Formononetin inhibits lipopolysaccharide-induced release of high mobility group box 1 by upregulating SIRT1 in a PPARδ-dependent manner

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Background. The release of high mobility group box 1 (HMGB1) induced by inflammatory signals acts as a cellular alarmin to trigger a chain of inflammatory responses. Although the inflammatory actions of HMGB1 are well studied, less is known about the therapeutic agents that can impede its release. This study investigated whether the isoflavonoid formononetin can modulate HMGB1 release in cellular inflammatory responses.
Methods. RAW264.7 murine macrophages were exposed to lipopolysaccharide (LPS) in the presence or absence of formononetin. The levels of HMGB1 release, sirtuin 1 (SIRT1) expression, and HMGB1 acetylation were analyzed by immunoblotting and real-time polymerase chain reaction. The effects of resveratrol and sirtinol, an activator and inhibitor of SIRT1, respectively, on LPS-induced HMGB1 release were also evaluated.
Results. Formononetin modulated cellular inflammatory responses by suppressing the release of HMGB1 by macrophages exposed to LPS. In RAW264.7 cells, formononetin significantly attenuated LPS-induced release of HMGB1 into the extracellular environment, which was accompanied by a reduction in its translocation from the nucleus to the cytoplasm. In addition, formononetin significantly induced mRNA and protein expression of SIRT1 in a peroxisome proliferator-activated receptor δ (PPARδ)-dependent manner. These effects of formononetin were dramatically attenuated in cells treated with small interfering RNA (siRNA) against PPARδ or with GSK0660, a specific inhibitor of PPARδ, indicating that PPARδ is involved in formononetin-mediated SIRT1 expression. In line with these effects, formononetin-mediated inhibition of HMGB1 release in LPS-treated cells was reversed by treatment with SIRT1-targeting siRNA or sirtinol, a SIRT1 inhibitor. By contrast, resveratrol, a SIRT1 activator, further potentiated the inhibitory effect of formononetin on LPS-induced HMGB1 release, revealing a possible mechanism by which formononetin regulates HMGB1 release through SIRT1. Furthermore, modulation of SIRT1 expression by transfection

of SIRT1- or PPARδ-targeting siRNA significantly counteracted the inhibitory effects of formononetin on LPS-induced HMGB1 acetylation, which was responsible for HMGB1 release.

Discussion. This study shows for the first time that formononetin inhibits HMGB1 release by decreasing HMGB1 acetylation via upregulating SIRT1 in a PPAR δ-dependent manner. Formononetin consequently exhibits anti-inflammatory activity. Identification of agents, such as formononetin, which can block HMGB1 release, may help to treat inflammation-related disorders.

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ABSTRACT

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INTRODUCTION

High mobility group box 1 (HMGB1), a non-histone DNA-binding protein, is a well-conserved nuclear protein that has multiple functions depending on its cellular location. In the nucleus, HMGB1 plays roles in DNA replication, transcription, recombination, and maintenance of chromosome stability (Stros, 2010). However, when released by stressed cells, HMGB1 plays a critical role in the inflammatory response and is a late proinflammatory marker in many diseases including sepsis (Andersson & Harris, 2010; Abdulahad et al., 2010; Sims et al., 2010; Stros, 2010; Zhang et al., 2009). Recent reports show that post-translational modifications of HMGB1, such as acetylation and phosphorylation, are associated with its translocation and release in inflammatory cells exposed to pathogen-related molecules including lipopolysaccharide (LPS) (Bonaldi et al., 2003; Ito et al., 2007; Youn & Shin, 2006). The importance of extracellular HMGB1 in the inflammatory response has been demonstrated in inflammatory conditions; a neutralizing anti-HMGB1 antibody and HMGB1 antagonists attenuate cellular damage induced by inflammation (Wang et al., 1999; Davé et al., 2009). These reports indicate the importance of pathways or molecules that regulate HMGB1 release from activated inflammatory cells.

Sirtuin 1 (SIRT1) is a type III histone deacetylase that controls multiple genetic programs by acting on histone and non-histone substrates (Xie et al., 2013). This protein is a vital regulator of various physiological and metabolic processes such as energy metabolism (Purushotham et al., 2009), aging (Tissenbaum & Guarente, 2001), apoptosis (Motta et al., 2004), mitochondrial biogenesis (Brenmoehl & Hoeflich, 2013), and the stress response (Brunet et al., 2004). Recent studies also demonstrate that SIRT1 is directly involved in cellular inflammatory responses by deacetylating inflammation-related transcription factors such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), which suppresses the transcription of diverse inflammation-responsive genes (Feige & Auwerx, 2008; Zhang &

Kraus, 2010). Furthermore, we demonstrated that transcriptional upregulation of SIRT1 by peroxisome proliferator-activated receptor δ (PPARδ) and PPARγ inhibits HMGB1 release by decreasing its LPS-induced acetylation, indicating that SIRT1 deacetylates HMGB1 (Hwang et al., 2012; Hwang et al., 2014). While genetic ablation of SIRT1 increases the secretion and expression of proinflammatory cytokines, SIRT1 activators prevent the production of tumor necrosis factor-α, monocyte chemoattractant protein-1, and interleukin (IL)-8 (Dong et al., 2014; Yang et al., 2007), highlighting the central role of SIRT1 in the regulation of cellular inflammatory responses.

Formononetin, a herbal isoflavonoid, was isolated from the medicinal plant *Astragalus membranaceus* and has a variety of biological activities including anti-tumor (Auyeung et al., 2012; Chen et al., 2011), wound healing (Huh et al., 2011), antioxidant (Mu et al., 2009), and anti-inflammatory (Krenn & Paper, 2009; Lai et al., 2013) effects. Specifically, formononetin inhibits inflammation-related gene expression by blocking the NF-κB and AP-1 signaling pathways in animal models of inflammatory diseases (Chen et al., 2007; Hämäläinen et al., 2007). In particular, synthetic derivatives of formononetin increase the activity of PPARδ, indicating this compound is useful to treat inflammation-related diseases (Zhao et al., 2017). Furthermore, we showed that activation of PPARδ and PPARγ by specific ligands induces SIRT1 expression in human coronary artery endothelial cells (Kim et al., 2012) and RAW264.7 cells (Hwang et al., 2014). Thus, we hypothesized that formononetin may modulate cellular inflammatory responses by inhibiting HMGB1 release via upregulation of SIRT1. Here, we show that formononetin reduces LPS-induced HMGB1 acetylation by upregulating SIRT1 in a PPARδ-dependent manner, thereby blocking HMGB1 release into the extracellular environment.

99 # MATERIALS & METHODS

100 ## Materials

101 Formononetin, actinomycin D (Act D), cycloheximide (CHX), Ponceau S solution,
102 resveratrol, sirtinol, MTT, LPS (Escherichia coli 0111:B4), and an anti-β-actin polyclonal
103 antibody were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). GSK0660 and the
104 luciferase assay system were purchased from Tocris Bioscience (Bristol, UK) and Promega
105 (Madison, WI, USA), respectively. Monoclonal antibodies specific for HMGB1 and PPARδ
106 were supplied by Epitomics (Burlingame, CA, USA). Monoclonal antibodies specific for
107 acetyl-lysine, lamin B, and α-tubulin as well as a polyclonal antibody specific for SIRT1 were
108 supplied by Santa Cruz Biotechnology (Dallas, TX, USA).
109
110 ## Cell culture
111 RAW264.7 murine macrophage-like cells were purchase from the Korean Cell Line Bank
112 (Seoul, Korea), and then maintained in DMEM (Dulbecco's modified Eagle medium)
113 containing antibiotics and 10% FCS at 37°C in a 5% humidified CO2 incubator.
114
115 ## Cell viability assays
116 RAW264.7 cells were stimulated with 30 μM formononetin for the indicated duration or the
117 indicated dose of formononetin for 24 h in 24-well plates. Thereafter, MTT assays and trypan
118 blue exclusion were performed to determine the cell viability. For trypan blue exclusion, the
119 collected cells were mixed with trypan blue solution (0.4%), and then viable cells were
120 determined by a hemocytometer. For MTT assay, the cells were incubated for final 2h in
121 medium containing MTT solution (0.1 mg/ml). Following removing the medium, the
122 absorbance at 570 nm was measured using formazan crystals solution dissolved in acidified
123 isopropanol.

Western blot analysis

Protein levels were analyzed by immunoblot as described previously (Hwang et al., 2015). Briefly, RAW264.7 cells washed with ice-cold PBS were lysed and aliquots of the resulting whole-cell lysates or conditioned media were analyzed by immunoblot with indicated antibodies. Immuno-reactive bands were detected using WesternBright ECL (Advansta Co., Melon Park, CA, USA).

Measurement of extracellular HMGB1

Levels of HMGB1 released into culture media were determined using a previously described method (Hwang et al., 2012). Briefly, the relative amounts of HMGB1 were determined in the conditioned media of RAW264.7 cells treated with the indicated reagents for the indicated durations. The 80% ice-cold acetone was used to precipitate the proteins in the conditioned media. After centrifugation, the pellets were obtained and washed with 80% ice-cold acetone. Following resuspension in SDS-PAGE sample buffer, the levels of HMGB1 released into culture media were analyzed by immunoblot.

Fractionation of nuclear and cytoplasmic proteins

Cellular fractions were prepared using a previously described method (Hwang et al., 2015). Briefly, RAW264.7 cells were washed in PBS, suspended in lysis solution for 15 min at 4°C to swell. Nonidet P-40 (final 0.1% concentration) was added to the lysates and then vortexed vigorously for 20 sec. Following centrifugation (13,000 × g) for 20 sec, the supernatant containing cytosolic fraction was obtained and the resulting pellet was lysed by a PRO-PREP Protein Extraction Solution. Following standing for 20 min on ice, the nuclear fraction (supernatant) was obtained by centrifugation.

Reporter gene assay

The luciferase construct containing mouse SIRT1 promoter was a gift from Dr. Toren Finkel (NIH, MD, USA). The promoter activity of SIRT1 was measured as described previously (Hwang et al., 2014). Briefly, 1 μg of the SIRT1 luciferase reporter plasmid and 0.5 μg of pSV β-Gal (SV40 β-galactosidase expression vector) were introduced into RAW264.7 cells by SuperFect reagent (Qiagen, Valencia, CA, USA). After 38 h, the cells were treated with GSK0660 for 30 min prior to stimulation with formononetin for 24 h. Then, the cells were lysed by adding the luciferase reporter lysis buffer (Promega) and then aliquots of the lysates were used to determine luciferase activity.

Small interfering RNA (siRNA)-mediated gene silencing

The indicated siRNA was introduced into RAW264.7 cells in serum-containing medium using SuperFect (Qiagen) as described previously (Hwang et al., 2014). Briefly, siRNA targeting scrambled non-specific sequences (Ambion, Austin, TX, USA), PPAR (Ambion), or SIRT1 designed against nucleotides (5'-TAATATCTGAGGCACTTCA-3' and 5'-TGAAGTGCCTCAGATATTA-3') of mouse (Bioneer, Daejeon, Korea) was introduced into the cells for 6 h. The cells were then cultured for further 38 h in fresh medium. At which point, the indicated reagents were added into the cells for the indicated durations. Gene silencing was analyzed by immunoblot.

Real-time polymerase chain reaction (PCR)

Levels of SIRT1 mRNA were analyzed by real-time PCR as described previously (Hwang et al., 2014). Briefly, total RNA was converted into cDNA by a reverse transcription kit (TOPscript RT DryMIX, Enzynomics, Seoul, Korea). Real-time PCR was carried out using equal amount of cDNA in a 20 μl reaction solution containing primers and 1 × SYBR PCR

mix (Takara Bio Inc., Otsu, Japan). The PCR condition: initial denaturation at 94°C for 20 min, followed by 42 cycles of 25 s at 95°C, 44 s at 58.2°C, and 40 s at 72°C. The primers were as follows: SIRT1, 5'-AGAACCACCAAAGCGGAAA-3' and 5'-TCCCACAGGAGACAGAAACC-3'; and GAPDH, 5'-CATGGCCTTCCGTGTTCCTA-3' and 5'-CCTGCTTCACCACCTTCTTGAT-3'. Levels of SIRT1 mRNA were analyzed by real-time PCR as described previously (Hwang et al., 2014). Briefly, total RNA was converted into cDNA by a reverse transcription kit (TOPscript RT DryMIX, Enzynomics, Seoul, Korea). Real-time PCR was carried out using equal amount of cDNA in a 20 μl reaction solution containing primers and 1 × SYBR PCR mix (Takara Bio Inc., Otsu, Japan). The PCR condition: initial denaturation at 94°C for 20 min, followed by 42 cycles of 25 s at 95°C, 44 s at 58.2°C, and 40 s at 72°C. The primers were as follows: SIRT1, 5'-AGAACCACCAAAGCGGAAA-3' and 5'-TCCCACAGGAGACAGAAACC-3'; and GAPDH, 5'-CATGGCCTTCCGTGTTCCTA-3' and 5'-CCTGCTTCACCACCTTCTTGAT-3'.

Co-immunoprecipitation

Immunoprecipitation was performed using a previously described method (Hwang et al., 2015). Briefly, the protein G Sepharose was added to whole-cell lysates to pre-clear and then the pre-cleared lysates were mixed with 1 μg of an anti-HMGB1 antibody. After incubation overnight at 4°C, the mixture was reacted with protein G Sepharose for 4 h. Mixtures were extensively washed with PBS and then boiled in gel-loading buffer. The immunoblot analysis was performed using an anti–acetyl-lysine antibody (Santa Cruz Biotechnology).

Statistical analysis

The significance in statistical analysis was evaluated by a one-way ANOVA.

198 # RESULTS

199 **Formononetin inhibits LPS-induced release of HMGB1 in RAW264.7 cells**

200 To determine the optimal concentration of formononetin, we determined the viability of
201 RAW264.7 cells treated with various concentrations of formononetin for 24 h or with 30 μM
202 formononetin for various durations. Treatment with concentrations of formononetin up to 30
203 μM did not elicit cytotoxic effects on RAW264.7 cells, and cell viability remained high
204 following treatment with 30 μM formononetin for up to 5 days (Figure 1). Thus, we selected
205 30 μM formononetin as the optimal concentration for subsequent experiments using
206 RAW264.7 cells.

207 Next, we examined whether formononetin affects LPS-induced HMGB1 release in
208 RAW264.7 cells to assess its anti-inflammatory activity. The level of HMGB1 released into
209 culture media was increased in cells exposed to LPS, and this increase was markedly reduced
210 in the presence of formononetin (Figure 2A). By contrast, neither LPS nor formononetin
211 affected the expression level of endogenous HMGB1. These results indicate that
212 formononetin affects LPS-induced HMGB1 release, but not HMGB1 expression.

213 HMGB1 is reported to translocate from the nucleus into the cytoplasm in response to
214 inflammatory signals such as LPS (Bonaldi et al., 2003; Youn & Shin, 2006). Therefore, we
215 examined whether formononetin affects this translocation of HMGB1 in LPS-stimulated
216 RAW264.7 cells. While translocation of HMGB1 into the cytoplasm was increased in cells
217 exposed to LPS, this was markedly suppressed by formononetin (Figure 2B). These results
218 suggest that formononetin decreases the release of HMGB1 by inhibiting its translocation in
219 LPS-primed RAW264.7 cells.

220

221 **Formononetin upregulates SIRT1 expression in RAW264.7 cells**

Formononetin increased protein expression of SIRT1 in RAW264.7 cells in a concentration- and time-dependent manner. SIRT1 protein expression was significantly increased in cells treated with 20–30 μM formononetin for 24 h (Figure 3A) and peaked at 12–24 h in cells treated with 30 μM formononetin (Figure 3B).

To elucidate the mechanisms by which formononetin induces SIRT1 expression, we determined the effects of Act D (a RNA synthesis inhibitor) and CHX (a protein synthesis inhibitor). While formononetin significantly increased mRNA expression of SIRT1 in a time-dependent manner (Figure 4A), this was significantly reduced in the presence of Act D or CHX (Figure 4B). These results indicate that de novo synthesis of mRNA as well as of proteins that act on the *SIRT1* gene promoter is indispensable for the induction of *SIRT1* mRNA by formononetin in RAW264.7 cells.

Formononetin induces SIRT1 expression via PPARδ in RAW264.7 cells

To further examine the mechanisms by which formononetin upregulates SIRT1 expression, we evaluated the role of PPARδ, a nuclear receptor that regulates the transcription of a variety of target genes (Kidani & Bensinger, 2012; Mangelsdorf et al., 1995), by transfecting RAW264.7 cells with siRNA against PPARδ. The protein level of PPARδ was reduced in cells transfected with PPARδ-targeting siRNA, but not in cells transfected with control siRNA composed of a pool of nonspecific sequences (Supplemental Figure 1). Transfection of PPARδ-targeting siRNA attenuated the induction of SIRT1 expression by formononetin, whereas transfection of control siRNA did not (Figure 4C). In line with these findings, GSK0660, a specific inhibitor of PPARδ, significantly attenuated the formononetin-induced increase in SIRT1 promoter activity (Figure 4D). These results suggest that formononetin upregulates SIRT1 expression via PPARδ at the transcriptional level.

SIRT1 is essential for inhibition of LPS-induced HMGB1 release by formononetin

To investigate the direct effect of SIRT1 on LPS-induced HMGB1 release, we examined the levels of SIRT1 protein and released HMGB1 in RAW264.7 cells exposed to LPS in the presence or absence of formononetin. A high level of HMGB1 was released upon LPS treatment, whereas this was reduced in the presence of formononetin. On the other hand, the level of SIRT1 protein was significantly suppressed in LPS-treated RAW264.7 cells. However, this LPS-mediated repression of SIRT1 was recovered in the presence of formononetin, indicating that SIRT1 is critical for modulation of LPS-induced HMGB1 release by formononetin (Figure 5A).

To further clarify the functional significance of formononetin-mediated upregulation of SIRT1 in RAW264.7 cells, we manipulated the expression and activity of SIRT1 using siRNA or chemicals. The levels of SIRT1 protein were diminished in cells transfected with SIRT1 siRNA, however control siRNAs had no effect on the levels of either protein (Supplemental Figure 2). Transfection of SIRT1-targeting siRNA significantly attenuated the inhibitory effect of formononetin on LPS-induced HMGB1 release (Figure 5B). Consistently, inhibition of SIRT1 activity by sirtinol also prevented inhibition of HMGB1 release by formononetin (Figure 5C). By contrast, activation of SIRT1 by resveratrol inhibited LPS-induced HMGB1 release. Furthermore, resveratrol treatment potentiated the inhibitory effects of formononetin, suggesting that SIRT1 plays a role in the suppression of HMGB1 release by formononetin (Figure 5D). These results indicate that formononetin inhibits LPS-induced HMGB1 release by regulating SIRT1 activity.

SIRT1-mediated deacetylation of HMGB1 underlies the inhibition of its release by formononetin

Inflammatory signal-mediated acetylation of HMGB1 is critical for its release into the extracellular compartment and acetylated HMGB1 is a substrate of SIRT1 (Bonaldi et al., 2003; Hwang et al., 2014; Rickenbacher et al., 2014); therefore, we evaluated whether formononetin affects LPS-induced acetylation of HMGB1. When RAW264.7 cells were stimulated with LPS for 6 h, the level of acetylated HMGB1 in an immunoprecipitate obtained using an anti-HMGB1 antibody was significantly enhanced. However, formononetin reduced this increase in acetylated HMGB1 in a concentration-dependent manner, indicating that formononetin is involved in the deacetylation of HMGB1 primed by LPS (Figure 6A).

To evaluate whether this inhibition of LPS-induced HMGB1 acetylation by formononetin correlates with the level of SIRT1 in RAW264.7 cells, we knocked down SIRT1. Transfection of SIRT1-targeting siRNA significantly prevented the decrease in acetylated HMGB1 by formononetin in LPS-exposed RAW264.7 cells (Figure 6B). Transfection of PPARδ-targeting siRNA elicited the same effect (Figure 6C). These results indicate that formononetin reduces HMGB1 acetylation via PPARδ-mediated upregulation of SIRT1, thereby inhibiting the release of HMGB1 into the extracellular milieu.

DISCUSSION

HMGB1 plays physiological and pathological roles by acting as an intracellular structural protein and an extracellular cytokine (Ueda & Yoshida, 2010; Andersson & Tracey, 2011). Although the roles of extracellular HMGB1 in the pathogenesis of inflammatory disease are well established, the regulatory mechanisms underlying HMGB1 release or therapeutic agents that can impede its release was not fully elucidated. Here, we showed that formononetin inhibited LPS-induced release of HMGB1 in RAW264.7 cells. This inhibition was mediated by PPARδ-dependent upregulation of SIRT1, a class III deacetylase involved in cellular inflammatory responses (Brunet et al., 2004; Yeung et al., 2004; Zhang et al., 2010). SIRT1 expression was also upregulated at the transcriptional level in RAW264.7 cells treated with formononetin. Modulation of SIRT1 expression and activity by siRNAs and chemicals abolished the inhibitory effect of formononetin on HMGB1 release. In addition, SIRT1 upregulated by formononetin deacetylated HMGB1, which inhibited release of HMGB1. This demonstrates that formononetin has anti-inflammatory actions in LPS-stimulated RAW264.7 cells. These results are in line with the previous finding that formononetin elicits anti-inflammatory effects by upregulating PPARγ expression in an animal model of LPS-induced acute lung injury (Ma et al., 2013). In addition, formononetin attenuates hydrogen peroxide- and IL-1β-induced activation of NF-κB in retinal ganglion cells and the insulinoma cell line INS-1, respectively (Jia et al., 2014; Wang et al., 2012). Although the molecular mechanisms underlying formononetin-mediated anti-inflammatory responses have not been fully elucidated until now, the present study clearly demonstrated that formononetin inhibits LPS-induced release of HMGB1 in the mouse macrophage cell line RAW264.7, suggesting that formononetin is a promising therapeutic agent for inflammation-related disorders.

The release of HMGB1 during inflammatory responses is closely linked with its post-translational modifications such as acetylation and phosphorylation (Bonaldi et al., 2003;

311 Youn & Shin, 2006). Consistent with previous studies, formononetin inhibited LPS-induced
312 acetylation of HMGB1, leading to suppression of its release. This effect of formononetin on
313 HMGB1 release was intimately correlated with the level of SIRT1 expression, indicating that
314 SIRT1 deacetylates HMGB1 and thereby inhibits its release. This result is in line with
315 previous reports indicating that SIRT1 deacetylates inflammation-related transcription factors
316 such as AP-1 and NF-κB, and thereby modulates the progression of inflammation by
317 suppressing the transcription of diverse inflammation-related genes (Yang et al., 2007; Yeung
318 et al., 2004; Zhang et al., 2010). These results provide a rationale for the use of SIRT1
319 activators as therapeutic agents in inflammatory diseases. In fact, a recent study demonstrated
320 that inflammatory diseases are closely associated with a reduced SIRT1 protein level (Xie et
321 al., 2013). Because release of HMGB1 is intimately correlated with its post-translational
322 modifications along with decreased SIRT1 expression, it may be possible to suppress
323 inflammatory reactions by inducing SIRT1 expression using formononetin.

324 Formononetin-mediated upregulation of SIRT1 was critical for inhibition of LPS-
325 induced HMGB1 release. SIRT1, a NAD^+-dependent deacetylase, is implicated in diverse
326 cellular processes, such as stress responses, aging, energy metabolism, and inflammation,
327 through its deacetylase activity (Brunet et al., 2004; Chen et al., 2005; Cohen et al., 2004;
328 Feige & Auwerx, 2008; Yeung et al., 2004; Zhang & Kraus, 2010; Zhang et al., 2010).
329 Although transcriptional regulation of SIRT1 in mammalian cells has been mainly established
330 in the context of energy metabolism-related pathways such as caloric restriction (Chen et al.,
331 2005; Cohen et al., 2004), transcription factors, including TLX, BRCA1, HIC1, and E2F1,
332 are also implicated in the regulation of SIRT1 expression (Chen et al., 2005; Iwahara et al.,
333 2009; Wang et al., 2006; Wang et al., 2008). However, the transcriptional regulation of SIRT1
334 is complex and the underlying mechanism is unclear. The nuclear hormone receptor PPARδ
335 was recently demonstrated to regulate SIRT1 expression in various cell lineages (Kim et al.,

2012; Okazaki et al., 2010). PPARδ was initially shown to promote SIRT1 expression in human hepatocytes via an unconventional mechanism in which specificity protein 1 plays a central role, rather than the PPAR-response element (Okazaki et al., 2010). PPARδ activation also induces SIRT1 expression in vascular endothelial cells (Kim et al., 2012). On the other hand, formononetin, a compound extracted from *S. flavescens* roots, significantly increases PPARδ activity in a concentration-dependent manner (Quang et al., 2013), indicating that transactivation of PPARδ by formononetin is linked to SIRT1 expression. This result is in line with our finding that formononetin induced SIRT1 expression in a PPARδ-dependent manner.

CONCLUSIONS

To our knowledge, this is the first report to show that formononetin inhibits HMGB1 release by upregulating SIRT1 transcription and thus inducing HMGB1 deacetylation in LPS-treated RAW264.7 cells. This novel finding has important implications for our understanding of the molecular mechanism underlying the transcriptional regulation of SIRT1 as well as the anti-inflammatory effect of formononetin. In light of these observations, formononetin-mediated enhancement of SIRT1 activity in macrophages is likely a new therapeutic strategy for inflammatory disorders.

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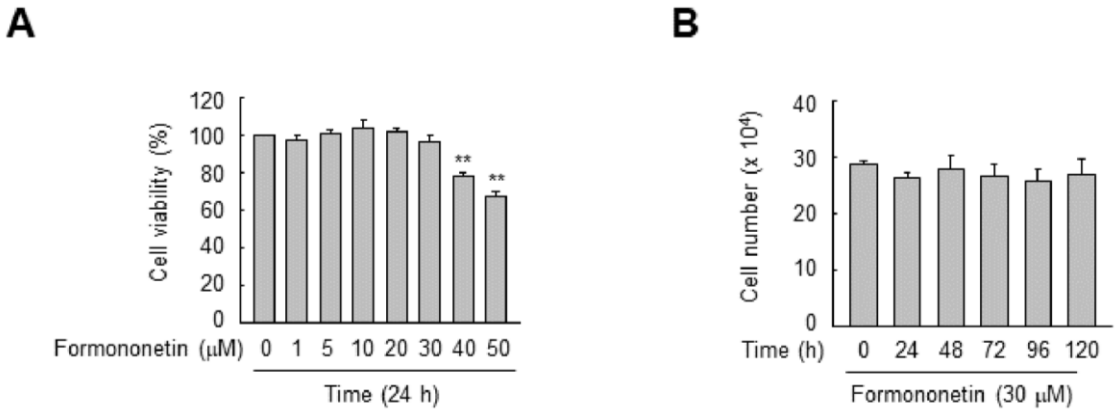


Figure 1 Effects of formononetin on the viability of RAW264.7 cells. (A and B) Cells cultured in serum-free medium for 16 h were treated with the indicated concentrations of formononetin for 24 h (A) or with 30 μM formononetin for the indicated durations (B). Cell viability was determined by the MTT (A) and trypan blue exclusion (B) assays. The results are plotted as the means ± SE (n=4). $^{**}p<0.01$ compared with the untreated group.

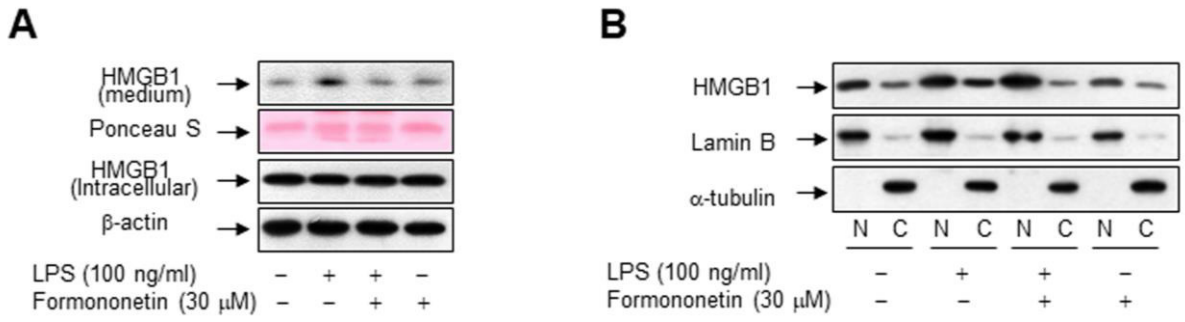


Figure 2 Effects of formononetin on the LPS-induced release and translocation of HMGB1 in RAW264.7 cells. (A) Cells cultured in serum-free medium for 16 h were stimulated with LPS in the presence or absence of formononetin for 24 h. Equal volumes of conditioned media or aliquots of whole-cell lysates were analyzed by immunoblotting. Ponceau S staining and β-actin were used as the loading controls. (B) Cells treated with LPS in the presence or absence of formononetin for 24 h were fractionated into nuclear (N) and cytosolic (C) fractions. The localization of HMGB1 was determined by Western blot analysis with the indicated antibodies.

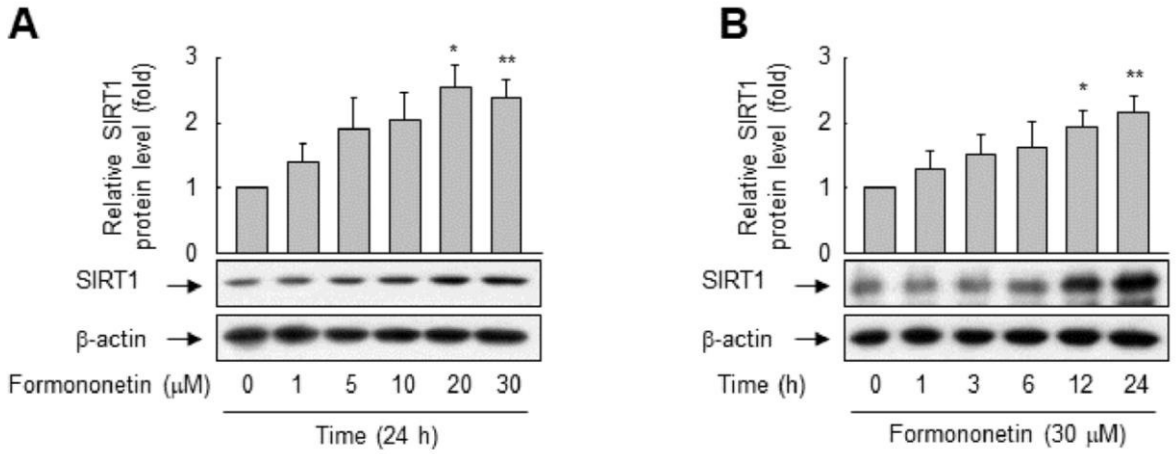


Figure 3 Effects of formononetin on expression of SIRT1 in RAW264.7 cells. (A and B) Cells cultured in serum-free medium for 16 h were incubated with various concentrations of formononetin for 24 h (A) or with 30 μM formononetin for the indicated durations (B). Aliquots of whole-cell lysates were analyzed by immunoblotting. Representative blots are provided. Fold changes in the SIRT1/β-actin ratio relative to that in the untreated group are shown as means ± SE (n=3). $^{*}p<0.05$, $^{**}p<0.01$ compared with the untreated group.

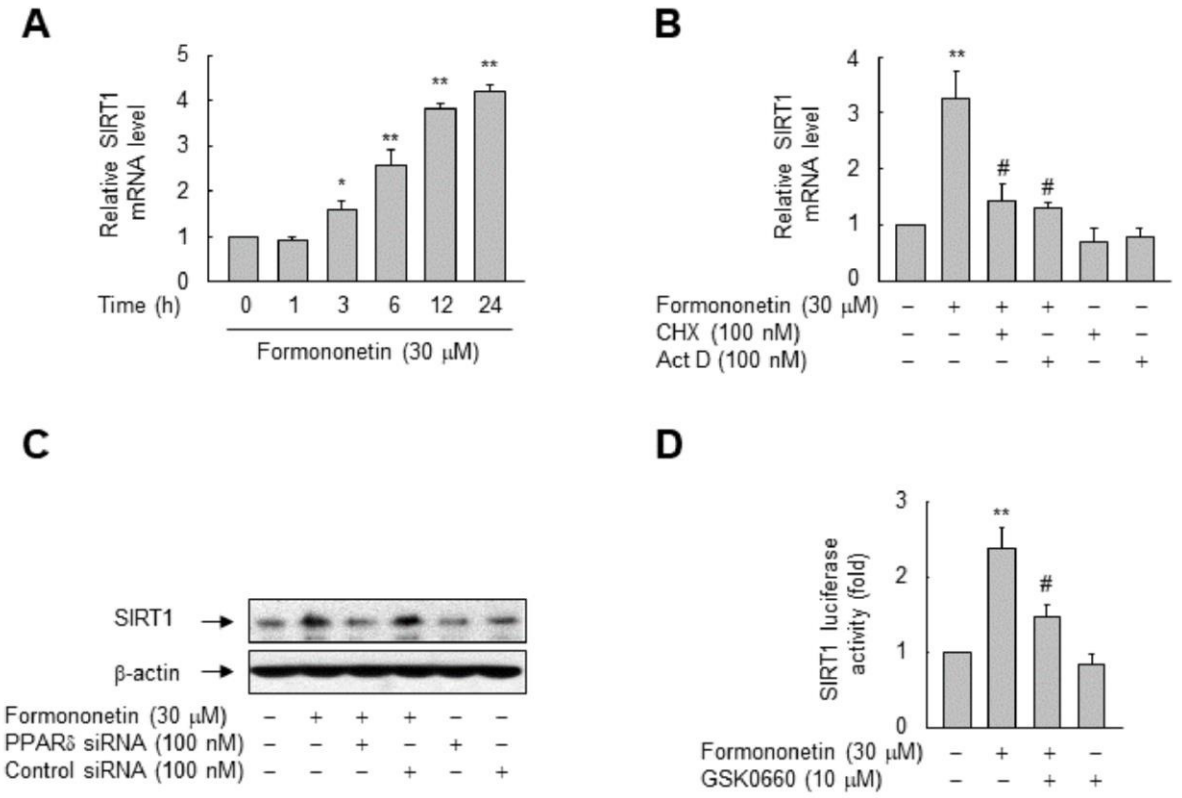


Figure 4 Involvement of PPARδ in formononetin-mediated upregulation of SIRT1 in RAW264.7 cells. (A and B) Cells cultured in serum-free medium for 16 h were stimulated with formononetin for the indicated durations (A) or incubated with CHX or Act D in the presence or absence of formononetin (B). After incubation for 24 h, total RNA was isolated and the levels of SIRT1 mRNA were analyzed by real-time PCR. The results are expressed as the means ± SE (n=3). (C) Cells transfected with PPARδ-targeting or control siRNA for 38 h were stimulated with formononetin for 24 h. Aliquots of whole-cell lysates were analyzed by immunoblotting. (D) Cells transfected with 1 μg of the SIRT1 luciferase reporter plasmid and 0.5 μg of pSV β-Gal for 38 h were pretreated with GSK0660 for 30 min and then exposed to formononetin for 24 h. Luciferase activity was normalized to β-galactosidase activity. The results are expressed as the means ± SE (n=3). $^{*}p<0.05$, $^{**}p<0.01$ compared with the untreated group; $^{\#}p<0.05$ compared with the formononetin-treated group.

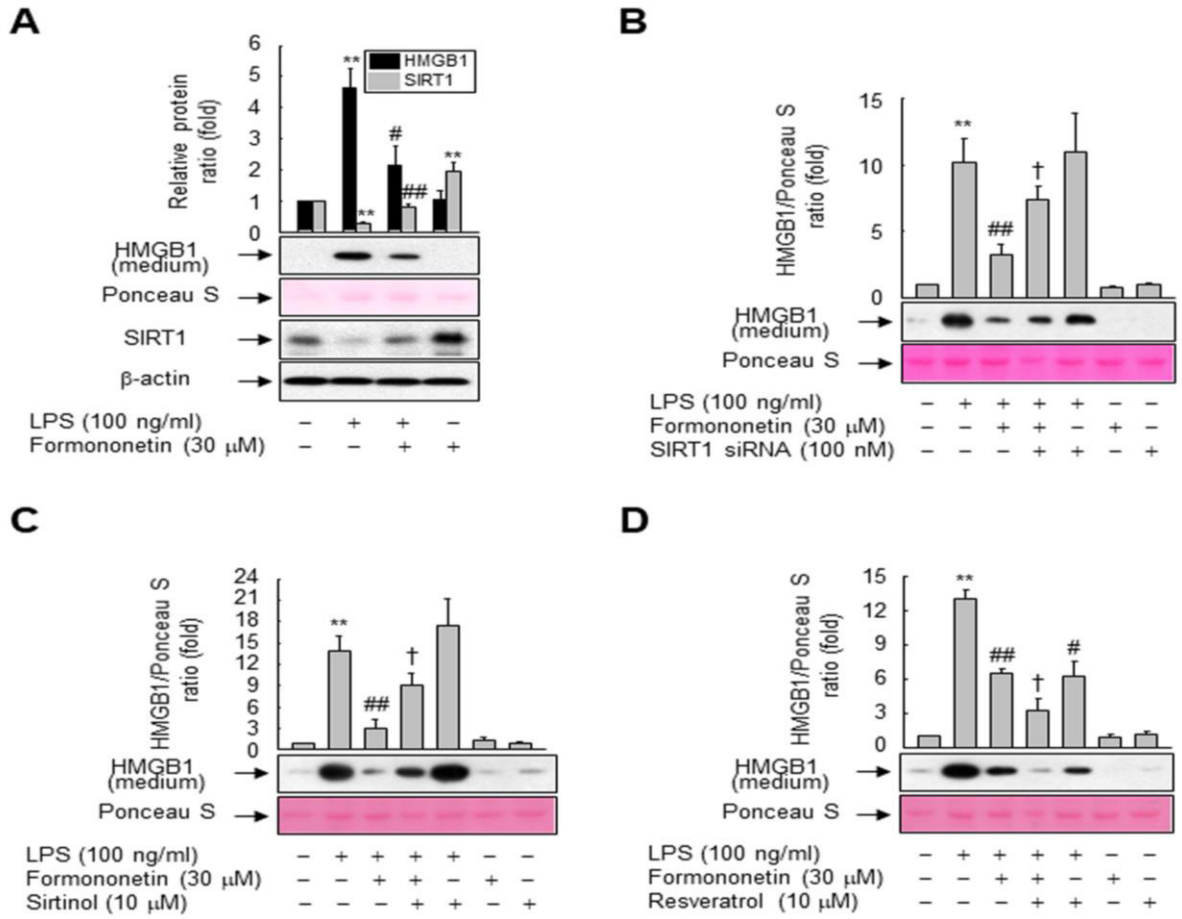


Figure 5 Involvement of SIRT1 in the formononetin-mediated inhibition of LPS-induced HMGB1 release. (A) RAW264.7 cells cultured in serum-free medium for 16 h were stimulated with LPS in the presence or absence of formononetin for 24 h. (B) Cells transfected with or without SIRT1-targeting siRNA for 38 h were exposed to LPS in the presence or absence of formononetin for 24 h. (C and D) Cells pretreated with sirtinol (C) or resveratrol (D) for 30 min were stimulated with LPS in the presence or absence of formononetin for 24 h. Aliquots of whole-cell lysates or equal volumes of conditioned media were analyzed by immunoblotting with the indicated antibodies. Ponceau S staining was used as a loading control. Representative blots are provided. The fold changes in the SIRT1/β-actin or HMGB1/Ponceau S ratio relative to that in the untreated group are shown as means ± SE (n=3). $^{**}p<0.01$ compared with the untreated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the LPS-treated group; $^{\dagger}p<0.05$ compared with the LPS plus formononetin-treated group.

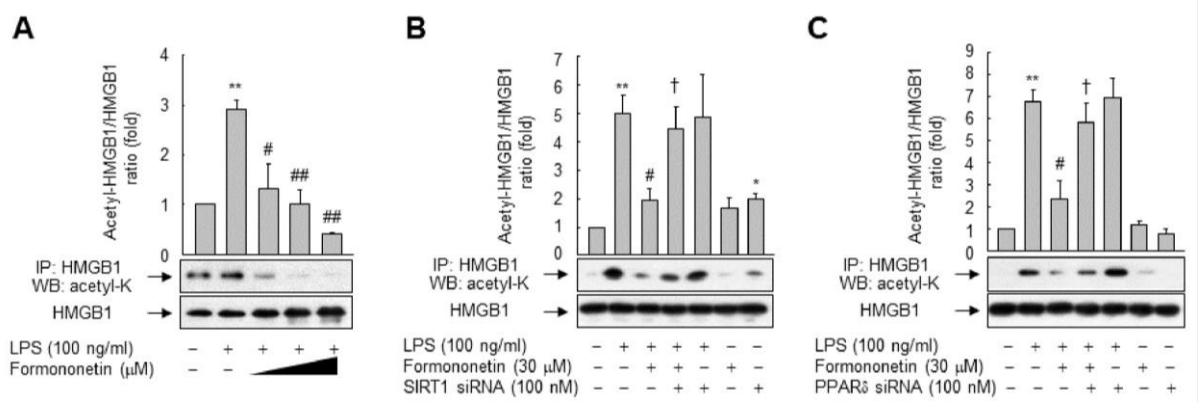


Figure 6 Effect of formononetin on LPS-induced HMGB1 acetylation. (A) RAW264.7 cells cultured in serum-free medium for 16 h were stimulated with LPS in the presence of increasing concentrations (10, 20, and 30 μM) of formononetin for 6 h. (B and C) Cells transfected with SIRT1-targeting siRNA (B) or PPARδ-targeting siRNA (C) for 38 h were exposed to LPS in the presence or absence of formononetin for 6 h. Whole-cell lysates were immunoprecipitated with an anti-HMGB1 antibody, and then acetylated HMGB1 was detected by immunoblot analysis with an anti-acetyl-lysine antibody. Representative blots are provided. The fold changes in the acetylated HMGB1/total HMGB1 ratio relative to that in the untreated group are shown as means ± SE (n=3). $^{**}p<0.01$ compared with the untreated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the LPS-treated group; $^{\dagger}p<0.05$ compared with the LPS plus formononetin-treated group.

Figure 1

Effects of formononetin on the viability of RAW264.7 cells.

(A and B) Cells cultured in serum-free medium for 16 h were treated with the indicated concentrations of formononetin for 24 h (A) or with 30 mM formononetin for the indicated durations (B). Cell viability was determined by the MTT (A) and trypan blue exclusion (B) assays. The results are plotted as the means ± SE (n=4). $^{**}p<0.01$ compared with the untreated group.

Figure 1

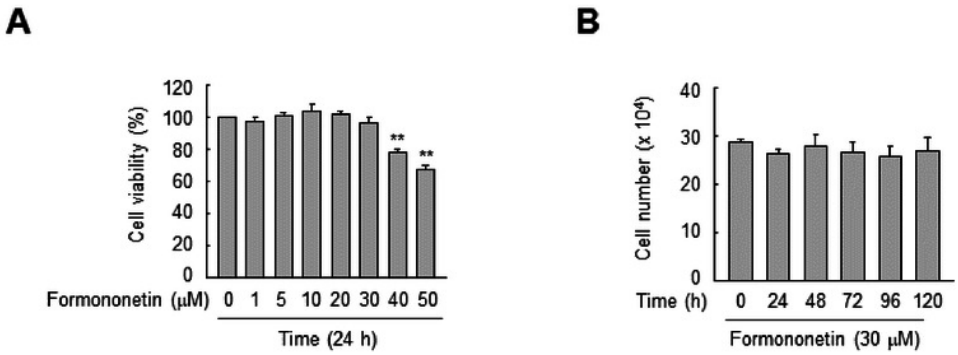


Figure 2

Effects of formononetin on the LPS-induced release and translocation of HMGB1 in RAW264.7 cells.

(A) Cells cultured in serum-free medium for 16 h were stimulated with LPS in the presence or absence of formononetin for 24 h. Equal volumes of conditioned media or aliquots of whole-cell lysates were analyzed by immunoblotting. Ponceau S staining and β-actin were used as the loading controls. (B) Cells treated with LPS in the presence or absence of formononetin for 24 h were fractionated into nuclear (N) and cytosolic (C) fractions. The localization of HMGB1 was determined by Western blot analysis with the indicated antibodies.

Figure 2

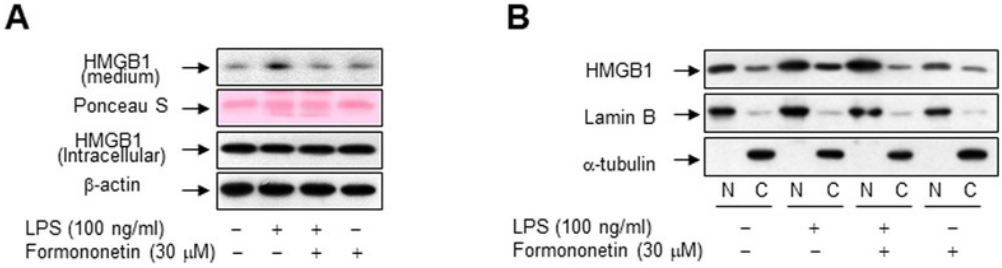


Figure 3

Effects of formononetin on expression of SIRT1 in RAW264.7 cells.

(A and B) Cells cultured in serum-free medium for 16 h were incubated with various concentrations of formononetin for 24 h (A) or with 30 mM formononetin for the indicated durations (B). Aliquots of whole-cell lysates were analyzed by immunoblotting. Representative blots are provided. Fold changes in the SIRT1/β-actin ratio relative to that in the untreated group are shown as means ± SE (n=3). $^{*}p<0.05$, $^{**}p<0.01$ compared with the untreated group.

Figure 3

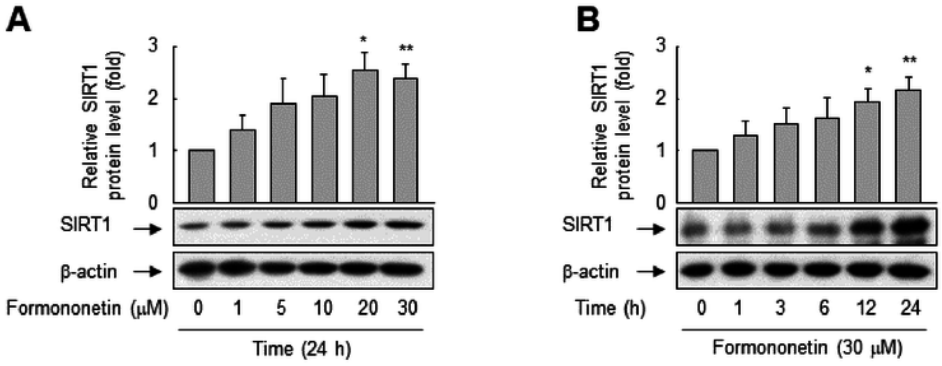


Figure 4

Involvement of PPARd in formononetin-mediated upregulation of SIRT1 in RAW264.7 cells.

(A and B) Cells cultured in serum-free medium for 16 h were stimulated with formononetin for the indicated durations (A) or incubated with CHX or Act D in the presence or absence of formononetin (B). After incubation for 24 h, total RNA was isolated and the levels of SIRT1 mRNA were analyzed by real-time PCR. The results are expressed as the means ± SE (n=3). (C) Cells transfected with PPARδ-targeting or control siRNA for 38 h were stimulated with formononetin for 24 h. Aliquots of whole-cell lysates were analyzed by immunoblotting. (D) Cells transfected with 1 μg of the SIRT1 luciferase reporter plasmid and 0.5 μg of pSV β-Gal for 38 h were pretreated with GSK0660 for 30 min and then exposed to formononetin for 24 h. Luciferase activity was normalized to β-galactosidase activity. The results are expressed as the means ± SE (n=3). $^{*}p<0.05$, $^{**}p<0.01$ compared with the untreated group; $^{\#}p<0.05$ compared with the formononetin-treated group.

Figure 4

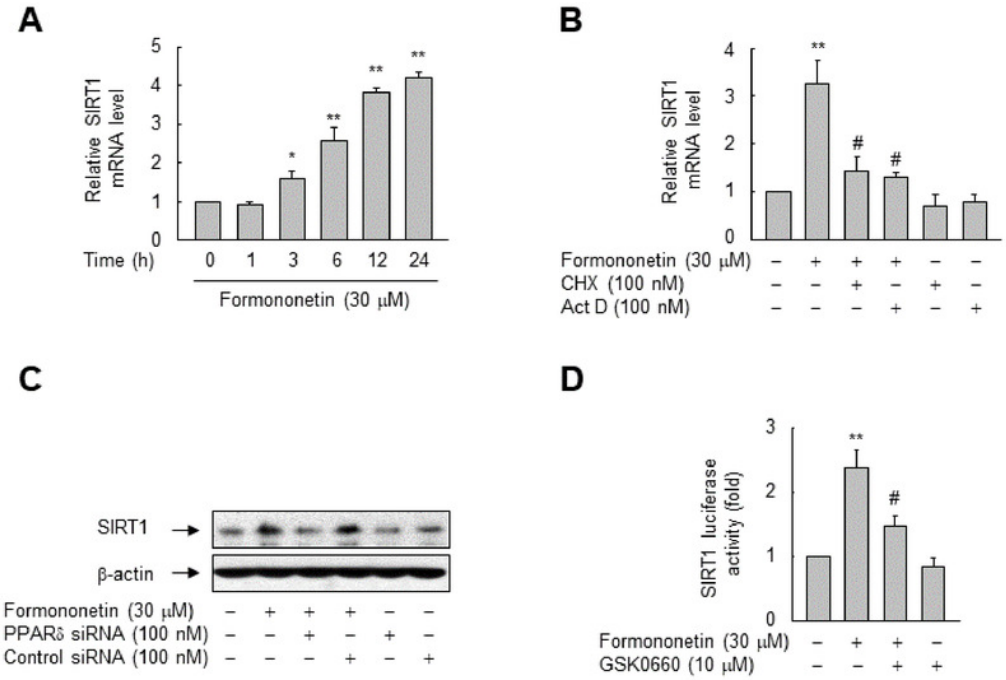

A
Relative SIRT1 mRNA level
Time (h)
0 1 3 6 12 24
Formononetin (30 μM)
B
Relative SIRT1 mRNA level
Formononetin (30 μM)
CHX (100 nM)
Act D (100 nM)
C
SIRT1
β-actin
Formononetin (30 μM)
PPARδ siRNA (100 nM)
Control siRNA (100 nM)
D
SIRT1 luciferase activity (fold)
Formononetin (30 μM)
GSK0660 (10 μM)

Figure 5

Involvement of SIRT1 in the formononetin-mediated inhibition of LPS-induced HMGB1 release.

(A) RAW264.7 cells cultured in serum-free medium for 16 h were stimulated with LPS in the presence or absence of formononetin for 24 h. (B) Cells transfected with or without SIRT1-targeting siRNA for 38 h were exposed to LPS in the presence or absence of formononetin for 24 h. (C and D) Cells pretreated with sirtinol (C) or resveratrol (D) for 30 min were stimulated with LPS in the presence or absence of formononetin for 24 h. Aliquots of whole-cell lysates or equal volumes of conditioned media were analyzed by immunoblotting with the indicated antibodies. Ponceau S staining was used as a loading control. Representative blots are provided. The fold changes in the SIRT1/β-actin or HMGB1/Ponceau S ratio relative to that in the untreated group are shown as means ± SE (n=3). $^{**}p<0.01$ compared with the untreated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the LPS-treated group; $^{\dagger}p<0.05$ compared with the LPS plus formononetin-treated group.

Figure 5

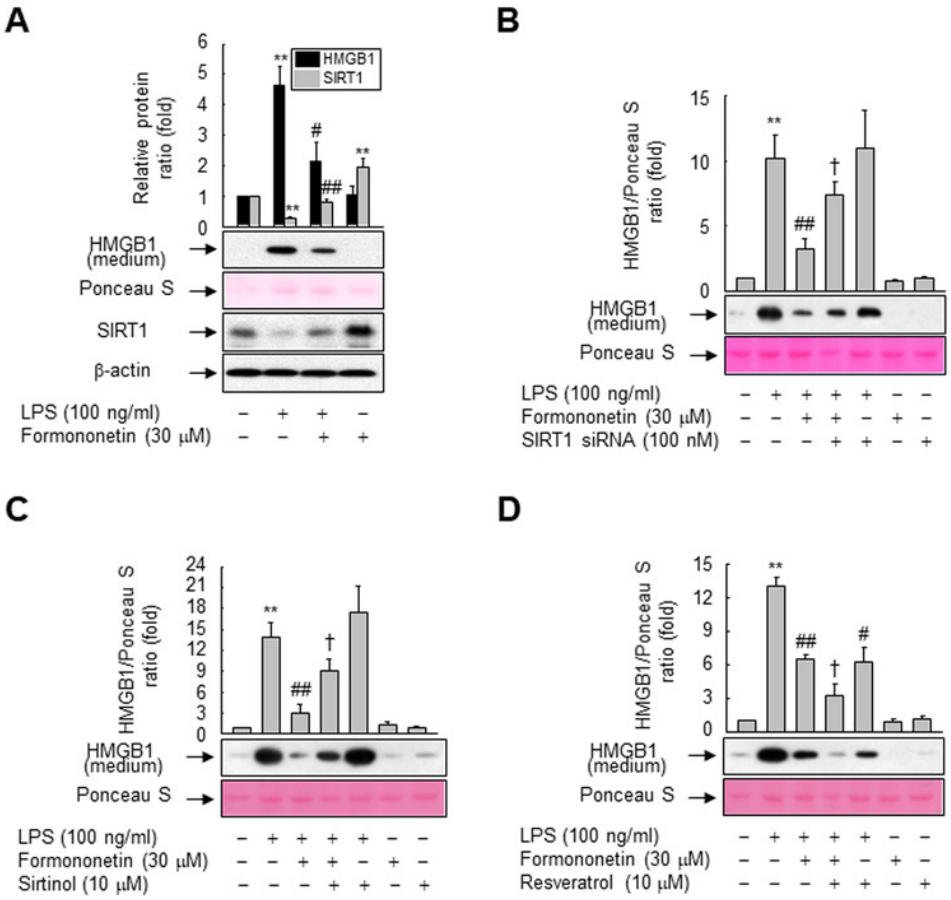
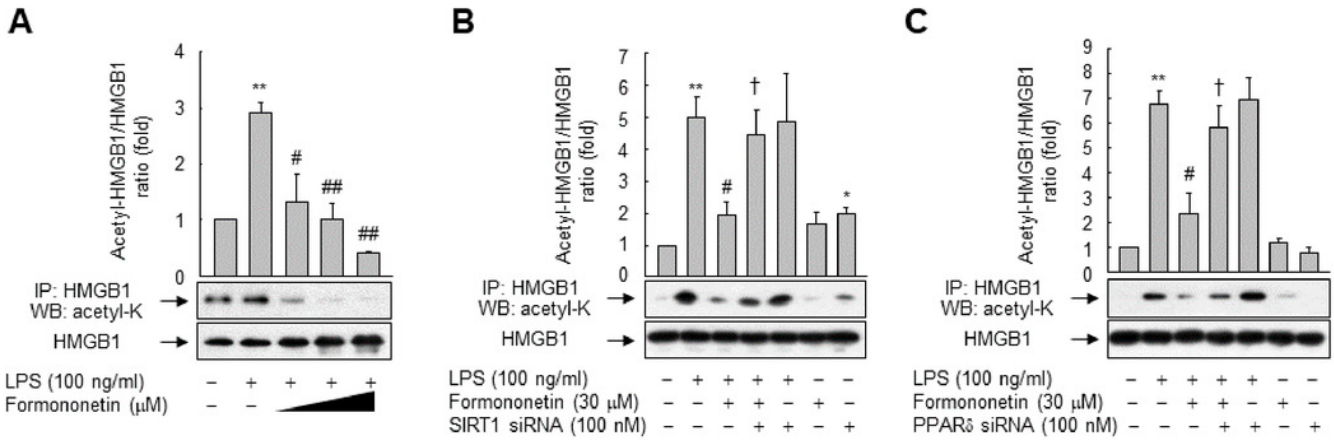

A
Relative protein ratio (fold)
HMGB1
SIRT1
HMGB1 (medium)
Ponceau S
SIRT1
β-actin
LPS (100 ng/ml)
Formononetin (30 μM)
B
HMGB1/Ponceau S ratio (fold)
HMGB1 (medium)
Ponceau S
LPS (100 ng/ml)
Formononetin (30 μM)
SIRT1 siRNA (100 nM)
C
HMGB1/Ponceau S ratio (fold)
HMGB1 (medium)
Ponceau S
LPS (100 ng/ml)
Formononetin (30 μM)
Sirtinol (10 μM)
D
HMGB1/Ponceau S ratio (fold)
HMGB1 (medium)
Ponceau S
LPS (100 ng/ml)
Formononetin (30 μM)
Resveratrol (10 μM)

Figure 6

Effect of formononetin on LPS-induced HMGB1 acetylation.

(A) RAW264.7 cells cultured in serum-free medium for 16 h were stimulated with LPS in the presence of increasing concentrations (10, 20, and 30 mM) of formononetin for 6 h. (B and C) Cells transfected with SIRT1-targeting siRNA (B) or PPARδ-targeting siRNA (C) for 38 h were exposed to LPS in the presence or absence of formononetin for 6 h. Whole-cell lysates were immunoprecipitated with an anti-HMGB1 antibody, and then acetylated HMGB1 was detected by immunoblot analysis with an anti-acetyl-lysine antibody. Representative blots are provided. The fold changes in the acetylated HMGB1/total HMGB1 ratio relative to that in the untreated group are shown as means ± SE (n=3). $^{**}p<0.01$ compared with the untreated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the LPS-treated group; $^{\dagger}p<0.05$ compared with the LPS plus formononetin-treated group.

Figure 6


A
Acetyl-HMGB1/HMGB1 ratio (fold)
IP: HMGB1
WB: acetyl-K
HMGB1
LPS (100 ng/ml)
Formononetin (μM)
B
Acetyl-HMGB1/HMGB1 ratio (fold)
IP: HMGB1
WB: acetyl-K
HMGB1
LPS (100 ng/ml)
Formononetin (30 μM)
SIRT1 siRNA (100 nM)
C
Acetyl-HMGB1/HMGB1 ratio (fold)
IP: HMGB1
WB: acetyl-K
HMGB1
LPS (100 ng/ml)
Formononetin (30 μM)
PPARδ siRNA (100 nM)