびHMGB1 mRNAの発現を抑制し、HO-1およびIL-10 mRNAの発現を上昇させた。全長アディポネクチンの抑制効果はHO-1阻害剤の存在下で完全に消失したが、IL-10の抑制効果は部分的にしか解除されなかった。さらに全長アディポネクチンの抑制効果とHO-1 mRNAの誘導はAMPキナーゼ阻害剤によって阻害された。以上の結果は全長アディポネクチンが、IL-10とは独立して、AMPキナーゼを介して誘導したHO-1の作用によってLPSによるHMGB1の細胞外への放出を抑制することが示唆された。

次に大腸菌を用いて組換えHMGB1を作成し、Niカラムとエンドトキシン除去カラムで精製した。RAW264細胞を抗LPS剤ポリミキシンB存在下において組換えHMGB1で刺激すると、その濃度に依存して炎症性サイトカインTNFα（Tumor necrosis factor α）mRNAの発現を増加させた。この作用は組換え体の加熱変性によって失われ、HMGB1アンタゴニストによっても阻害されるので特異的な受容体の関与が示唆された。受容体アンタゴニスト共存下でHMGB1の活性を調べたところ、HMGB1の反応はTLR4アンタゴニストにより強く、RAGE (Receptor for advanced glycation end products)アンタゴニストにより弱く阻害されたが、TLR1/2アンタゴニストによっては影響されなかった。以上の結果よりRAW264細胞においてHMGB1は主にTLR4を介して炎症性サイトカインの発現を亢進させることが明らかとなった。

全長あるいは球状アディポネクチンの前処理はHMGB1によるTLR４に直接結合するMyD88 (Myeloid differentiation primar response 88)アダプタータンパク質を介するTNFαとIL-1β遺伝子発現およびMyD88非依存性に調節されるC-X-C chemokine (CXCL)10遺伝子発現の亢進を濃度依存性に抑制した。また球状アディポネクチンの前処理もTLR4 mRNAの発現を減少させ、HO-1 mRNAの発現を誘導した。そこでAMPキナーゼとHO-1の阻害剤を適用したが、AMPキナーゼ阻害剤は両アディポネクチンによる炎症性サイトカイン発現の抑制を解除したが、HO-1阻害剤は効果がなかった。両アディポネクチンの作用が、HMGB1によるMyD88を介するサイトカインと介さないサイトカインの発現亢進を供に抑制するので、以上の結果は全長および球状アディポネクチンはAMPキナーゼを介してHO-1非依存性に、おそらくはTLR4の発現を抑制することでHMGB1の炎症性シグナルを減じていることが示唆された。

以上のように、脂肪細胞分泌因子アディポネクチンはマクロファージにおける細菌内毒素LPSによる核内タンパク質HMGB1の放出をAMPキナーゼ／heme oxygenase-1依存性に抑制し、HMGB1受容体として機能するTLR4の発現をおそらくは抑制することでHMGB1

のシグナルを減弱させていると考えられた。非感染性炎症である肥満においてもアディポネクチンの低下がHMGB1の放出やサイトカイン誘導能を増強させていることが推察され、生活習慣病のターゲットとしてHMGB1が注目される。