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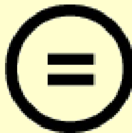

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Doctoral Dissertation

The effects of metformin on the development of endotoxin tolerance in macrophages

Department of Biomedical Sciences
Graduate School, Chonnam National University

Mei Yin

February 2023

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Department of Biomedical Science
Graduate School, Chonnam National University

Mei Yin

Supervised by Professor Bae, Hong-Beom

A dissertation submitted in partial fulfillment of the requirements Doctor of Philosophy in Medicine

Committee in Charge:

Lee, Jae-Hyuk	____________________
Kwak, Sang Hyun	____________________
Park, In-Kyu	____________________
Jo, Jihoon	____________________
Bae, Hong-Beom	____________________

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The effects of metformin on the development of endotoxin tolerance in macrophages

Mei Yin

Department of Biomedical Science
Graduate School, Chonnam National University
(Supervised by Professor Bae, Hong-Beom, M.D., Ph.D.)

Abstract

Background: It was reported that AMP-activated protein kinase (AMPK) is involved in the suppression of the development of endotoxin tolerance, one of the causes of immunosuppression induced by sepsis. However, the mechanism by which AMPK inhibits the development of endotoxin tolerance has not been clearly elucidated. In the present study, the author investigated the mechanisms by which metformin, an AMPK activator, is involved in the inhibition of development of endotoxin tolerance.

Methods: Bone marrow-derived macrophages and Raw 264.7 cells were used *in vitro* experiments. To develop endotoxin tolerance, the cells were stimulated with an initial lipopolysaccharide (LPS) (100 ng/ml) for 24 h, washed, and incubated with fresh media for 2 h, followed by a 2nd LPS challenge for 4 h. 7 weeks old Male C57BL/6 mice were received cecal ligation and puncture (CLP) for *in vivo* test. After 72 hours of CLP, the splenocytes were harvested and stimulated with LPS for 4 h. The expression of TGF-β1, TNF-α and IL-6 were measured by ELISA or western blot.

Results: LPS increased the production of TGF-β1 in bone marrow-derived macrophages.

Metformin and resveratrol, AMPK activators, inhibited the production of TGF-β1 induced by LPS. However, knock-down of AMPKα1 in Raw 264.7 cells inhibited the suppressive effect of metformin on LPS-induced TGF-β1 production. Furthermore, addition of recombinant TGF-β1 to culture media decreased the production of LPS-induced TNF-α and IL-6, which was reversed by metformin. The phosphorylation of Smad2, a downstream protein in TGF-β, was increased by LPS, which was inhibited in cell treated with TGF-β neutralizing antibody and metformin. Knock-down of Smad2 inhibited the development of endotoxin tolerance, evidenced by an increase in 2nd LPS-induced TNF-α production. Splenocytes from CLP mice increased the expression of TGF-β1 and decreased the production of LPS-induced TNF-α and IL-6. However, treatment of metformin or TGF-β neutralizing antibody suppressed the decrease in LPS-induced TNF-α and IL-6 production in splenocytes of CLP mice.

Conclusion: These results indicate that AMPK activation inhibits the LPS-induced production of TGF-β1, which is involved in the suppression of the development of endotoxin tolerance in macrophages.

Introduction

Sepsis-3 defined sepsis as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. The dysregulated host response includes not only excessive inflammation, but also immunosuppression [2]. Moreover, both hyper-inflammation and immune-paralysis occur simultaneously from the onset of sepsis [3]. Improvement of antibiotics administration, fluid resuscitation, and multiple organ support therapies decreased mortality in the initial phase of sepsis but long-term mortality is still high [4, 5], which is due to later state of prolonged immune suppression [6]. Endotoxin tolerance is one the most important mechanisms of immune suppression [7].

Macrophage endotoxin tolerance is a condition often encountered in sepsis, in which macrophages pre-exposed to lipopolysaccharide (LPS) reduce the expression of inflammatory cytokines response to subsequent LPS stimulation, which is well known as one of the major mechanisms contributing to the development of immunosuppression caused by sepsis [8, 9]. Prolonged immunosuppression during sepsis reduces the ability to eradicate infectious pathogens and contributes to increased secondary infections and long-term morbidity and mortality [10]. Tolerized cells such as monocytes and macrophages exhibit an altered immune reaction to endotoxin challenges compared to non-tolerized cells, characterized by a shift towards reduced pro-inflammatory cytokines (TNFα, IL-6, IL-1β) and increased expression of anti-inflammatory cytokines (IL-10, TGF-β) [11-13]. Recent studies have shown that the expression of inflammatory cytokines such as TNFα and IL-6 is significantly reduced after LPS re-stimulation compared to single LPS stimulation in humans and mice [14].

The transforming growth factor-beta (TGF-β) is a pleiotropic cytokine that includes three isoforms (TGF-β1, TGF-β2 and TGF-β3) in mammals and plays pivotal roles in regulating cell

proliferation and differentiation, inflammation, angiogenesis, and tissue repair in various cell types [15]. Of these, TGF-β1, the most relevant member of immune regulation in mammals, binds to TGF-β receptors (TGFBR)2 followed by the recruitment and subsequent phosphorylation of TGFBR1, which induces downstream protein Smad2 and Smad3 phosphorylation. The phosphorylated Smad2/3 recruit and translocate Smad4 to nucleus for regulating transcription of target genes. TGF-β can also activate other signaling cascades through Smad-independent pathways, including extracellular-signal-regulated kinase, c-Jun-N-terminal kinase, TGF-β-activated kinase 1 and p38 mitogen-activated protein kinase pathways [16]. Although the effect of TGF-β1 is known to be highly context-dependent [17], it has been reported that TGF-β1 down-regulates LPS-induced inflammatory cytokine production in macrophage and monocyte [18]. TGF-β1 increased expression of Src homology 2 (SH2) domain-containing inositol-5-phosphatase 1 (SHIP1) and IL-1 receptor associated kinase-M (IRAK-M) in monocytic cell line through a Smad4-dependent pathway [19], and endotoxin tolerance was closely associated with increase in expression of negative regulators such as IRAK-M, suppressor of cytokine signaling 1 (SOCS1) and SHIP1, and lack of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) activation [20].

Adenosine monophosphate-activated protein kinase (AMPK), a well-known cellular sensor of energy, is a heterotrimeric complex consisting of a catalytic α-subunit and two regulatory β and γ subunits [21]. Under energy-depleted conditions or stressful circumstances including hypoxia, ischemia, heat shock, and glucose denudation, AMPK switches on catabolic pathways and turns off ATP-consuming processes to maintain energy homeostasis [22]. Besides acting as a sensor of energy, a growing number of studies are demonstrating that AMPK may play a beneficial role in conditions such as inflammation and bacterial infections. For instance, metformin suppressed LPS-induced inflammation of mouse colon smooth muscle cells by

down-regulating the secretion of TNF-α, IL-1α and phosphorylation nuclear factor-κB [23]. Berberine and 5-aminoimidazole-4-carbox-amide-1-D-ribofuranoside (AICAR), another AMPK activators, inhibited the release of TNF-α and IL-6 in LPS-stimulated bone marrow neutrophils, as well as in bronchoalveolar lavage fluid of mice lung injured by LPS [24]. Previous studies have reported that stearoyl lysophosphatidylcholine increased Ca^{2+}/CaM-dependent protein kinase kinase β (CaMKKβ)/AMPK activity, thereby inhibiting the secretion of high mobility group box 1 (HMGB1), which is known to be closely related to the severity and mortality of sepsis [25]. Moreover, it has been reported that resveratrol can inhibit the development of endotoxin tolerance by suppressing the LPS-induced expression of interleukin-1 receptor-associated kinases (IRAK)-M and Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1) through increased AMPK activity [20]. Another study also indicated AMPK activation inhibited the development of LPS-induced endotoxin tolerance by repressing the accumulation of immunosuppressive transcriptional factor hypoxia-inducible factor-1α (HIF-1α) [26].

Increasing evidence has shown that AMPK inhibits the TGF-β signaling pathway in multiple cell populations, including cancer cells [27], peritoneal mesothelial cells [28], and airway smooth muscle cells [29]. However, it is not clear whether inhibition of the TGF-β signaling pathway by AMPK is associated with suppression of the development of endotoxin tolerance. Hence, in this study, I investigated the effect of AMPK on LPS-induced TGF-β1 production and TGF-β1 signaling pathway and whether inhibition of the TGF-β1 pathway by AMPK is involved in suppressing the development of endotoxin tolerance.

Materials and Methods

Mice

Male C57BL/6 mice (7 weeks old, 20-25 g weight) were purchased from Samtako (Daejeon, Korea). The mice were housed in cages within controlled temperature and light, and allowed free access to food and water. All experimental procedures mentioned in this study were approved by the Animal Care and Ethics Committee of Chonnam National University Medical School (CNU IACUC-H-2022-9).

Reagents and antibodies

Metformin and LPS (*E.coli* O55:B5) were purchased from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin (BSA) was obtained from BioShop (Burlington, ON, Canada), and Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were purchased from WELGENE (Gyeongsan, Korea). Antibodies for specific proteins including p-Smad2, Smad2 and AMPKα1 were supplied from Cell Signaling Technology (Beverly, MA, USA). Antibody specific for β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemicals including compound C and SB431542 were obtained from Selleck Chemicals (Houston, TX, USA). Antibody for TGF-β, recombinant mouse TGF-β1 and TGF-β neutralizing antibody were available from R&D systems (Minneapolis, MN, USA). Shigatoxin1 (13C4) (Santa Cruz, CA, USA) was used as IgG control for TGF-β neutralizing antibody.

Cell culture

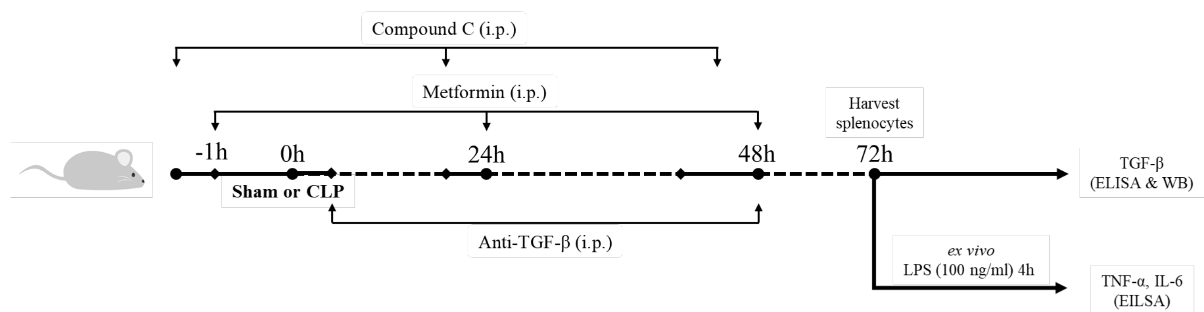
Bone marrow-derived macrophages (BMDM) were obtained as previously described [30]. Briefly, bone marrow was harvested from mice femurs and tibias by flushing with cold DMEM media.

Red cells were removed with red blood cell (RBC) lysis buffer and the remaining cells were then cultured in a petri dish with complete growth media containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% L929 cell culture supernatant as a source of granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce hematopoietic cell differentiation into macrophages. On the 5th day, the culture media were replaced with fresh complete growth media, then on the 7th day, fully differentiated macrophages were harvested. The percentage of $CD11b^+$ $F4/80^+$ macrophages measured by flow cytometry is more than 95%. RAW 264.7 cell was purchased from ATCC. The cells were cultured in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in 5% CO_2. The development of endotoxin tolerance in macrophages was performed as previously described [20]. Briefly, to induce endotoxin tolerance in macrophages, the cells were cultured in LPS (100 ng/ml; 1st LPS) for 24 h, washed twice, and then maintained in DMEM medium for 2 h before being treated with a second dose of LPS (100 ng/ml; 2nd LPS). To induce macrophage activation with LPS, the cells were treated with LPS (100 ng/ml; 2nd LPS) only once and cultured for 4 h.

Cecal ligation and puncture (CLP) model and drugs treatment

Cecal ligation and puncture was performed as previously described [31]. Briefly, the mice were anesthetized with sevoflurane. Then, a midline laparotomy was performed, and the cecum was exteriorized and ligated at a 1 cm site from the distal tip of the cecum. The ligated portion of the cecum was perforated with two holes using a 21-gauge needle and squeezed gently to extrude a small amount of fecal content from the punctured cecum. The cecum was then returned to abdominal cavity and the incision was closed using two layers of sutures. In the sham group, animals were anesthetized and the cecum was exteriorized without ligation or puncture. According to each experiment design, mice were injected intraperitoneally (i.p.) with

TGF-β neutralizing antibody or IgG control at a dose of 2 mg/kg at 30 min, 48 h after CLP. Metformin (50 mg/kg) was injected i.p. 1 h before CLP and 24 and 48 h after CLP. Compound C (5 mg/kg), an AMPK inhibitor, was injected i.p. every 30 min before metformin treatment. The spleens were isolated and lysed 72 h after sham or CLP surgery, and the splenocytes suspensions were prepared using 100 μm and 40 μm nylon cell strainer, and RBCs were removed with lysis buffer. The cells were washed and re-suspended in RPMI 1640 media and plated in 12 well tissue culture plates (1.5×10^6 cells/ml). The cells were analyzed by western blots or ELISA as described in the figure legends. The scheme of drug administration and sample preparation were shown as follows.



Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-6 and TGF-β1 in cell culture media or splenocytes were measured using mouse ELISA kits purchased from R&D Systems, all procedure performed according to manufacturer's instructions.

Western blot

Cells were harvested on ice in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, , 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 2 mM EDTA) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Beverly, MA, USA). Protein lysates were centrifuged and protein concentration was determined using

Pierce™ BCA protein assay kit (Thermo Fisher SCIENTIFIC, Waltham, MA, USA). Lysates were resolved on 10%–12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Burlington, MA, USA, 0.45μm pore size) for immunoblotting. Bands were detected using Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA) and imaged with GE Healthcare Life Sciences AI600 Imager.

RNA interference

Raw 264.7 cells (2 x 10^5 cells/well) in 12-well plates were incubated and the cells were transfected using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. Cells were administered with 100 nM of siRNA duplexes (Thermo Fisher SCIENTIFIC, Waltham, MA, USA) specific for mouse AMPKα1, Smad2, or scrambled siRNA for 48 h. The transfected cells were analyzed by western blots or ELISA as described in the figure legends.

Statistical Analysis

Data are expressed as the mean ± standard deviation (S.D.). The Shapiro-Wilk test Shapiro-Wilk test was used to determine whether the data were normally distributed. Statistical significance was evaluated by a Student's *t*-test between two groups or using a one-way analysis of variance (ANOVA) with Tukey's post hoc test between more than two groups if values were normally distributed. Mann–Whitney U test or Kruskal–Wallis H-test were used for comparisons of two groups or multiple groups, respectively if values were not normally distributed. All analyses were performed using SPSS software version 21.0 (IBM, Chicago, IL, USA). A value of $P < 0.05$ was considered significant.

Results

AMPK activation inhibits the production of TGF-β1 induced by LPS.

In initial experiments, I determined whether LPS increased the production of TGF-β1 in BMDM. The cells were stimulated with LPS for different times, and then examined the production of TGF-β1 in cell lysates and culture supernatants (Fig. 1A and B). The production of TGF-β1 increased in a time-dependent manner after LPS challenge, which increased from 8 h and continued to increase until 24 h after LPS administration. The production of TGF-β1 by LPS also increased in a dose-dependent fashion (Fig. 1C and D). Previous studies have reported that AMPK negatively regulates the TGF-β-induced signaling pathway in various cell populations [32]. In this experiment, I investigated whether AMPK inhibits the production of TGF-β1 by LPS in addition to inhibition of the TGF-β1 signaling pathway in macrophages. The AMPK activator, metformin, was administered to macrophage culture media 1 h before the LPS challenge. As shown in Fig. 2A, the release of TGF-β1 from macrophages increased significantly 8 h after exposure to LPS, but inclusion of metformin in culture media significantly suppressed TGF-β1 release from cells. Metformin also inhibited LPS-induced TGF-β1 release from cells in a dose-dependent manner (Fig. 2B). Fig. 2C shows that metformin also inhibited the expression of TGF-β1 induced by LPS. Also, resveratrol, which has different AMPK activation mechanisms, inhibited the production of TGF-β1 by LPS (Fig. 2D and E). In addition, TGF-β1 protein level was increased in AMPKα1 siRNA-treated cells compared to the control siRNA-treated group after LPS challenge. Of note, the inhibitory effect of metformin on LPS-induced TGF-β1 release by Raw 264.7 cells was reduced in AMPKα1 knock-down cells (Fig. 3A and B).

AMPK activation regulates the development of endotoxin tolerance by LPS-induced TGF-β1 in macrophages

In my previous study, it was shown that AMPK activation increases the production of inflammatory cytokines by LPS re-stimulation in tolerized macrophages [20]. In this experiment, I also reproduced that metformin inhibits the development of endotoxin tolerance by long-term exposure to LPS, evidenced by the increase in TNF-α production by re-stimulation with LPS in tolerized macrophages (Fig. 4A). A previous study reported that TGF-β1 suppresses LPS-induced inflammatory cytokine production in macrophages and monocyte [18]. To examine the effect of TGF-β1 on immune suppression in macrophages, various doses of recombinant mouse TGF-β1 (rmTGF-β1) were administered to culture media before LPS challenge. As shown in Fig. 4B, rmTGF-β1 diminished the production of TNF-α in a dose-dependent manner. However, metformin increased the LPS-induced production of TNF-α and IL-6 in macrophages treated with rmTGF-β1 (Fig. 4C and D). Next, in order to investigate whether TGF-β1 released by LPS is involved in the development of endotoxin tolerance, BMDM were cultured with TGF-β neutralizing antibody or TGF-β type I receptor (SB431542). TGF-β neutralizing antibody was treated 1 h before (pre) or 4 h after (post) 1st LPS administration, or SB431542 was added to culture media 1h before 1st LPS administration. As shown in Fig. 5A-D, TNF-α and IL-6 production were significantly increased in macrophages stimulated only once with LPS (2nd LPS only), and TNF-α and IL-6 production were significantly reduced when the macrophages previously exposed to primary LPS and re-stimulated with secondary LPS (1st LPS/2nd LPS). However, the TGF-β neutralizing antibody and SB431542 increased the production of TNF-α and IL-6 in macrophages re-stimulated with LPS (1st LPS/2nd LPS). In this experimental setting, since TGF-β1 expression increased 8 h after LPS stimulation, I investigated whether TGF-β1 is involved in endotoxin tolerance that occurs earlier. As shown in Fig. 6, a tolerized state was

induced even after 6 h of LPS administration. However, inclusion of TGF-β neutralizing antibody in culture media did not inhibit the decrease in LPS-induced TNF-α production in tolerized macrophages. In the canonical TGF-β pathway, TGF-β binds to TGFBR2 and TGFBR1 is recruited to phosphorylation of the downstream protein Smad2 [16]. To determine the effect of AMPK on TGF-β pathway activation by LPS, I measured changes in phosphorylation of Smad2 by the LPS incubation time first. As shown in Fig. 7A and C, consistent with the increase in LPS-induced TGF-β1 production (Fig. 1A and B), the phosphorylation of Smad2 increased from 8 h after LPS administration. The addition of TGF-β neutralizing antibody to culture media inhibited LPS-induced Smad2 phosphorylation. Metformin also suppressed LPS-induced Smad2 phosphorylation (Fig. 7C and D). In addition, SB431542 diminished the phosphorylation of Smad2 by LPS (Fig. 7E). The effect of metformin on inhibiting LPS-induced Smad2 phosphorylation was abolished in AMPKα1 knock-down in RAW 264.7 cells (Fig. 8A and B). Furthermore, knock-down of Smad2 in RAW 264.7 cells increased the production of TNF-α by LPS re-stimulation in tolerized macrophages (Fig. 9A and B). These data indicate that AMPK inhibits LPS-induced TGF-β1 production and subsequent activation of its signaling pathway, which is involved in the suppression of endotoxin tolerance development.

AMPK activation and neutralization of TGF-β restore immune function impairment in mice subjected to CLP.

Previous studies have shown that both pro-inflammatory and anti-inflammatory responses occur at the onset of sepsis, and if sepsis persists, complications such as secondary or opportunistic infection due to immune system failure and persistent immunosuppression can lead to late patient death [33, 34]. Similarly, it has been reported that mice subjected to CLP showed

an early hyperinflammatory condition, followed by an endotoxin tolerance state 48-72 h after CLP surgery [35, 36]. To measure the effect of TGF-β1 on the immune-tolerant state that occurs in the CLP model, mice were subject to CLP, and spleens were harvested and analyzed after 72 hours. As shown in Fig. 10A and B, the production of TGF-β1 increased in splenocytes of CLP mice compared to control mice. I also found that the production of pro-inflammatory cytokines, such as TNF-α and IL-6, were reduced upon ex vivo LPS stimulation of splenocytes from CLP-treated mice. (Fig. 10C and D). Because, *in vitro* experiments, metformin-induced AMPK activation suppressed the development of endotoxin tolerance, I investigated whether metformin could improve CLP-induced impairment of immune function. Mice were subjected to CLP for 72 hours, and metformin or compound C were administered. As shown in Fig. 11A and B, TNF-α and IL-6 production decreased by splenocytes from CLP mice after *ex vivo* LPS stimulation, but metformin inhibited the decrease in production of TNF-α and IL-6 by LPS stimulation in splenocytes from CLP mice. This effect of metformin was inhibited by compound C, an inhibitor of AMPK. Of note, metformin suppressed TGF-β1 production increased by CLP, which was inhibited by compound C (Fig. 11C). In addition, a TGF-β neutralizing antibody increased the production of TNF-α and IL-6 by LPS in splenocytes from mice subjected to CLP (Fig. 11D and E).

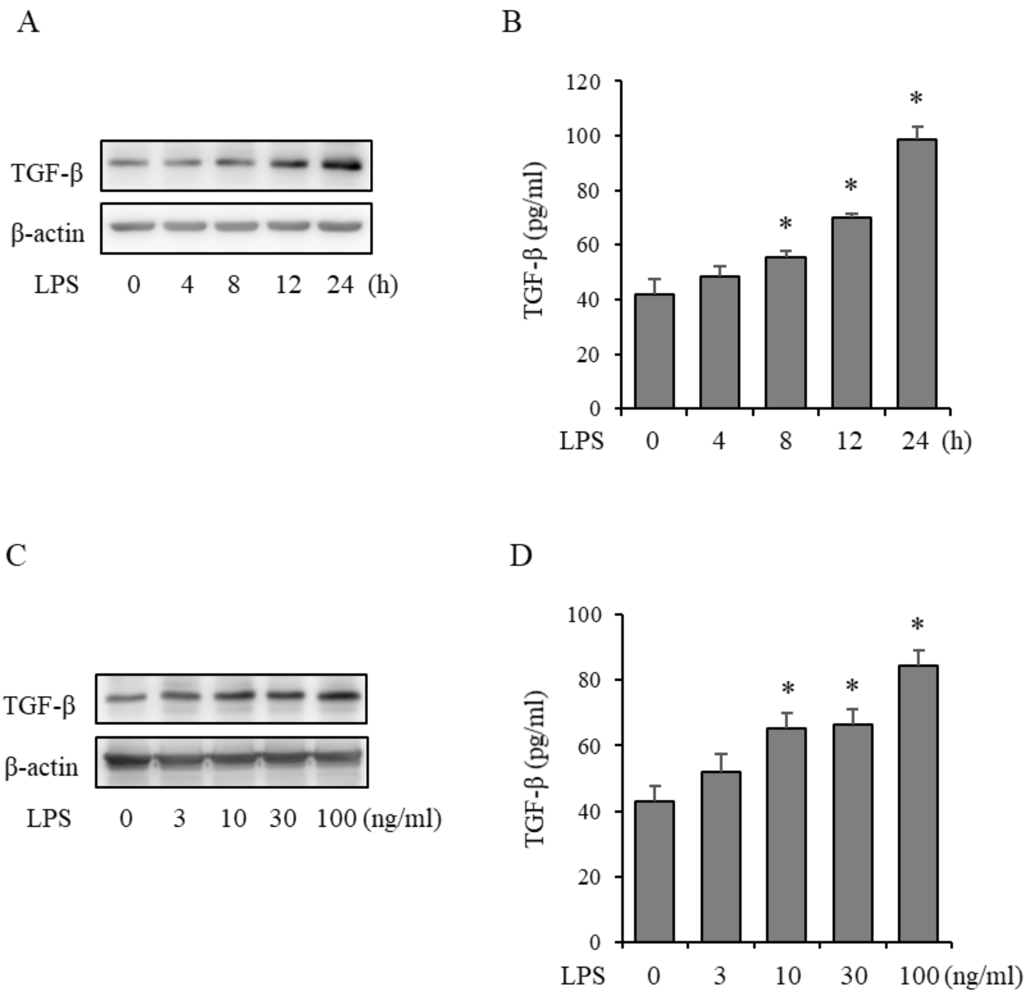


Figure 1. LPS increases the production of TGF-β1 in bone marrow-derived macrophages.

Bone marrow-derived macrophages (BMDM) were cultured with LPS (100 ng/ml) for the indicated times (0, 4, 8, 12, and 24 h) or at the indicated doses for 24 h. (A and C) The cell lysates were subjected to western blotting, (B and D) the level of TGF-β1 in cell culture media was measured by ELISA. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$ compared to control.

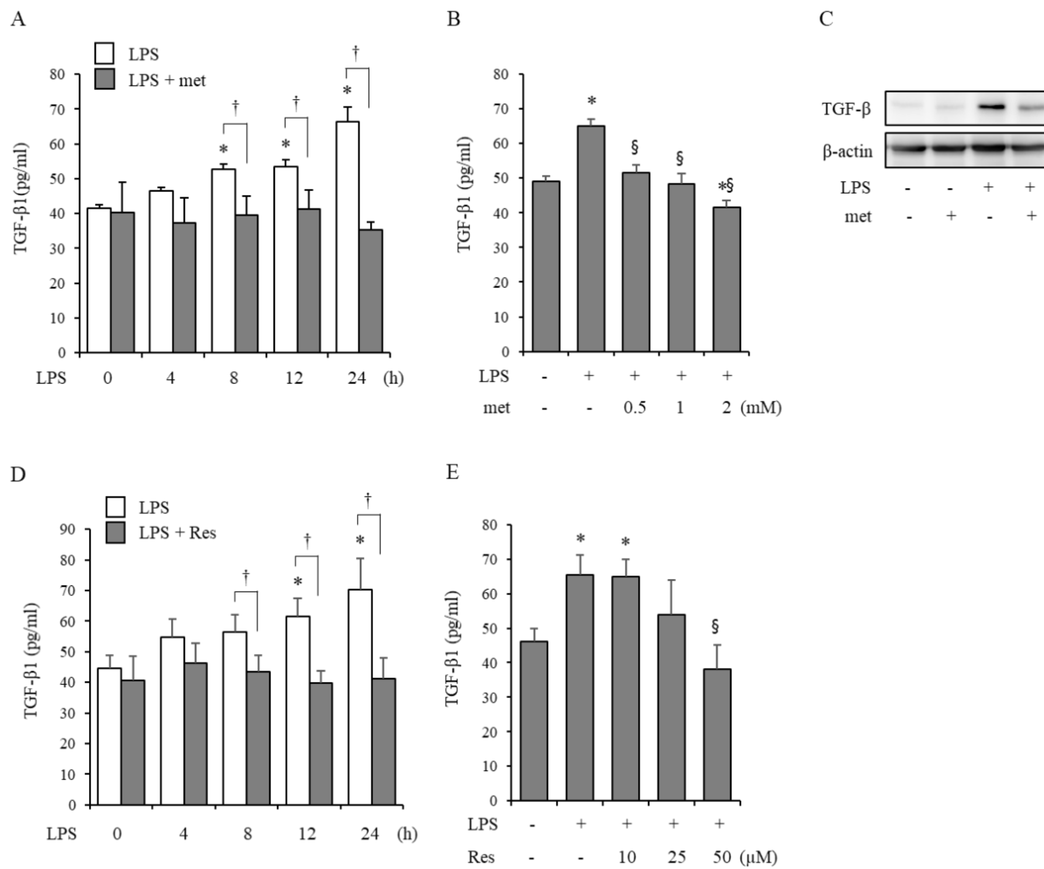


Figure 2. AMPK activation inhibits the production of TGF-β1 induced by LPS.

(A) BMDM were cultured with metformin (met; 0 or 2 mM) 1 h before LPS (100 ng/ml) treatment for the indicated times (0, 4, 8, 12, and 24 h). (B) The cells were treated with metformin (0, 0.5, 1, and 2 mM) 1h prior to LPS (100 ng/ml) exposure for 24 h. (C) BMDM were pre-treated with metformin (0 or 2 mM) 1 h prior to LPS (100 ng/ml) exposure for 24 h, then the cell lysates were collected for western blot. Representative western blots using antibodies specific for TGF-β1 and β-actin are shown. (D and E) The experimental method was the same as A and B above, and resveratrol (Res) was used in doses of 50 μM (D) or 0, 10, 25 and 50 (E). Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$ compared to the control; § $P < 0.05$ compared to LPS only group; † $P < 0.05$.

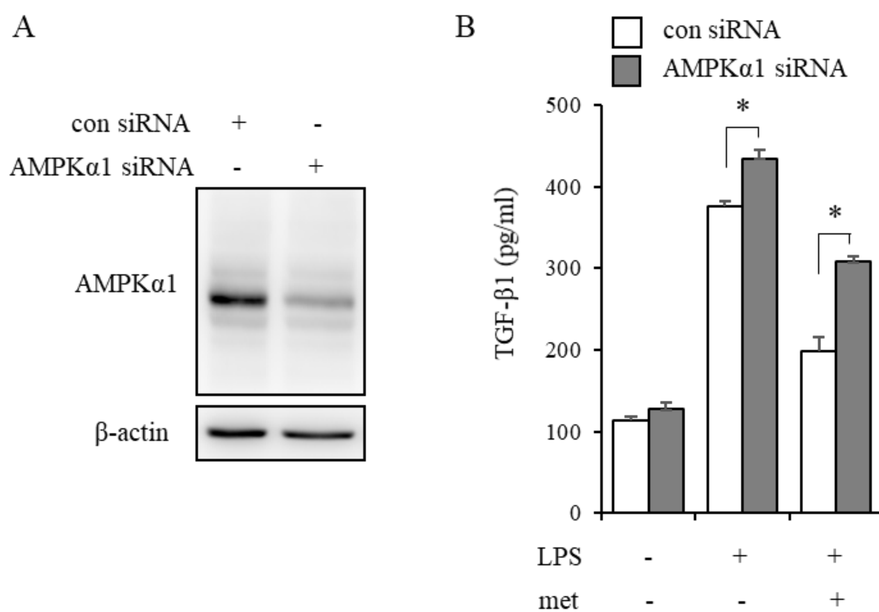


Figure 3. Knockdown of AMPK suppresses the inhibitory effect of metformin on the production of TGF-β1 by LPS.

(A) Representative western blots of Raw 264.7 cells transfected with AMPKα1 siRNA or control siRNA are shown. (B) The cells were incubated with metformin (0 or 2 mM) 1 h prior to adding LPS (0 or 100 ng/ml) for 24 h. The TGF-β1 protein level in cell culture media was measured by ELISA. * $P < 0.05$.

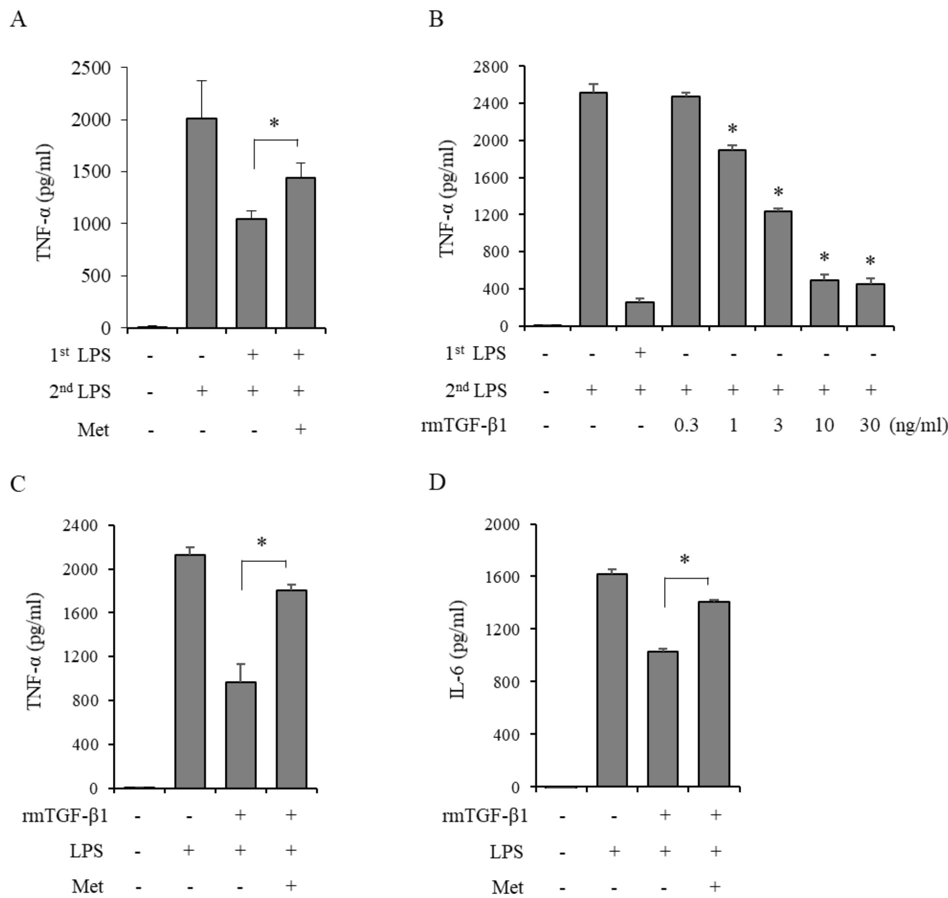


Figure 4. AMPK is involved in the development of endotoxin tolerance by LPS-induced TGF-β1 in macrophages.

(A and B) BMDM cells were exposed to 1st LPS (0 or 100 ng/ml) for 24 h, washed twice, and then incubated in culture media for 2 h, followed by the addition of 2nd LPS (0 or 100 ng/ml) to culture media for 4 h. The level of tumor necrosis factor (TNF)-α in cell culture media was measured by ELISA. Each bar represents the mean ± S.D. (n = 4). (A) Metformin (0 or 2mM) was treated 1 h before 1st LPS challenge. * $P < 0.05$ compared to 1st LPS/ 2nd LPS group. (B) Recombinant mouse TGF-β1 (rmTGF-β1) was added to serum-free culture media at indicated doses 16 h before 2nd LPS exposure. * $P < 0.05$ compared to 2nd LPS only group. (C and D) BMDM were treated with rmTGF-β1 (0 or 10 ng/ml) in serum free media for 16 h and followed by LPS (0 or 100 ng/ml) exposure for 4 h. Metformin (0 or 2 mM) was treated 1 h prior to inclusion of rmTGF-β1. The level of TNF-α and interleukin (IL)-6 in cell culture media were measured by ELISA. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$.

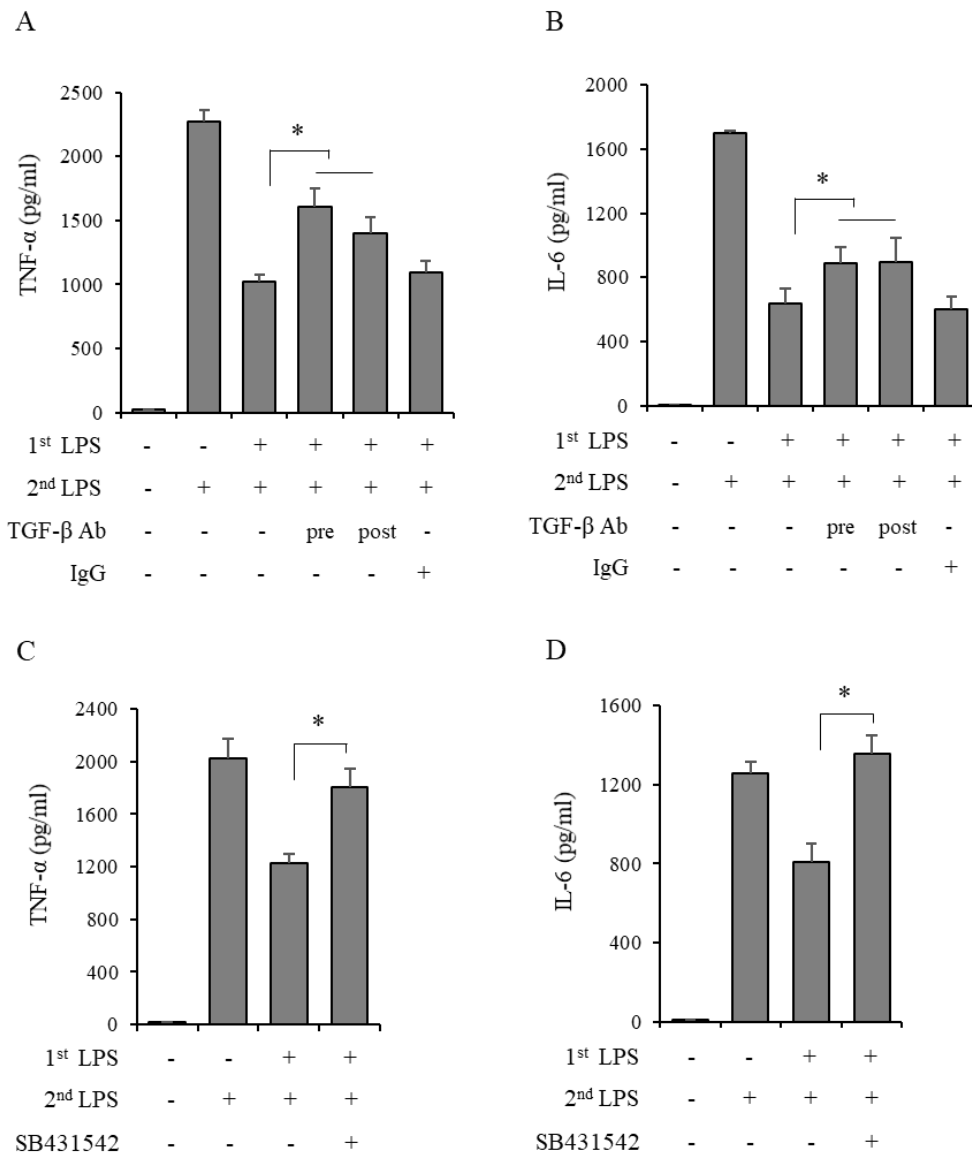


Figure 5. Inhibition of TGF-β1 ameliorates endotoxin tolerance in macrophages.

(A-D) BMDM cells were exposed to 1st LPS (0 or 100 ng/ml) for 24 h, washed twice, and then incubated in culture media for 2 h, followed by the addition of 2nd LPS (0 or 100 ng/ml) to culture media for 4 h. (A and B) TGF-β neutralizing antibody (0 or 1 μg/ml) or Control IgG were treated 1 h before (pre) or 4 h after (post) 1st LPS stimulation. (C and D) SB431542 (0 or 3 μM) were treated 1 h before 1st LPS exposure. The level of TNF-α and IL-6 in cell culture media was measured by ELISA. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$ compared to 1st LPS/ 2nd LPS group.

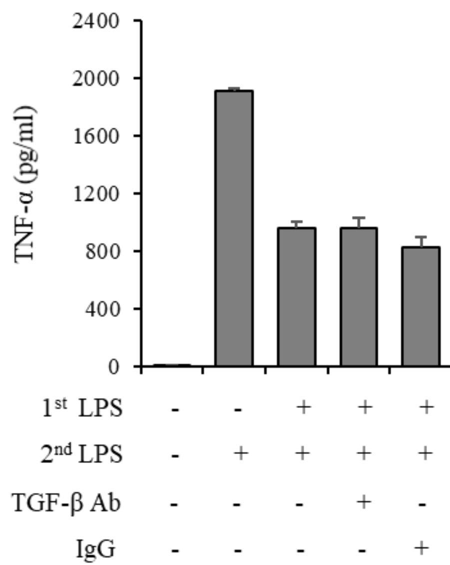


Figure 6. TGF-β1 is not involved in the early development of endotoxin tolerance.

BMDM cells were exposed with 1st LPS (0 or 100 ng/ml) for 6 h, washed twice, and then incubated in culture media for 2 h, followed by the addition of 2nd LPS (0 or 100 ng/ml) to culture media for 4 h. TGF-β neutralizing antibody (0 or 1 μg/ml) was treated 1 h before 1st LPS exposure. The level of TNF-α in cell culture media was measured by ELISA. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$.

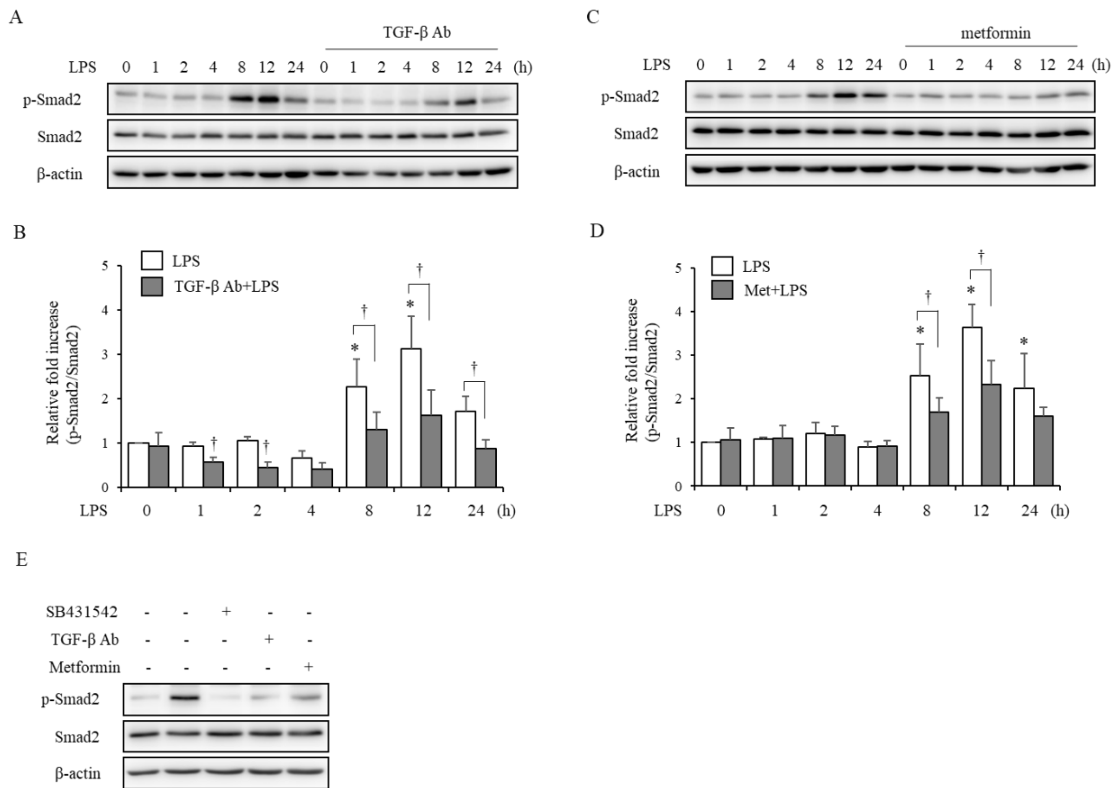


Figure 7. AMPK activation reduces LPS-induced Smad2 phosphorylation.

(A-D) BMDM were exposed to 100 ng/ml of LPS for the indicated time point (0, 1, 2, 4, 8, 12 and 24 h). TGF-β neutralizing antibody (1μg/ml) or Metformin (2 mM) were added to culture media 1 h before LPS stimulation. (A and C) The cell lysates were subjected to western blotting and representative western blots using antibodies specific for phosphor-Smad2, Smad2 and β-actin are shown. (B and D) Relative p-Smad2 protein levels were determined after normalization to Smad2. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$ compared to the control group; † $P < 0.05$. (E) BMDM cells were treated with SB431542 (3 μM) or Anti-TGF-β antibody (1 μg/ml) or metformin (2 mM) 1 h before inclusion of LPS (100 ng/ml) for 12 h. Representative western blots are shown.

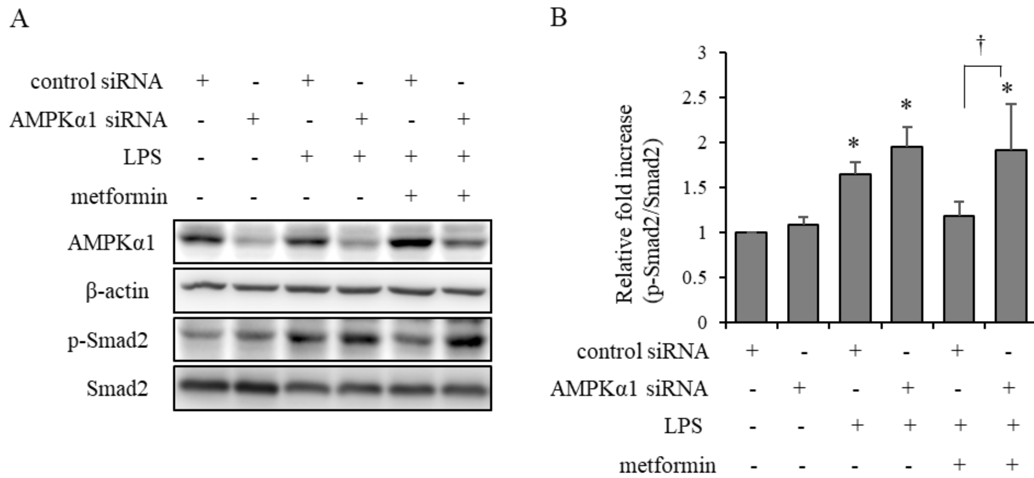


Figure 8. Knock-down of AMPK reduces metformin effect on suppression of Smad2 phosphorylation induced by LPS.

Raw264.7 cells transfected with AMPKα1 siRNA or control siRNA were treated with 2 mM of metformin for 1 h before incubation with LPS (100 ng/ml) for 24 h. (A) The cell lysates were subjected to western blotting and representative western blots using antibodies specific for AMPKα1, phosphor-Smad2, Smad2 and β-actin are shown. (B) Relative phosphor-Smad2 protein levels were determined after normalization to Smad2. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$ compared to the control group; † $P < 0.05$.

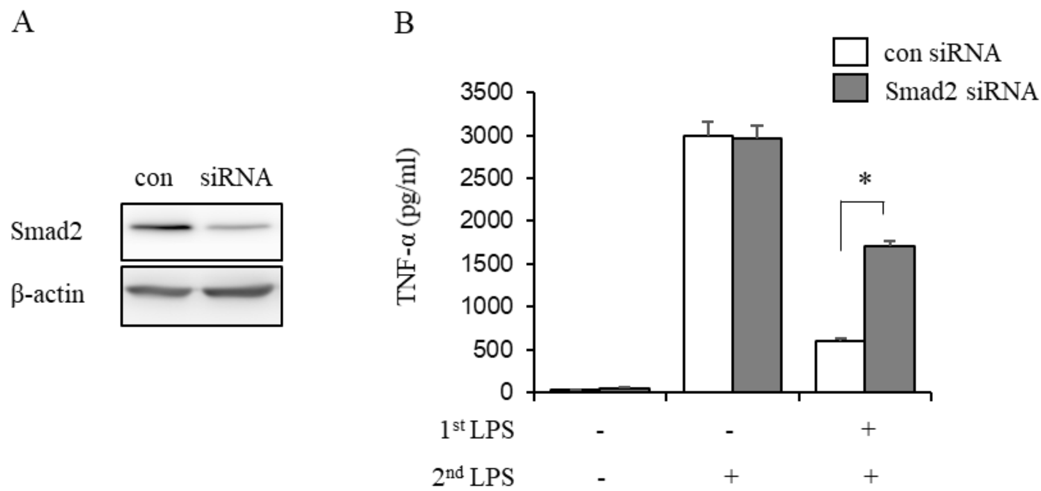


Figure 9. Knockdown of Smad2 suppresses the development of endotoxin tolerance.

(A) Raw 264.7 cells were transfected with control siRNA or siRNA specific to Smad2. The transfection effect of Smad2 siRNA was determined by western blot. Representative western blots are shown. (B) After transfection, the cells were exposed with 1st LPS (0 or 100 ng/ml) for 24 h, washed twice, and then incubated in culture media for 2 h, followed by the addition of 2nd LPS (0 or 100 ng/ml) to culture media for 4 h. The level of TNF-α in cell culture media was measured by ELISA. Each bar represents the mean ± S.D. (n = 4). * $P < 0.05$ compared to the control siRNA group.

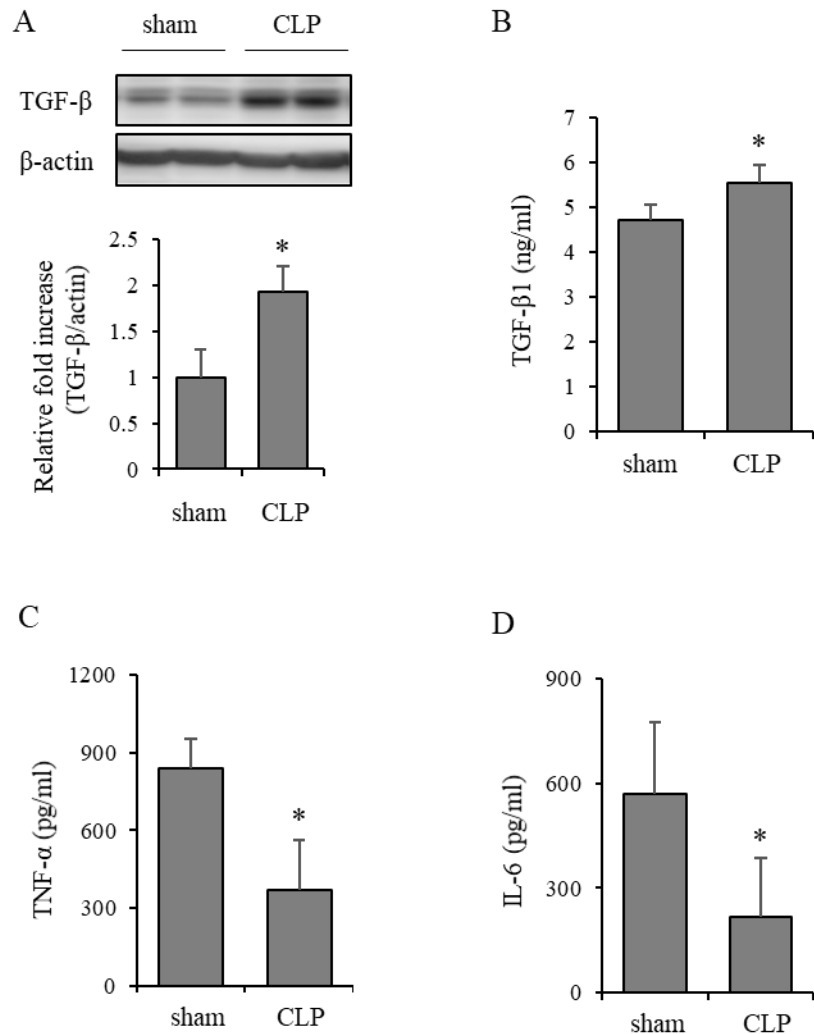


Figure 10. CLP-induced TGF-β production contributes to immune function impairment in mice subjected to CLP.

Mice were subjected to cecal ligation and puncture (CLP). The mouse spleen was collected 72 hours after CLP. (A) Representative western blots for TGF-β1 expressions in splenocytes and bars showing the relative TGF protein level to actin are shown (n = 4 mice). (B) TGF-β1 expressions in splenocytes were measured in ELISA (n=5 mice). * $P < 0.05$ compared to the sham group. (C and D) Splenocytes isolated from the mice of the sham or CLP group were stimulated with LPS (100 ng/ml) for 4 h *ex vivo* and then TNF-α and IL-6 in culture media were measured by ELISA (n=5 mice). * $P < 0.05$.

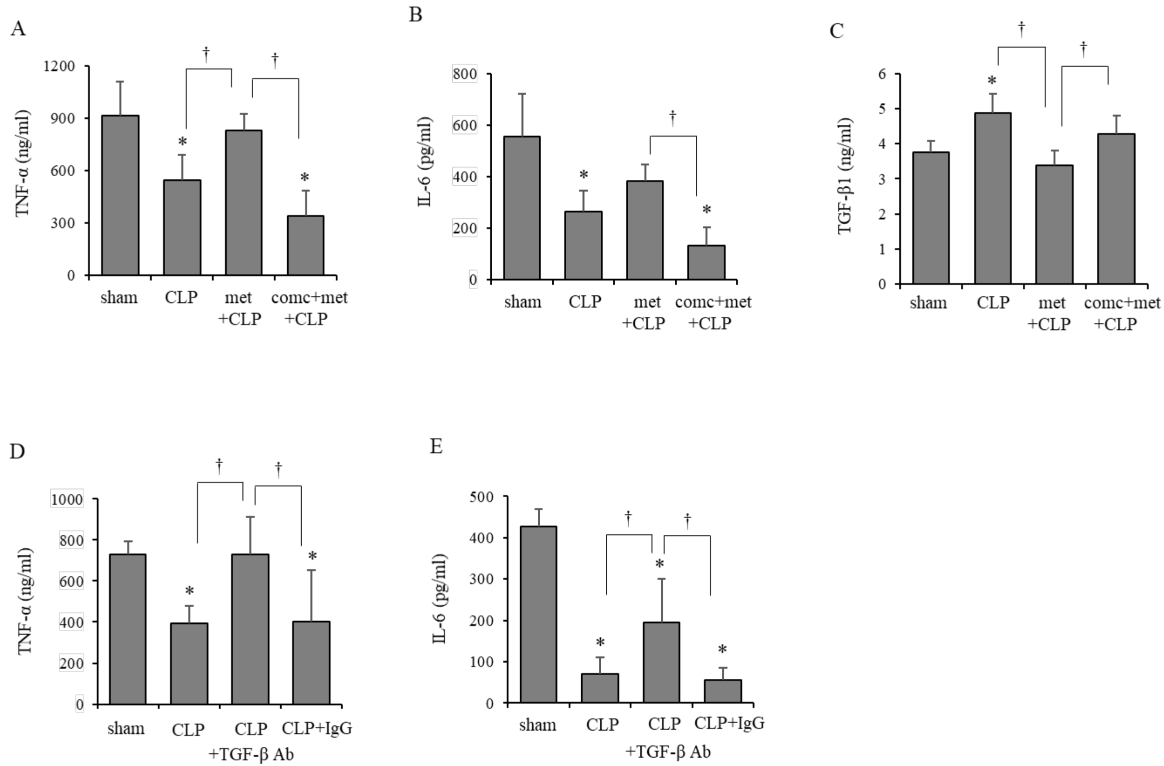


Figure 11. AMPK improves TGF-β-induced immune function impairment in mice subjected to CLP.

(A-C) Metformin (met; 0 or 50 mg/kg) was injected (i.p.) 1 h prior to CLP, and injected at 24 h intervals over a total of 3 times, compound c (comc; 0 or 5 mg/kg) was injected (i.p.) 30 min prior to each administration of metformin. (D and E) TGF-β1 neutralizing antibody or control IgG were injected (i.p.) in 30 min and 48 h after CLP. (A, B, D and E) Splenocytes isolated from the mice of each group were stimulated with LPS (100 ng/ml) for 4 h *ex vivo* and then TNF-α and IL-6 in culture media were measured by ELISA (n=5 mice). (C) TGF-β1 expressions in splenocytes from the mice of each group were measured by ELISA (n=5 mice). * $P < 0.05$ compared to sham group; † $P < 0.05$.

Discussion

This study demonstrates that LPS-induced TGF-β1 production is involved in the development of endotoxin tolerance, which is suppressed by metformin-induced AMPK activation. Prolonged exposure to LPS suppressed the inflammatory response to LPS re-stimulation by increasing the production of TGF-β1 from macrophages. Metformin suppressed the LPS-induced production of TGF-β1, and inhibited the immunosuppressive effect of TGF-β1 evidenced by the increased production of TNF-α and IL-6 by LPS re-stimulation in cells pretreated with TGF-β1 or LPS. Metformin also suppressed LPS-induced Smad2 phosphorylation in macrophages and knock-down of Smad2 inhibited the development of endotoxin tolerance. These results show a potential mechanism by which metformin modulates the development of endotoxin tolerance.

In the present study, the production of TGF-β1, which is the most relevant member of immune regulation among three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) in mammals [15], was increased by LPS exposure in a time- and dose-dependent fashion and metformin or resveratrol suppressed the increase in LPS-induced TGF-β1 production. Activation of AMPK is known to be regulated by upstream kinases such as LKB1 or CaMKKβ [22]. It has been reported that metformin increases AMPK activity through interaction with LKB1 and AMPK but resveratrol at the dose used in this experiment increases AMPK activity through interaction with CaMKKβ and AMPK [20, 37]. Although these two drugs have different mechanisms of action involved in AMPK activation, the fact that both drugs were involved in the inhibition of TGF-β1 production indicates that AMPK contributed to the inhibition of the LPS-induced TGF-β1 production in cultured macrophages. Moreover, siRNA-mediated knockdown of AMPKα1 increased LPS-induced TGF-β1 production and diminished the inhibitory effect of metformin

on LPS-induced TGF-β1 production in cultured macrophages.

A recent study showed that LPS enhanced TGF-β1 production in cultured macrophages [38] and TGF-β1 increased expression of negative regulators such as SHIP1 and IRAK-M in monocytic cell line through a Smad4-dependent pathway [19]. In the present study, TGF- β1 blunted the inflammatory response to LPS, and TGF-β1 neutralizing antibody or TGF-β1 type 1 receptor inhibitor (SB431542) suppressed the development of endotoxin tolerance. In addition, metformin inhibited the immunosuppression caused by rmTGF-β1. These experimental results indicate that metformin inhibits the development of endotoxin tolerance by inhibiting not only TGF-β1 production but also the TGF-β1 signaling pathway. Of note, a previous study has demonstrated that AMPK activation by metformin suppressed TGF-β1 signaling pathway including Smad2/3 phosphorylation, which contributed to the inhibition of cancer cell migration and epithelial-to-mesenchymal transition [27].

In the present experimental setting, LPS significantly increased the release of TGF-β1 and phosphorylation of Smad2 from 8 h after administration of LPS to the culture media, which were inhibited in cultured macrophages treated with metformin. Moreover, siRNA-induced knockdown of Smad2 suppressed the development of endotoxin tolerance, evidenced by the increase in the production of TNF-α by 2nd LPS in cultured macrophages. A previous study has shown that the onset of endotoxin tolerance is induced very rapidly after LPS administration (1 h after LPS exposure) and can persist up to 5 days after LPS exposure [39]. However, in the present experiment, the decrease in TNF-α production by 2nd LPS stimulation was suppressed even when the TGF-β neutralizing antibody was treated 4 h after 1st LPS stimulation, consistent with the result that the increase in TGF-β1 production appears about 8 h after LPS stimulation. Also, a tolerized state was induced in macrophages 6 h after LPS stimulation, consistent with

the previous study [39], but the TGF-β neutralizing antibody did not inhibit the development of endotoxin tolerance. These results suggest that TGF-β1-related induction of endotoxin tolerance is involved later rather than early tolerance induction.

In the previous study [20], I reported that AMPK activation by resveratrol suppressed the expression of negative regulators such as IRAK-M and SHIP1 in macrophages and improved the survival rate of LPS-induced tolerized mice subjected to mice. In the present study, I found that the TGF-β1 expression of splenocytes increased in mice subjected to CLP and the splenocytes isolated from CLP mice exhibited an immune impairment state after stimulation with LPS *ex vivo* conditions, evidenced by the decrease in the production of TNF-α and IL-6 by LPS stimulation compared to the splenocytes of sham mice. In particular, the administration of metformin or TGF-β neutralizing antibody inhibited CLP-induced TGF-β1 production and the diminishment of the production of TNF-α and IL-6 by LPS stimulation in splenocytes of CLP mice. These results indicate that AMPK activation suppressed the development of immune impairment in CLP mice through the inhibition of TGF-β1 expression.

In previous studies [20, 26], AMPK activation suppressed the expression of negative regulators such as IRAK-M and SHIP1 or immunosuppressive transcriptional factor HIF-1α in macrophages treated with LPS, which was associated with inhibition of the development of endotoxin tolerance and improved survival rate of LPS-induced tolerized mice subjected to mice. In this study, AMPK activation negatively regulates TGF-β1 production and its signaling pathway, by which AMPK suppressed the development of endotoxin tolerance in macrophages. In summary, AMPK activation negatively regulates TGF-β1 production and its signaling pathway, by which AMPK suppressed the development of endotoxin tolerance. These results suggest that AMPK activators may be appropriate agents in conditions where

immunosuppression occurs, such as sepsis.

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대식세포에서 내독소 내성 발생에 미치는 metformin의 영향

윤 매

전남대학교 대학원 의과학과

(지도교수 : 배 홍 범)

(초록)

배경: 과거 연구에 따르면 AMPK(AMP-activated protein kinase)는 패혈증에서 발생하는 면역 억제의 원인이 되는 내독소 내성(endotoxin tolerance) 발달 억제에 관여하는 것으로 알려져 있다. 그러나 AMPK가 내독소 내성 발생을 억제하는 기전은 자세히 알려져 있지 않으며, 본 연구에서 저자는 AMPK 활성제인 metformin이 내독소 내성 발달 억제에 관여하는 기전을 조사하고자 하였다.

방법: 골수유래대식세포 및 Raw 264.7 세포가 실험에 사용되었으며, 내독소 내성을 유도하기 위해, 세포에 내독소 (LPS)를 24 시간 배양하고 이어서 새로운 배지로 2 시간 배양한 후 두번째 LPS를 4시간 처치하고 결과를 측정하였다. 마우스에 패혈증을 유도하기 위하여 맹장 결찰 및 천자(CLP)를 시행하였고, 72시간 후, 비장 세포를 분리하여 LPS로 4시간 동안 자극하였다. TGF-β1, TNF-α 및 IL-6의 발현은 ELISA 또는 western blot으로 측정하였다.

결과: LPS는 골수유래대식세포에서 TGF-β1의 생산을 증가시켰으나, AMPK 활성제인 metformin 또는 resveratrol은 LPS에 의해 유도되는 TGF-β1의 생성을 억제하였다. AMPKα1을 녹다운(knock-down)시킨 Raw 264.7 세포에서는 LPS에 의한 TGF-β1 생성 증가를 억제시키는 metformin의 효과를

감소시켰다. 또한, 재조합 TGF-β1을 처치할 경우 LPS에 의한 TNF-α 및 IL-6의 생성이 감소하였으나, 이러한 효과는 metformin에 의해 역전되었다. TGF-β의 downstream target인 Smad2의 인산화는 LPS에 의해 증가되었으며, TGF-β 중화 항체와 metformin은 이를 억제하였다. 또한, Smad2의 knockdown은 내독소 내성의 발생을 억제하였다. CLP 마우스의 비장세포에서는 TGF-β1의 발현이 증가되었고, CLP 마우스의 비장세포에 LPS를 처리할 경우 TNF-α 및 IL-6의 생성이 대조군에 비해 감소되었다. 그러나 metformin 또는 TGF-β 중화 항체를 투여할 경우 CLP 마우스의 비장세포에서 발생하는 LPS에 의한 TNF-α 및 IL-6 생성 감소가 억제되었다.

결론: 이러한 실험 결과는 AMPK 활성화가 LPS로 유도된 TGF-β1 생성을 억제하며, 이런 기전을 통하여 대식세포에서 내독소 내성 발달 억제에 관여함을 보여주고 있다.