Regulation of Inflammatory Response by 3-Methyladenine Involves the Coordinative Actions on Akt and Glycogen Synthase Kinase 3 \( \beta \) Rather than Autophagy

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Regulation of Inflammatory Response by 3-Methyladenine Involves the Coordinative Actions on Akt and Glycogen Synthase Kinase 3β Rather than Autophagy

Yi-Chieh Lin,* Hsuan-Cheng Kuo,* Jang-Shiun Wang,* and Wan-Wan Lin*,†

3-Methyladenine (3-MA) is one of the most commonly used inhibitors in autophagy research today. However, rather than inhibiting class III PI3K that is involved in autophagy suppression, 3-MA might also interfere with class I PI3K and consequently augment autophagy flux. In this study, we aim to get a thorough understanding on the action mechanisms of 3-MA in TLR4-mediated inflammatory responses in RAW264.7 macrophages and, moreover, to decipher the action of 3-MA in modulation of autophagy. We found that 3-MA could enhance LPS-induced NF-κB activation and production of TNF-α, inducible NO synthase (iNOS), cyclooxygenase-2, IL-1β, and IL-12. In contrast, 3-MA suppressed LPS-induced IFN-β production and STAT signaling. Studies revealed that 3-MA can, through inhibition of Akt as a result of class I PI3K interference, positively regulate p38, JNK, and p65, but negatively regulate TANK-binding kinase 1 and IFN regulatory factor 3 mediated by TLR4. As glycogen synthase kinase 3β (GSK3β) is an important Akt substrate, we further explored its involvement in the actions of 3-MA. 3-MA was found to enhance LPS-induced NF-κB activation, iNOS, and pro–IL-1β expression, and these actions were reversed by either GSK3β inhibitors or small interfering GSK3β. Lastly, we demonstrated that 3-MA acts as an autophagy inducer in RAW264.7 macrophages, but the stimulating effects on NF-κB activation and iNOS and cyclooxygenase-2 expression were not affected in LPS-stimulated macrophages with small interfering autophagy protein-5 treatment. These results not only shed new light on the action mechanisms of 3-MA to differentially regulate inflammatory outcomes derived from TLR4-mediated MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β pathways, but also highlight the necessity to check autophagy status upon taking 3-MA as a general autophagy inhibitor. The Journal of Immunology, 2012, 189: 000–000.

Innate immunity serves as the first line of defense against invading microorganisms. TLR4 is a pattern recognition receptor that can recognize bacterial LPS and plays a critical role in Apc cells for host defense and activation of adaptive immune response (1). After activation, TLR4 can induce MyD88- and Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF)–dependent upstream signals, leading to production of proinflammatory and anti-inflammatory mediators, respectively (2, 3). To achieve these actions, TGF-activated kinase 1 (TAK1), IκB kinase (IKK), p38 MAPK, JNK, TANK-binding kinase 1 (TBK1), NF-κB, AP-1, and IFN regulatory factor 3 (IRF3) are involved with different preferences for target gene transcription.

Recently, there were reports suggesting that activation of PI3K/Akt/glycogen synthase kinase 3β (GSK3β) pathways upon the early phase of TLR4 stimulation can modulate the MyD88 and TRIF signaling pathways of TLR4. It was demonstrated that Akt can negatively regulate MyD88/NF-κB pathways and limit LPS-induced production of inflammatory mediators (4–7). Studies further showed that inhibition of GSK3β following Akt activation contributes to inhibit NF-κB and is involved in the regulation of TLR-mediated cytokine production (5, 6–10). Nevertheless, the crosstalk between PI3K/Akt and NF-κB pathways remains controversial. Despite more findings supporting Akt being a negative regulator on NF-κB, some studies indicate Akt can positively regulate NF-κB–dependent gene expression via increase of p65 transactivation activity or via activation of IKK and p38 (11–13). The controversy mainly is due to cell-type or stimulation differences. In contrast to the major negative role in MyD88-mediated proinflammatory response, TRIF–dependent innate immunity response seems to be enhanced by Akt itself (14). A very recent study demonstrated Akt being a downstream molecule of TRIF–TBK1 and playing an important role in the activation of IRF3 and IFN-β gene expression (15). Besides the NF-κB pathway, PI3K/Akt was also reported to regulate MAPKs in a negative manner. Akt itself can inhibit JNK and p38 MAPK through inhibition of apoptosis signal-regulated kinase 1 (16).

Autophagy is a ubiquitous biological process essential for maintaining cellular homeostasis among eukaryotes. The cellular events during autophagy follow distinct stages, and the first step is vesicle nucleation (formation of phagophore), which is differentially regulated by PI3Ks. Activation of class I PI3K and its downstream molecule the mammalian target of rapamycin (mTOR) lead to autophagy suppression. In contrast, activation of class III PI3K (Vps34) positively regulates vesicle nucleation (17).

*Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan; and †Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 11031, Taiwan.

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Address correspondence and reprint requests to Dr. Wan-Wan Lin, Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan. E-mail address: wwflaura1119@ntu.edu.tw.

Abbreviations used in this article: Atg5, autophagy protein-5; COX-2, cyclooxygenase-2; GSK3, glycogen synthase kinase 3; IKK, IκB kinase; iNOS, inducible NO synthase; IRF3, IFN regulatory factor 3; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; poly I:C, polyinosinic-polycytidylic acid; p60RSK, p90 ribosomal S6 kinase; p70S6K, p70 ribosomal protein S6 kinase; siRNA, small interfering RNA; TAK1, TGF-activated kinase 1; TBK1, TANK-binding kinase 1; tLC3, tandem fluorescent-tagged LC3 construct; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β.

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phagy has been shown to intersect with innate immune responses at multiple levels and link to the elimination of intracellular pathogens (18–21). It was shown that TLR4 and TLR7 are autophagy sensors and can induce autophagy by suppressing the interaction between Beclin 1 and Bcl-2 to control the replication of pathogens (22, 23). Moreover, autophagy was shown to be essential for TLR7-dependent production of type I IFNs and proinflammatory cytokines, implicating a positive regulatory role of autophagy in innate immune responses (24). Yet, paradoxically, there are other studies pointed out that absent or downregulation of autophagy results in enhanced production of proinflammatory molecules (25–27). Thus, the distinct roles of autophagy in inflammatory responses implicate that autophagy serves as a dual functional regulator in innate immunity to ensure homeostatic balance of immune responses.

3-Methyladenine (3-MA) is a widely used pharmacological inhibitor of autophagy today, as it is able to inhibit autophagosome formation by interfering with class III PI3K (28). Nevertheless, some nonspecific effects of 3-MA independently of autophagy inhibition have been reported, including inhibition of class I PI3K and mTOR (29), suppression of protein degradation, and cell migration and invasion (30, 31). The preferential action of 3-MA in distinct PI3Ks was later on shown to be associated with the cellular setting (32). It was found that prolonged treatment of 3-MA can promote autophagic flux under nutrient-rich conditions, whereas 3-MA is capable of suppressing starvation-induced autophagy. Given that autophagy is in close relationship with innate immunity and its involvement or not in the actions of 3-MA in macrophage-mediated inflammatory response remains elusive, we thus explore the effects and the action mechanisms of 3-MA on LPS-induced inflammatory responses in murine macrophages and also elucidate the involvement or not of autophagy in these events.

Materials and Methods

**Cell culture**

Murine RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA), and cultured in DMEM complete medium containing 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37˚C in a humidified atmosphere of 5% CO2 in air. Primary murine bone marrow-derived macrophages were prepared as we previously described (33).

**Reagents**

LPS, 3-MA, MTT, protease inhibitor cocktails, SB415286, SB216763, and Akt inhibitor were obtained from Calbiochem (La Jolla, CA). Murine IFN-β was obtained from PBL InterferonSource (Piscataway, NJ). Abs directed against IKK, p38, JNK1, IκBα, TBK1, JAK1, STAT1, STAT3, GSK3β, cyclooxygenase-2 (COX-2), and HRP-coupled anti-rabbit and anti-mouse secondary Abs were purchased from Santa Cruz Bio-technology (Santa Cruz, CA). Anti-NLRP3 Ab was purchased from Abcam (Cambridge, UK). Ab directed against phosphorylated JAK1 (Y102/103) was obtained from Invitrogen (Biosource International, Nivelles, Belgium). Abs against β-actin and phosphorylated GSK3β (Y216) were purchased from Cell Signaling Technology (Beverly, MA). Ab against phosphorylated TBK1 were from BD Biosciences Pharmingen (Franklin Lakes, NJ). LC3 Ab was purchased from MBL (Woburn, MA). Abs specific to the phosphorylated p38 (Thr180/Tyr182), JNK (Thr183/Tyr185), ERK (Thr202/Tyr204), IκBα (Ser32/36), and JAK1 (Thr185/187), IF3 (Ser63), p65 (Ser536), STAT1 (Tyr701), STAT3 (Tyr705), p70 ribosomal protein S6 kinase (pS6K) (Thr389), p90 ribosomal S6 kinase (p90RSK) (Ser380), and GSK3β (Ser9), as well as Ab against p70S6K were purchased from Cell Signaling Technology (Beverly, MA). Ab against IF3 was from Epitomics (Burlingame, CA). Ab against autophagy protein-5 (Atg5) was purchased from Abcam (Cambridge, UK). Ab directed against phosphor-ylated JAK1 (Y102/103) was obtained from Invitrogen (Biosource International, Nivelles, Belgium). Abs against β-actin and phosphorylated GSK3β (Y216) were purchased from Cell Signaling Technology (Beverly, MA). Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). DMEM, trypsin-EDTA, and antibiotic penicillin/streptomycin were from Invitrogen (Rockville, MD). The RNA-Beef isolation reagent was purchased from Tel-Test (Friendswood, TX).

The ECL reagent (Western Lightening Chemiluminescence Reagent Plus) was from PerkinElmer (Wellesley, MA). The FastStart SYBR Green Master was from Roche Applied Science (Nutley, NJ).

**MTT assay**

Murine RAW264.7 macrophages (10^7/ml) plated in 96-well plates were incubated with indicated agents at 37˚C for 24 h. MTT (5 mg/ml) was added for 1 h and then the culture medium was removed. The formazan granules generated by live cells were dissolved in DMSO and shaken for 10 min. The OD values at 550 and 630 nm were measured using a microplate reader. The net absorbance (OD550−OD630) indicates the enzymatic activity of mitochondria and implicates the cell viability.

**Transfection and luciferase assay**

The iNOS promoter plasmid was kindly provided by Dr. C. K. Glass (Department of Medicine, University of California, San Diego, La Jolla, CA). The 2.3-kb cDNA for murine iNOS promoter was ligated into the luciferase reporter vector. The NF-κB reporter plasmid containing three binding sites (pGL2-ELMA-luciferase) was provided by Dr. S. L. Hsieh (Yang-Ming University, Taipei, Taiwan). The HA-p65 plasmid was provided by Dr. Hiroaki Sakurai (Tanabe Seiyaku, Osaka, Japan). The β-galactosidase expression plasmid (pCR3αZc; Pharmacia, Stockholm, Sweden) was used for normalization of transfection efficiency. All plasmids were prepared using an endotoxin-free plasmid preparation kit (Qiagen, Germantown, MD). Following the commercial standard protocol, RAW264.7 cells were transfected with 0.25 μg each plasmid by using Lipofectamine 2000 reagent (Invitrogen). After the indicated treatments, RAW264.7 cells were lysed in reporter lysis buffer (Promega). Subsequently, the lysates were reacted with commercial luciferase substrate in a luciferase assay system kit (Promega) and then were assayed by microplate lumino-meter (Packard Instrument, Meriden, CT).

**Real-time RT-PCR with SYBR green detection**

The expression of murine iNOS, COX-2, IL-12β, IL-1β, and TNF-α mRNAs were determined by real-time RT-PCR analysis. After treatment as indicated, cells were homogenized with 500 μl RNA-Bea isolation reagent (Tel-Test), and total RNA was extracted following the commercial standard protocol. Reverse transcription was performed using an RT-PCR kit (Promega, Heidelberg, Germany), and 5 μg total RNA was reversely transcribed to cDNA following the commercial standard procedures. The specific primers for iNOS were forward primer 5’-CAG TCT GGC TGT ACA AAC CTT-3’ and reverse primer 5’-CAT TGG AAG TAA GGC AGT TGG-3’. The specific primers for COX-2 were forward primer 5’-GAG AGA AGG AAA TGG CAG CAG AA-3’ and reverse primer 5’-GCC TTC CAG TAT TGA GGA GAA CAGA-3’. The specific primers for IL-1β were forward primer 5’-TTC AGG GGA ACA TTA GGCAG-3’ and reverse primer 5’-TGG GCT GGT GCT TCA TTC AT-3’. The specific primers for IL-12β were forward primer 5’-CAG AAG TCA ACC ATC TTC TGG GTT-3’ and reverse primer 5’-CCG GAG TAA TTT GGT GCT CCA CAC-3’. The specific primers for TNF-α were forward primer 5’-ATG AGA AGT TCC AAA ATG GCC-3’ and reverse primer 5’-TCT AGG TGG TTT GGT AGC-3’. Real-time PCR was performed using the ABI Prism 7900 (Applied Biosystems), and PCR fluorescence detection was performed in 96-well plates using FastStart SYBR Green Master (Roche Applied Science) as we previously described (34).

**RNA interference**

The small interfering RNA (siRNA) for Atg5 and GSK3β contained four RNA sequences in a SmartPool selected from the National Center for Biotechnology Information RefSeq Database using a proprietary algorithm. The control is a pool of four functional nontargeting siRNAs with guanine cytosine content comparable to that of the functional siRNA but lacking specificity for the known gene target. We transfected cells with the 100 nM siRNA for 72 h by DharmaFECT 1 (Dharmacon, Chicago, IL) followed by drug treatment and then evaluated the gene-silencing effects by Western blot analysis.

**Immunoblotting analysis**

After indicated treatment, the medium was aspirated. Cells were rinsed with ice-cold PBS and lysed in whole-cell lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, 2 mM EDTA, 2 mM NaF, 2 mM Na2VO4, 1 mM PMSF, and protease inhibitor cocktails). After cell harvest, total cell lysates were centrifuged at 12,500 rpm, 4˚C for 15 min. The protein concentrations were determined using the Bio-Rad protein assa (Bio-Rad). Equal amounts of the soluble protein were electrophoresed on 8–10% SDS-PAGE and transferred to Immobilon-P...
(Millipore). Non-specific binding was blocked with 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature. After immunoblotting with the primary Abs, membranes were washed three times with TBST and incubated with HRP-conjugated secondary Ab for 1 h. After washing three times with TBST, the protein bands were detected with ECL detection reagent (PerkinElmer). To ensure equal amounts of sample were applied for electrophoresis and immunoblotting, β-actin was used as an internal control. Each immunoblotting trace shown in the figures was representative of at least three reproducible experiments.

**Fluorescence microscopy analysis**

The tandem fluorescent-tagged LC3 construct (tfLC3), which was generated by Dr. T. Yoshimori’s group (35), was purchased from Addgene (Cambridge, MA). Transfection of the plasmid in RAW264.7 macrophages was performed by using Lipofectamine 2000 reagent (Invitrogen). After 24 h of transfection, cells were treated with agents, fixed with 4% paraformaldehyde, and examined by a fluorescent microscope (Zeiss AXIO Imager A1; Carl Zeiss).

**Statistical evaluation**

Values were expressed as the mean ± SEM of at least three independent experiments, which were performed in duplicate. ANOVA was used to assess the statistical significance of the differences, and a p value <0.05 was considered statistically significant.

**Results**

3-MA enhances LPS-induced inflammatory responses in RAW264.7 macrophages

To investigate the effects of 3-MA on innate inflammatory response, LPS-stimulated RAW264.7 macrophages were used in this study. Cell viability was first examined after 3-MA and LPS treatment. Low concentration of 3-MA (1 mM), either alone or in company with LPS, did not cause significant effect on cell viability, whereas higher concentration of 3-MA (3 mM) decreased cell viability (Fig. 1A). Notably, the cytotoxicity of 3-MA (3 mM) can be diminished by LPS (1 mg/ml) cotreatment.

First of all, we determined the effects of 3-MA on LPS-induced inflammatory response. Results revealed that LPS-induced iNOS protein expression (Fig. 1B) and iNOS promoter activation (Fig. 1C) were enhanced by 3-MA in a concentration-dependent manner. To exclude the interference of cell viability on the inflammatory responses, 3-MA (1 mM) was adopted as the optimal experimental condition in the following experiments. Likewise, the transcription of several proinflammatory genes, including iNOS, pro-IL-1β, IL-12β, TNF-α, and COX-2, was also enhanced by 3-MA after LPS stimulation (Fig. 1D). Consistently, COX-2 and pro-IL-1β protein expression induced by LPS was increased by 3-MA addition (Fig. 1E). Altogether, these data suggest that 3-MA could enhance the inflammatory responses in LPS-activated RAW264.7 macrophages.

Effects of 3-MA on LPS-induced upstream signaling pathways and NF-κB activation

Because NF-κB was reported to be one of the main transcription factors mediating inflammatory response downstream of TLR4, we evaluated the effect of 3-MA on NF-κB activation. As shown in the luciferase reporter assay, 3-MA could concentration-dependently augment NF-κB activation under LPS stimulation (Fig. 2A). However, 3-MA exerted no effect on NF-κB activity in p65-overexpressing RAW264.7 macrophages (Fig. 2B). We next analyzed the signaling cascade downstream of TLR4 activation. In the aspect of MyD88-dependent pathway, which provides stronger signals than the TRIF pathway for IKK and MAPK activation, 3-MA did not exert significant influence on the phosphorylation of IKK or IκBα degradation. In contrast, although the intensities of LPS-induced phosphorylation of TAK1 and p65 were not much affected at early time points (≤10 min), both phosphorylations seemed to sustain longer under 3-MA treatment compared with those treated with LPS alone (Fig. 2C). Similarly, we found that 3-MA also can increase the phosphorylation of p38 and JNK, two signals downstream of TAK1, at the late stage of LPS activation (30–120 min) (Fig. 2C).

In the aspect of the TRIF-dependent pathway, unlike what have been observed in the MyD88-dependent pathway, 3-MA did not increase the phosphorylation of TBK1 and IRF3. To be more exact, 3-MA even slightly reduced the phosphorylation of TBK1 and IRF3 at the early stage of LPS stimulation (Fig. 2D). It was known that 3-MA can interfere with class I PI3K (29, 32) and that class I PI3K is a direct regulator of endocytosis (36). Because LPS-induced MyD88- and TRIF-dependent pathways are triggered by TLR4 located in the plasma membrane and endosome, respectively, we wondered if enhancing TAK1 downstream signals but inhibiting TBK1 downstream signals caused by 3-MA are associated with endocytotic interference. To access this possibility, we pretreated RAW264.7 cells with 3-MA and then stimulated with a direct activator of the TRIF pathway, polyinosinic-polycytidylic acid (poly I:C; 25 μg/ml), which can bind to TLR3 on the endosomal membrane and activate the TRIF pathway independent of the process of endocytosis. As shown in Fig. 2E, 3-MA (1 mM) could still inhibit poly I:C-induced IRF3 phosphorylation. Thus, we suggest that 3-MA (1 mM) may negatively regulate LPS-induced IRF3 activation through a mechanism independent of TLR4 endocytosis. Together, these findings indicate that 3-MA can differentially regulate MyD88- and TRIF-dependent signaling pathways downstream of TLR4 activation.

3-MA inhibits PI3K–Akt–GSK3β signaling pathway

Previous studies showed that 3-MA inhibits not only class III PI3K, but also interferes with class I PI3K. Next, we determined the effects of 3-MA on PI3K/Akt/GSK3β pathways. Akt was phosphorylated under LPS stimulation, and 3-MA exhibited an inhibitory effect in this event (Fig. 3A). Given that the PI3K/Akt pathway could activate mTOR, which in turn activates p70S6K, we next examined p70S6K phosphorylation in LPS-activated macrophages. As expected, 3-MA inhibited p70S6K phosphorylation under LPS stimulation, in accordance with its inhibitory action on Akt (Fig. 3A). These results indicate that 3-MA indeed is a negative regulator of PI3K/Akt/mTOR pathway.

PI3K-dependent activation of Akt leads to phosphorylation of GSK3β, which is active in resting cells but inactivated by phosphorylation (9). Because 3-MA has been demonstrated to inhibit PI3K/Akt in LPS-stimulated RAW264.7 macrophages, it inspires us to determine if 3-MA also activates GSK3β following its effect on Akt inhibition. As shown in Fig. 3B, 3-MA alone decreased the inactivating phosphorylation of GSK3β at Ser9, indicating that 3-MA could activate GSK3β. LPS (1 μg/ml) stimulation was shown to increase the inactivating GSK3β phosphorylation, meaning that LPS itself exerts an inhibitory effect on GSK3β activity. The GSK3β-activating effect of 3-MA also existed under LPS stimulation, as 3-MA significantly decreased LPS-induced Ser9-inactivating phosphorylation. GSK3 is also known to be phosphorylated at a tyrosine residue (Tyr279 in GSK3α and Tyr272 in GSK3β), and several studies pointed out that this Tyr phosphorylation also contributes to GSK3 activity (37). However, in our study, we did not detect any effects of 3-MA and LPS on this phosphorylation (Fig. 3B), which is consistent with previous findings in LPS-stimulated macrophages (38).

Notably, the ATP-competitive GSK3 inhibitor SB216763, which is supposed to inhibit GSK3β activity, appeared to decrease the inactivating phosphorylation at Ser9 (Fig. 3B). This phenomenon
has also been observed in previous studies (39) and was ascribed
to the nonspecific effect of GSK3 inhibitors on p90RSK, another
upstream kinase of GSK3\(\beta\) (40). To understand if 3-MA could
decrease GSK3\(\beta\) Ser9 phosphorylation through inhibiting p90RSK,
we explored p90RSK phosphorylation. As shown in Fig. 3C, 3-MA
did not affect p90RSK phosphorylation induced by LPS. Collect-
ively, these results demonstrate that 3-MA could inhibit PI3K/Akt
signaling and in turn activate GSK3\(\beta\).

FIGURE 1. 3-MA augmented LPS-stimulated inflammatory responses in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with 3-MA at the concentrations indicated for 30 min, followed by the stimulation with LPS (1 \(\mu\)g/ml). (A) After LPS treatment for 24 h, the cell viability was assessed by MTT assay. (B) Total cell lysates were subjected to SDS-PAGE and immunoblotting for iNOS. The increased iNOS expression was normalized with \(\beta\)-actin level and expressed as fold of control response of LPS without 3-MA treatment. (C) RAW264.7 macrophages were transfected with iNOS promoter construct followed by treatment with LPS (1 \(\mu\)g/ml) and/or 3-MA at the concentrations indicated. After 24 h of incubation, the luciferase activity was determined, normalized with \(\beta\)-galactosidase activity, and expressed relative to the control group. (D) RAW264.7 macrophages were pretreated with 3-MA (1 mM) for 30 min prior to stimulation with LPS (1 \(\mu\)g/ml). After incubation for different periods, total RNA was extracted and reversely transcribed for quantitative PCR analyses of iNOS, COX-2, IL-12, IL-1\(\beta\), and TNF-\(\alpha\) mRNA. Values were normalized to \(\beta\)-actin gene expression and expressed relative to the control group. Data were the mean \(\pm\) SEM from three independent experiments. (E) RAW264.7 macrophages were treated with LPS (1 \(\mu\)g/ml) and/or 3-MA (1 mM) as indicated, followed by immunoblotting to determine protein expression. *\(p < 0.05\), indicating significant effects of 3-MA as compared with control (A) or LPS response alone (B–D).
Akt but not GSK3β is involved in differentially regulating LPS-induced p38, JNK, p65, and IRF3 activation

Akt signaling has been shown to negatively regulate MyD88-dependent pathway, whereas it positively regulates the TRIF-dependent pathway upon TLR4 activation (4–7, 14, 15). Because 3-MA could significantly attenuate LPS-induced Akt phosphorylation, we wonder if the differentially regulatory effects of 3-MA on LPS signaling as shown previously in Fig. 2C–E were mediated by Akt. As shown in Fig. 4A, pharmacological inhibition of Akt by Akt inhibitor (10 μM) enhanced the phosphorylation of p38, JNK, and p65 in response to LPS stimulation, whereas it diminished the phosphorylation of IRF3. Such phenomena appear to correspond with those affected by 3-MA upon LPS stimulation as shown above.

Recently, GSK3β was proposed as a key downstream target of Akt to regulate TLR-mediated cytokine production (9). Thus, to understand if 3-MA regulation of LPS-induced signaling is associated with GSK3β activation, we tested a GSK3β inhibitor and found that 3-MA–induced enhancement of JNK and p38 phosphorylation were not affected by SB216763 (10 μM). LPS-induced IKK phosphorylation was not affected by 3-MA or SB216763 (Fig. 4B). These results suggest that GSK3β activation by 3-MA, unlike Akt, is not involved in regulating TLR4 signaling of p38 and JNK.

3-MA augments LPS-induced proinflammatory responses through GSK3β-dependent NF-κB activation

Next, we demonstrated if 3-MA–enhanced inflammatory responses and NF-κB activation were associated with GSK3β activation. To this end, pharmacological GSK3β inhibitors (SB216763 and SB415286) were used. We found that NF-κB activity triggered by LPS itself was inhibited by SB216763 and SB415286, suggesting that GSK3β plays a positive regulatory role in LPS-induced NF-κB activation. Moreover, 3-MA–induced NF-κB enhancement under LPS stimulation was also significantly blunted by both GSK3β inhibitors (Fig. 5A). Confirming results using a kinase inhibitor, LPS-elicited NF-κB activity either in the presence or absence of 3-MA was reduced in cells pretreated with si-GSK3β (Fig. 5B). To address if GSK3β-mediated NF-κB results from p65 phosphorylation, we determined this via immunoblotting. As a result, SB216763 did not change LPS-induced p65 phosphorylation, unlike the effect of p38 inhibitor (SB203580) (Fig. 5B).

In agreement with the response of NF-κB, SB216763 can suppress the enhancing effect of 3-MA on the LPS-induced iNOS and pro–IL-1β protein expression (Fig. 5C). In parallel, GSK3
inhibition significantly reduced the level of LPS-induced IL-1β mRNA expression. 3-MA–augmented IL-1β mRNA expression in response to LPS stimulation was also inhibited by GSK3β inhibitors (Fig. 5D). Consistently, iNOS and pro–IL-1β protein expression induced by LPS was markedly reduced in cells treated with GSK3β siRNA (Fig. 5E).

To understand if current findings for GSK3β-mediated proinflammatory response display the cell-type specificity, we tested the effects of 3-MA and SB216763 in primary bone marrow-derived macrophages. As expected, protein levels of proinflammatory mediators like NLRP3, iNOS, COX-2, and pro–IL-1β were induced by LPS, and all of these effects were diminished in the presence of GSK3β inhibitor. Moreover, as in the response shown in RAW264.7 macrophages (Fig. 5C), 3-MA further enhanced pro–IL-1β expression under LPS stimulation, and this effect of LPS plus 3-MA is still sensitive to GSK3β inhibitor (Fig. 5F). In summary, we suggest that 3-MA–augmented LPS-induced proinflammatory response, at least in part, is ascribed to the positive regulation of the GSK3β-dependent NF-κB pathway.

3-MA negatively regulates the JAK/STAT1 pathway

Upon activation of TLR4, the transcription of type I IFN can be induced via the TRIF-dependent pathway, and it in turn via an autocrine action leads to the activation of the type I IFN receptor-mediated JAK/STAT pathway. Next, we were interested to understand whether 3-MA also influences the LPS-induced JAK/STAT signaling pathway, type I IFN production, and their relevance to GSK3β activation. We found that 3-MA appeared to diminish LPS-triggered JAK1, STAT1, and STAT3 phosphorylation (Fig. 6A). In parallel, 3-MA could attenuate LPS-induced IFN-β protein expression, and this effect was not altered by SB216763 (Fig. 6B), suggesting the noninvolvement of GSK3β in regulating molecular events underlying the inhibitory actions of 3-MA for IFN-β gene transcription.

In addition to inhibit IFN-β production, we examined if 3-MA might also affect JAK/STAT signaling at the receptor level. Thus, we stimulated RAW264.7 macrophages with exogenous IFN-β and determined the effect of 3-MA on STAT phosphorylation. Results showed that 3-MA failed to affect STAT1 phosphorylation upon IFN-β treatment, but can inhibit IFN-β–mediated STAT3 phosphorylation (Fig. 6C). Both effects of 3-MA were still present when treating SB216763, which itself has no significant effects in IFN-β–induced STAT1 and STAT3 phosphorylation. These results suggest the ability of 3-MA to interrupt LPS-induced IFN-β signaling through both indirect (i.e., inhibition of IFN-β production) and direct (i.e., inhibition of IFN-β–mediated STAT3 phosphorylation) mechanisms.
Autophagy activation by 3-MA is not involved in NF-κB activation

Although 3-MA is a commonly used autophagy inhibitor today, recent evidence has suggested that this pharmacological agent had other off-target effects, including the augmentation of autophagic flux (32, 41). Thus, we aimed to understand the role of autophagy in the action of 3-MA in activated macrophages. Because lipid conjugation of LC3 leading to the conversion of the soluble form of LC3 (LC3-I) to the lipidated form (LC3-II) and resulting in formation of the autophagosome is a widely used marker of autophagy, we determined LC3-II level and intracellular LC3 staining. As shown in Fig. 7A, 3-MA enhanced the conversion from LC3-I to LC3-II regardless of the presence or not of LPS, indicating its ability to induce autophagy. We also used tfLC3
construct to confirm 3-MA–induced autophagic flux. This double-tagged LC3 construct can monitor the maturation processes of autophagosome and autolysosome with different fluorescent signals (42). As the greenish fluorescent signal would be quenched under low pH condition, yellow puncta represents autophagosome, whereas red puncta represents autolysosome. As shown in Fig. 7B, RAW264.7 macrophages under starvation with treatment of HBSS exhibited increased red puncta formation compared with the control group. In contrast, bafilomycin A1, which inhibits acidification inside the lysosome and thus impairs autolysosomal maturation, only increased yellow puncta. Under 3-MA and/or LPS treatment, obvious red puncta were detected. These data suggest that 3-MA is a positive regulator of autophagy in RAW264.7 macrophages.

To further understand if the enhancing effect of 3-MA on LPS-induced inflammatory response was associated with autophagy, we adopted the siRNA approach to abrogate autophagy. Deprivation of Atg5 by siRNA significantly increased LPS-triggered NF-κB activation, and under this condition, the enhancing effect of 3-MA on LPS-induced NF-κB activation was still observed (Fig. 7C). Inconsistency of NF-κB activation, 3-MA–induced increase of iNOS and COX-2 protein expression (Fig. 7D) and COX-2 mRNA expression (Fig. 7E) caused by LPS were also present in autophagy-compromised cells. These findings suggest that the proinflammatory actions of 3-MA in LPS-stimulated macrophages are unrelated to autophagy induction. Taken together, 3-MA actions in macrophages shown in this study are summarized in Fig. 8.

Discussion
3-MA is a widely used autophagy inhibitor that was known to take effect via interference with class III PI3K, hVps34 (43). Nevertheless, induction of autophagy and other cellular actions via its inhibition on class I PI3K and independent of autophagy inhibition were also demonstrated (29–32). Based on these conflict actions of 3-MA and much scientific research in the field of autophagy commonly using 3-MA for autophagy inhibition, it is indeed worthwhile taking notice of the actual action mechanisms of 3-MA. Thus, in this study, we determined the action of 3-MA in macrophages with respect to the regulation of both inflammatory response and autophagy. Moreover, the link from Akt, GSK3β, and autophagy to inflammatory signals (e.g., NF-κB, MAPKs, and STATs) was elucidated in this study to provide a new insight on the action of 3-MA.

To investigate how 3-MA affects inflammatory responses, we used LPS-stimulated murine RAW264.7 macrophages as a cellular model. According to our finding, it showed that 3-MA inhibited LPS-induced phosphorylation of Akt as well as one of the downstream substrates of mTOR, p70S6K (Fig. 3A). These findings indicate that 3-MA may exert inhibitory effects on class I PI3K-mediated signaling. Before investigating the role of 3-MA in LPS-induced inflammatory responses, the concentration-gradient response of 3-MA in cell viability was assessed (Fig. 1A). Moreover, to exclude the interference of cell viability on 3-MA–affected inflammatory responses, 3-MA (1 mM) was chosen as the optimal experimental condition.

To date, there is a large body of evidence suggesting that PI3K/Akt pathway plays a negative regulatory role in TLR-triggered inflammation, particularly in MyD88-dependent pathways like p38, JNK, p65, and NF-κB (5, 16, 44, 45). Consistently in this study, we also observed that 3-MA enhanced the production of various LPS-induced proinflammatory responses, including the transcriptional expression of iNOS, COX-2, TNF-α, IL-12, and IL-1β (Figs. 1, 5F), as well as the activation of p65, p38, JNK, and NF-κB (Fig. 2). We further conducted an experiment using Akt inhibitor to confirm a negative regulation loop of Akt on MAPKs and STATs was elucidated in this study to provide a new insight on the action of 3-MA.

FIGURE 6. 3-MA negatively regulated JAK/STAT signaling pathway. RAW264.7 macrophages were pretreated with 3-MA (1 mM) and/or SB216763 (10 μM) for 30 min as indicated prior to the stimulation with LPS (1 μg/ml) or IFN-β (10 ng/ml). After incubation for different periods as indicated, the total cell lysates were subjected to SDS-PAGE followed by immunoblotting for STAT1, JAK1, and STAT3 (A, C). In some experiments, secreted IFN-β in the culture medium was determined by ELISA (B). *p < 0.05 indicates significant suppression of IFN-β protein expression by 3-MA.
inhibition of Akt, which is a negative regulator of p38, and the other is mediated by the increase of TAK1 activation (see below).

TAK1 is recognized as an upstream signaling molecule of MAPKs and NF-κB signaling in response to LPS stimulation. However, it has been suggested that TAK1 is more important for p38 and JNK activation than for IKK activation (2, 46). Phosphorylation of TAK1 is dispensable for IKK activation upon TLR4 activation in certain cellular contexts, indicating that the role of TAK1 as an adaptor protein rather than its kinase activity accounts for activation of IKK (47). In our case, TAK1 phosphorylation

**FIGURE 7.** Autophagy induced by 3-MA does not contribute to regulate NF-κB and inflammation response. (A) RAW264.7 macrophages were treated with 3-MA (1 mM) and/or LPS (1 μg/ml) for different periods as indicated. Total cell lysates were subjected to immunoblotting for LC3. (B) RAW264.7 macrophages were transfected with tflC3 plasmid and treated with 3-MA (1 mM), LPS (1 μg/ml), bafilomycin A1 (BA; 50 nM), or HBSS for 8 h. Cells were prepared and subjected to fluorescence microscopy. (C) RAW264.7 macrophages were transfected with either small interfering Atg5 (si-Atg5) or control siRNA and were also transfected with NF-κB reporter construct followed by pretreatment with 3-MA (1 mM) for 30 min prior to LPS (1 μg/ml) stimulation. After LPS incubation for 24 h, the luciferase activity was determined, normalized with β-galactosidase activity, and expressed relative to the control group. (D and E) RAW264.7 macrophages were transfected with si-Atg5 or control siRNA. Then cells were treated with 3-MA (1 mM) and LPS (1 μg/ml) for 24 h (D) or 3 h (E). Expression levels of iNOS, COX-2, and Atg5 proteins (D) as well as COX-2 mRNA (E) were determined. *p < 0.05, indicating significant increasing actions of 3-MA on the LPS response in control siRNA cells, #p < 0.05, indicating significant increasing actions of 3-MA alone and on the LPS response in si-Atg5 cells.
after 60 min was enhanced by 3-MA, whereas IKK phosphorylation occurring as fast as 10 min and lasting to 120 min was unaffected (Fig. 2C). It is possible that similar to previous findings, increased activity of TAK1 in 3-MA–treated cells has a greater influence on MAPKs than on IKK. We speculate TAK1 might be involved to mediate the effect of 3-MA in enhancing the late-phase response (>60 min) of p38, JNK, and p65.

In contrast to what has been observed in the MyD88-dependent pathway, 3-MA appeared to negatively regulate the TRIF-dependent pathway, because phosphorylation of TBK1 and IRF3 as well as IFN-β production were all dampened upon 3-MA treatment (Figs. 2D, 2E, 6B). Recent studies pointed out that Akt plays a dual role in regulating TLR4 signaling, in which Akt serves as a negative regulator of MyD88-dependent signaling, whereas it positively regulates the TRIF-dependent pathway (48). With respect to the TRIF-dependent pathway, Akt was shown to act downstream of TBK1 but upstream of IRF3, in which Akt can positively regulate activation of IRF3 (15). Consistent with this notion, our results showed the ability of the Akt inhibitor to diminish IRF3 phosphorylation upon LPS stimulation (Fig. 4A). Thus, the action of 3-MA in TRIF-mediated responses is primarily ascribed to inhibition of Akt.

Although many have suggested the negative regulation of Akt on NF-κB activation, the detailed mechanism underlying this phenomenon still remains poorly understood. Recently, GSK3β has become the most characterized candidate downstream of Akt that participates in its linkage to NF-κB. It was shown that Akt can negatively regulate GSK3β activity, while GSK3β can activate NF-κB (49, 50). In this respect, as reported by Martin et al. (49), GSK3β inhibition led to increased binding of CREB to CBP, thus decreasing the association of NF-κB p65 to CBP. However, neither the phosphorylation of p65 (Ser276 or Ser536) nor the DNA binding of NF-κB p65 and p50 was affected by GSK3β activity (49). As demonstrated in Fig. 3B, 3-MA, either alone or in the presence of LPS, decreased inactivating Ser9 phosphorylation of GSK3β, indicating the role of 3-MA in promoting GSK3β activation in the absence of Akt inhibition. In agreement with previous findings that GSK3β is involved to augment production of various proinflammatory cytokines (49), inhibition of GSK3β activity via pharmacological inhibitor of GSK3 or genetic silencing could reverse 3-MA–enhanced proinflammatory signaling, including NF-κB activation (Fig. 5A), iNOS, and pro–IL-1β protein expression (Fig. 5C, 5E) and pro–IL-1β mRNA expression (Fig. 5D).

It has been established that type I IFN possesses anti-inflammatory potential (51). Upon TLR4 activation, several different mechanisms orchestrate in the regulation of type I IFN production. Activated TRIF-dependent pathways can mediate transcription of IFN-β via IRF3. As mentioned above, activated Akt contributes to IRF3 activation; therefore, it is reasonable that inhibition Akt by 3-MA can diminish transcription of IFN-β (Fig. 6B) and in turn reduce the signaling evoked by type I IFN (i.e., activation of JAK1, STAT1, and STAT3) (Fig. 6A). In addition, unlike a previous report showing that GSK3β can negatively regulate IFN-β by promoting c-Jun degradation (52), our study ruled out this possibility because GSK3β inhibitor failed to reverse the inhibitory action of 3-MA on IFN-β production (Fig. 6B). Furthermore, we dissected the direct effect of 3-MA on IFN-β and found that STAT3 rather than STAT1 activation was inhibited by 3-MA (Fig. 6C). The possible mechanism for 3-MA–suppressed STAT3 activation is that mTOR can positively regulate STAT3 (53), and 3-MA could inhibit mTOR. It is reasonable that the decrease in STAT3 activation by 3-MA may be partly mediated by mTOR inhibition. Besides IFN-β, the engagement of STAT3 pathway can also be induced by IL-6 and IL-10 (54). Currently, we cannot rule out the effect of 3-MA on both cytokine production and signaling transduction. Taken together, our current data suggest that both the decreased cytokine production of IFN-β as well as the dampened mTOR activity contribute to the reduction of phosphorylated STATs by 3-MA.

To date, many studies have suggested an intimate interplay between autophagy and TLR-mediated inflammatory responses. TLRs can mediate autophagy (23, 55, 56), whereas conversely, the autophagy process or autophagy proteins themselves can also influence inflammatory responses (57–59). In this study, we found that actions of 3-MA stand at the convergence of TLR-induced inflammation and autophagy. Consistent with the abilities of 3-MA to inhibit Akt and subsequent mTOR activation, 3-MA indeed promoted autophagic flux in our study, as indexed by the increased LC3-II expression (Fig. 7A) and autolysosome formation (Fig. 7B). It has been well established that autophagy played a negative regulatory role in innate immune responses (57). However, in our study, silencing of Atg5 failed to affect the effects of 3-MA on NF-κB activation (Fig. 7C), iNOS and COX-2 protein expression (Fig. 7D), or COX2 gene expression (Fig. 7E). We thus suggest that the 3-MA–upregulated autophagy flux is not involved in its effect on LPS-induced inflammatory response.

In conclusion, our results demonstrate that 3-MA is capable of regulating various LPS-induced inflammatory responses through Akt- and GSK3β-mediated signaling pathways in murine macrophages (Fig. 8). They involve the interference with class I PI3K, inhibition of Akt, and activation of GSK3β, which all orchestrate cooperatively in modulating the signaling downstream of activated TLR4. In contrast, by suppressing the PI3K/Akt pathway, 3-MA can promote autophagy flux, but does not contribute to balance NF-κB activation or other inflammatory responses enhanced by 3-MA. These results not only shed new light on the action mechanisms of 3-MA to differentially regulate inflammatory outcomes derived from TLR4-mediated MyD88 and TRIF pathways, but also highlight the necessity to check autophagy status upon taking 3-MA as a general autophagy inhibitor.
Disclosures

The authors have no financial conflicts of interest.

References


