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Lithium Attenuates IFN-β Production and Antiviral Response via Inhibition of TANK-Binding Kinase 1 Kinase Activity

Lijuan Wang,1 Lei Zhang,1 Xueying Zhao, Meng Zhang, Wei Zhao, and Chengjiang Gao

Lithium salt is a widely used glycogen synthase kinase-3β inhibitor and effective drug for the treatment of psychiatric diseases. However, the effects of lithium in innate immune responses, especially in cellular antiviral responses, are unknown. In this study, we show that lithium chloride attenuates LPS-, polyinosinic-polycytidylic acid–, and Sendai virus–induced IFN-β production and IFN regulatory factor 3 activation in macrophages in a glycogen synthase kinase-3β–independent manner. The ability of the lithium to inhibit IFN-β production was confirmed in vivo, as mice treated with lithium chloride exhibited decreased levels of IFN-β upon Sendai virus infection. In vitro kinase assay demonstrates that lithium suppresses TANK-binding kinase 1 kinase activity. Consistently, lithium significantly enhanced the replication of vesicular stomatitis virus in vitro and in vivo. Severe infiltration of monocytes and tissue damage were observed in the lungs of control mice, compared with lithium-treated mice after virus infection. Our findings suggest lithium as an inhibitor of TANK-binding kinase 1 and potential target for the intervention of diseases with uncontrolled IFN-β production. Furthermore, lithium attenuates host defense to virus infection and may cause severely adverse effects in clinical applications. *The Journal of Immunology*, 2013, 191: 4392–4398.

Pattern-recognition receptors, including TLRs and retinoic acid–inducible gene I (RIG-I)–like helicase receptors (RLRs), play pivotal roles in defense against viral infection (1, 2). Once recognition of microbial components such as LPS, polyinosinic-polycytidylic acid [poly(I:C)], and viral RNA occurs, TLRs and RLRs are activated and initiate a series of signaling events leading to production of type I IFN (IFN-α/β) and proinflammatory cytokines (1, 2). TLR3/4 recruit Toll/IL-1R domain–containing adaptor–inducing IFN-β (TRIF) and promote activation of TANK-binding kinase 1 (TBK1)/IκB kinase ε (IKKe), leading to the phosphorylation, dimerization, and nuclear translocation of IFN regulatory factor (IRF)3 and the induction of IFN-β (3). RLRs comprise three cytoplasmic DExD/H-box RNA helicases, that is, RIG-I, melanoma differentiation–associated gene 5, and laboratory of genetics and physiology 2 (4). RIG-I and melanoma differentiation–associated gene 5 recognize viral RNAs and poly(I:C) in the cytoplasm and subsequently initiate the TBK1/IRF3 pathway and induce IFN-β production (4).

Lithium is widely used as a long-term mood stabilizer in the treatment of psychiatric diseases (5). The basis of the therapeutic action of lithium remains unclear, but accumulating evidence indicates that it stems largely from its inhibition of glycogen synthase kinase-3β (GSK3β) (6), a multifunctional kinase that plays important roles in cellular processes, including glycogen metabolism, cell proliferation, neuronal function, oncogenesis, and development (7, 8). Recent studies have provided evidence for the involvement of GSK3β in innate immune responses (9). For example, GSK3β has been demonstrated as a crucial regulator for the balance between pro- and anti-inflammatory cytokines following TLR stimulation (10). GSK3β negatively regulates TLR4-mediated IFN-β production by regulating c-Jun expression (11). GSK3β physically associated with TBK1 in a viral infection–dependent manner, promoted TBK1 self-association and autophosphorylation, and potentiated virus–triggered activation of IRF3 and production of IFN-β (12).

Lithium salts (such as lithium chloride [LiCl]) and SB216763 are widely used GSK3β inhibitors (8). However, a number of conflicting results have been reported using LiCl and SB216763 (13, 14). In these cases, LiCl has been demonstrated to exert its function through a GSK3β–independent mechanism. Unlike SB216763, inhibitory effects of lithium are not solely specific for enzymes belonging to the GSK family. Lithium also inhibits at least three other enzymes, that is, pyruvate kinase, polyphosphate 1-phosphatase, and inositol monophosphatase (15–17).

In this study, we demonstrate that lithium attenuates TLR3/4- and RIG-I–mediated IFN-β production and antiviral response via inhibition of TBK1 kinase activity, with a GSK3β–independent manner.

**Materials and Methods**

**Mice and reagents**

C57BL/6J mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in...
LiCl, KCl, and SB216763 were used at a final concentration of 100 ng/ml, respectively.

Cell culture

Female C57BL/6J mice (4–6 wk old) were used for the preparation of primary mouse macrophages, and thioglycolate-elicited mouse peritoneal macrophages were prepared as described (18, 19). The HEK293 cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured at 37°C under 5% CO₂ in DMEM supplemented with 10% FCS (Invitrogen-Life Technologies), 100 U/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich). LPS, poly(I:C), LiCl, KCl, and SB216763 were used at a final concentration of 100 ng/ml, 20 μg/ml, 10 mM, and 10 μM, respectively.

Small interfering RNA transfection

For transient silencing, duplexes of small interfering RNA (siRNA) were transfected into cells with the GenePORTER 2 transfection reagent (Gene Therapy Systems, San Diego, CA) according to the standard protocol. Target sequences for transient silencing were 5’-GUCCUAGGACACCAAACAA-3’ (siRNA 1) and 5’-GGAGAGCCCAAUGUUCU-3’ (siRNA 2) for GSK3β; the scrambled control sequence was 5’-UUCCUGAACGUGUCACGUU-3’.

ELISA

An ELISA kit for IFN-β was purchased from PBL Biomedical Laboratories (Piscataway, NJ). ELISA kits for TNF-α and IL-6 were purchased from Invitrogen/BioSource (Carlsbad, CA).

RNA quantification and Western blot

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The sequences of primers were 5’-ATGAGTGGTGTTGCCAGGC-3’, 5’-TGCACCTTCTAAATACGATGA-GATICA-3’ for IFN-β and 5’-TGTACCAACTGGGACGACA-3’, 5’-CTGGGTACATCCTTCACCAGGT-3’ for β-actin. For Western blot, cells were lysed with M-PER protein extraction reagent (Pierce, Rockford, IL) supplemented with a protease inhibitor mixture, and then protein concentrations in the extracts were measured with a bicinchoninic acid assay (Pierce). Equal amounts of extracts were separated by SDS-PAGE and then transferred onto nitrocellulose membranes for immunoblot analysis as described previously (18, 19).

Assay of luciferase reporter gene expression

HEK293 cells were cotransfected with indicated luciferase reporter plasmid and pRL-TK Renilla luciferase plasmid using jetPEI transfection reagent (Polyplus-Transfection). Luciferase activities were measured with a Dual-Luciferase Reporter assay system (Promega) according to the manufacturer’s instructions as described previously (18, 20). Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

TBK1 kinase activity assay

LiCl, KCl, or SB216763 was added to magnesium/ATP buffer containing 50 ng recombinant TBK1 and myelin basic protein (MBP). After incubation for 1 h, TBK1 kinase activity was assayed with ADP-Glo and a TBK1 kinase enzyme system (Promega) according to the manufacturer’s instructions as described (20).

Vesicular stomatitis virus plaque assay and detection of virus replication

Vesicular stomatitis virus (VSV) plaque assay was performed as described (18, 20). Peritoneal macrophages (2 × 10⁵) were transfected with LiCl, KCl, or SB216763 for 36 h prior to VSV infection (MOI of 0.1). At 1 h postinfection, cells were washed with PBS three times and then medium was added. The supernatants were harvested at 24 h after washing. The supernatants were diluted 1:106 and then used to infect confluent HEK293 cells cultured on 24-well plates. At 1 h postinfection, the supernatant was removed and 3% methylcellulose was overlaid. At 3 d postinfection, overlay was removed and cells were fixed with 4% formaldehyde for 20 min and stained with 0.2% crystal violet. Plaques were counted, averaged, and multiplied by the dilution factor to determine viral titer as log₁₀ (PFU/ml). Total cellular RNA was extracted from macrophages infected with VSV, and VSV RNA replicates were examined by quantitative RT-PCR. A LightCycler (ABI Prism 7000) and a SYBR RT-PCR kit (TaKaRa) were used for quantitative RT-PCR analysis. Data are normalized to β-actin expression in each sample. Primers used for VSV replicates were as follows: 5’-ACGGCGTACTTCCAGATGG-3’ and 5’-CTCCTGCCAAGA-TCCAGGT-3’.

Viral pathogenesis in mice

C57BL/6J mice (females, 8 wk old) were i.v. infected with VSV (5 × 10⁶ PFU/mouse) or SeV (1000 tissue culture-infective dose [50%]/ml, 200 μl/mouse) as described (21). For lithium treatment, LiCl (100 mg/kg) was i.v. administered each day. The virus titers in lungs were determined by standard plaque assays. Lungs from control or virus-infected mice were dissected, fixed in 10% phosphate-buffered formalin, embedded into paraffin, sectioned, stained with H&E solution, and examined by light microscopy for histologic changes.

Statistical analysis

All data are presented as means ± SD of three or four experiments. Analysis was performed using a Student t test. A p value <0.05 was considered statistically significant.

Results

Lithium negatively regulates TLR- and RIG-I–induced IFN-β production

To investigate the possible functions of lithium on TLR- and RIG-I–induced IFN-β production, mouse peritoneal macrophages were pretreated with LiCl and SB216763, followed by stimulation with LPS (TLR4 ligand), poly(I:C) (TLR3 ligand), or infection with SeV, a type of ssRNA virus recognized by RIG-I (4). LiCl treatment significantly attenuated LPS-, poly(I:C)-, and SeV-induced IFN-β production (Fig. 1A). On the contrary, SB216763 greatly enhanced secretion of IFN-β (Fig. 1A), which is consistent with a previous report (11). As a negative control, KCl, another chloride, had no effects on IFN-β production (Fig. 1A). Consistently, LPS- and SeV-induced IFN-β mRNA level was also greatly decreased in the presence of LiCl (Fig. 1B). Furthermore, LiCl inhibited LPS-, poly(I:C)-, and SeV-induced IFN-β secretion in a dose-dependent manner (Fig. 1C). The concentration of LiCl as low as 2 mM could significantly inhibit IFN-β expression (Fig. 1C). Similarly, LPS- and SeV-induced IFN-β mRNA expression was also inhibited with LiCl treatment in a dose-dependent manner (Fig. 1D).

To further investigate whether lithium attenuates IFN-β production in vivo, IFN-β secretion in blood was measured in mice i.v. infected with SeV. As shown in Fig. 1E, mice administered with LiCl exhibited a significant decrease in the level of IFN-β in the serum, indicating that lithium inhibits IFN-β production in vivo. Collectively, these results demonstrate that lithium attenuates IFN-β production both in vitro and in vivo.

Lithium inhibits IRF3 activation

IRF3 is the key transcription factor that mediates the expression of IFN-β in TLR3/4- and RIG-I–mediated signal transduction (22). We then observed the effect of lithium on IRF3 activation using an IRF3 luciferase assay. The IRF3 reporter used in this study includes two plasmids (20). One is a fusion expression plasmid that IRF3 fused to the GAL4-DNA binding domain. Another is a luciferase reporter with a promoter fragment that allows GAL4-DNA binding domain. Therefore, activation of IRF3 will allow IRF3-GAL4 binding. Therefore, activation of IRF3 will allow IRF3-GAL4 binding.
binding and drive the expression of the luciferase. TRIF-, RIG-I–TBK1-, and IKKe-induced IRF3 activation was substantially attenuated by LiCl treatment, but not by KCl and SB216763 (Fig. 2A). As a negative control, LiCl had no effects on IRF3-induced IRF3 activation (Fig. 2A).

Next, we investigated the effects of LiCl on phosphorylation of IRF3. LPS-induced phosphorylation of IRF3 was greatly inhibited by LiCl, but not by KCl and SB216763 (Fig. 2B). Similarly, SeV-induced phosphorylation of STAT1 at Tyr 701, which is IFN-β–dependent (23). To investigate whether the effect of lithium is consistent for IFN-mediated signaling, we examined the effects of LiCl on STAT1 phosphorylation. As shown in Fig. 2B, LiCl treatment substantially attenuated LPS- and poly(I:C)-induced phosphorylation of STAT1 at Tyr 701. Taken together, these data suggest that lithium attenuated TLR- and RIG-I–induced IRF3 activation and subsequent IFN-β signaling.

Lithium attenuates IFN-β production and IRF3 activation in a GSK3β-independent manner

It has been reported that GSK3β negatively regulated TLR4-mediated production of IFN-β (11). As expected, GSK3β inhibitor SB216763 greatly enhanced IFN-β secretion. However, LiCl had the opposite effects. Furthermore, LiCl-mediated inhibition of LPS- and SeV-induced IFN-β production was not abrogated by SB216763 (Fig. 3A). Collectively, these data indicate that LiCl and SB216763 play different roles in the regulation of TLR4/3- and RIG-I–induced IFN-β production in primary macrophages. Given the facts that SB216763 did not inhibit IFN-β expression and that LiCl inhibited IFN-β expression, these data also suggest that LiCl may inhibit IFN-β expression in a GSK3β-independent manner.
NF-κB facilitates proinflammatory cytokine expression through modulating GSK3β both greatly inhibited MyD88- and TRIF-induced NF-κB expression and KCl had no effects. Additionally, LiCl and SB216763 both greatly inhibited LPS-induced TNF-α and IFN-β expression. As shown in Fig. 3F, LiCl-mediated inhibition of LPS-induced IRF3 phosphorylation was not abrogated by GSK3β knockdown (Fig. 3E). Collectively, these data indicate that LiCl attenuates IFN-β expression and IRF3 activation in a GSK3β-dependent manner.

To determine whether lithium specifically attenuates the production of IFN-β, we investigated its regulatory roles in the production of proinflammatory cytokines. As shown in Fig. 3F, LiCl and SB216763 both greatly inhibited LPS-induced TNF-α and IL-6 production in peritoneal macrophages pretreated with DMSO, LiCl, KCl, or SB216763 and then stimulated with LPS. (B) ELISA analyses of TNF-α and IL-6 production in peritoneal macrophages pretreated with DMSO, LiCl, KCl, or SB216763 and then stimulated with LPS. (C) Western blot analyses of phosphorylated IRF3 and total IRF3 in peritoneal macrophages. (D) ELISA analyses of TNF-α and IL-6 production in peritoneal macrophages pretreated with DMSO, LiCl, KCl, or SB216763 and then stimulated with LPS. (E) Western blot analysis of GSK3β expression in mouse peritoneal macrophages (Fig. 3B). GSK3β siRNA2, which has a higher efficiency to knock down GSK3β expression, was used in the following experiments. As shown in Fig. 3C and 3D, GSK3β knockdown significantly increased LPS-induced IFN-β production in mouse peritoneal macrophages, inconsistent with the previous report (11). SB216763-mediated enhancement of LPS-induced IFN-β production was abrogated by GSK3β knockdown (Fig. 3C, 3D). However, LiCl-mediated inhibition of LPS-induced IFN-β production was not abrogated by GSK3β knockdown (Fig. 3C, 3D). Furthermore, LiCl-mediated inhibition of LPS-induced IRF3 phosphorylation was not abrogated by GSK3β knockdown (Fig. 3E). Collectively, these data indicate that LiCl attenuates IFN-β expression and IRF3 activation in a GSK3β-dependent manner.

To determine whether lithium specifically attenuates the production of IFN-β, we investigated its regulatory roles in the production of proinflammatory cytokines. As shown in Fig. 3F, LiCl and SB216763 both greatly inhibited LPS-induced TNF-α and IL-6 expression and KCl had no effects. Additionally, LiCl and SB216763 both greatly inhibited MyD88- and TRIF-induced NF-κB activation (Fig. 3G). Published work has shown that GSK3β facilitates proinflammatory cytokine expression through modulating NF-κB transcription factor activity (10). LiCl and SB216763, as GSK3β inhibitors, negatively regulate proinflammatory cytokine expression and NF-κB activation in a GSK3β-dependent manner.

Lithium inhibits TBK1 kinase activity

To determine the molecular targets of lithium in TLR3/4- and RIG-I-induced IFN-β production, the effects of LiCl on IFN-β promoter activation mediated by TRIF, RIG-I, TBK1, IKKe, and IRF3 were examined in luciferase assays. LiCl treatment significantly inhibited TRIF-, RIG-I-, TBK1-, and IKKe-induced IFN-β promoter activation (Fig. 4A). IRF3-induced IFN-β promoter activation remains unchanged by LiCl treatment (Fig. 4A). Therefore, we conclude that lithium targeted molecules upstream of IFN-β and downstream of TBK1 to inhibit IFN-β production. TBK1 is essential for TLR- and RIG-I-induced IRF3 activation and IFN-β expression (24). We investigated the effect of lithium on TBK1 kinase activity by evaluating recombinant TBK1-mediated activation of MBP. TBK1 substantially activated MBP and LiCl substantially inhibited MBP activation in a dose-dependent manner (Fig. 4B). As negative controls, KCl and SB216763 had no effects on TBK1 kinase activity (Fig. 4C). Collectively, these results demonstrate that lithium inhibited TBK1 kinase activity.

Lithium inhibits host antiviral responses

Type I IFNs play critical roles in the immune responses against viral infection (25). The fact that lithium attenuates IFN-β production prompted us to investigate the function of lithium in...
antiviral immunity. VSV, a type of ssRNA virus recognized by RIG-I (4), was used to infect macrophages. Plaque assays of HEK293 cells infected with VSV showed that LiCl substantially increased viral replication in a dose-dependent manner in the presence or absence of poly(I:C) (Fig. 5A). Similarly, VSV RNA replicates in macrophages were greatly increased in LiCl-treated cells as measured by quantitative RT-PCR (Fig. 5A). In sharp contrast, KCl and SB216763 could not increase viral replication (Fig. 5B). These data suggest that lithium inhibited cellular antiviral responses.

We next investigated the physiological and pathological relevance of these regulatory effects of lithium in the context of SeV and VSV infection in vivo. Severe infiltration of monocytes and tissue damage were observed in the lungs of control mice, compared with lithium-treated mice after SeV or VSV infection (Fig. 6A). In accordance with the above results, VSV virus titer in the lungs of LiCl-treated mice was higher than control mice after VSV infection (Fig. 6B). IFN-β production in the serum from mice administered LiCl significantly decreased compared with control mice after SeV infection (Fig. 1E). Taken together, these data demonstrate that lithium inhibits the cellular antiviral response by inhibiting TBK1 kinase activity and IFN-β expression, thus facilitating viral invasion of the immune system.

**Discussion**

TBK1 plays pivotal roles in the immune responses against viral infection. TBK1 mediates the activation of IRF3 and IRF7, leading to the induction of type I IFN (IFN-α/β) in response to stimulation via TLR or viral infection (3, 4). As a critical kinase in IFN-β production, TBK1 must be tightly regulated to maintain immune homeostasis. TBK1 activity can be regulated in different levels, such as phosphorylation, ubiquitination, and modulation of its kinase activity (26). SHIP1 and protein phosphatase Mg2+/Mn2+-dependent 1B decrease the levels of phosphorylated TBK1 and inhibit IFN-β production (27, 28). E3 ligases MIB1/2 and Nrdp1 activate TBK1 by promoting its lysine 63 (K63)-linked polyubiquitination (29, 30). A20 regulatory complex including ubiquitin-editing enzyme A20, Tax1-binding protein 1, and A20 binding inhibitor of NF-κB 1 cooperate to antagonize K63-linked polyubiquitination of TBK1 (31-33). NLR protein NLRP4 recruits the E3 ligase DTX4 to TBK1 for K48-linked polyubiquitination, which leads to degradation of TBK1 (34). Previously, we reported that TRAF-interacting protein, a virus-induced E3 ligase, negatively regulates antiviral response by promoting proteasomal degradation of TBK1 (20). Glucocorticoid dexamethasone and resveratrol (3’,4’,5’-trihydroxy-trans-stilbene, a polyphenol found in grapes and other plants) could suppress the kinase activity of VSV.
TBK1 (35, 36). In this study, we demonstrate that lithium attenuates TLR3/4- and RIG-I–induced IFN-β production and antiviral responses by directly inhibiting TBK1 kinase activity.

IKKε is another kinase playing a role in IRF3 activation. Although IKKε and TBK1 have similar biochemical properties in vitro, they have different functions in vivo (37–39). TBK1 is essential for the activation of IRF3 in vivo whereas IKKε is not. In fact, IKKε is required for the activation of IFN-stimulated genes in vivo and is not required for IFN expression (39). Thus, we focused on the effects of lithium in TBK1-dependent IFN-β production in this study. Nevertheless, we investigated the effect of lithium on IKKε-induced signal transduction in vitro. Interestingly, LiCl attenuates IKKε-induced IFN-β and IRF3 luciferase gene activation (Figs. 2A, 4A). However, the potential mechanisms of lithium in IKKε-induced signaling need to be further investigated.

Magnesium (Mg2+) and ATP are both crucial for GSK3 kinase activity. SB216763 and lithium inhibit GSK3 kinase activity in different mechanisms. SB216763 is a potent and selective ATP-competitive inhibitor of GSK3. Lithium inhibits GSK3 by competition with native Mg2+ for metal-binding sites, but not ATP (40). Recently, lithium was considered as a nonspecific GSK3 inhibitor. It has been reported that lithium regulated multiple signaling pathways in a GSK3-independent manner. For example, lithium can inhibit function of voltage-dependent sodium channels and attenuate BMP-2 signaling through a GSK3-independent mechanism (13, 14). LiCl enhanced TRAIL-induced apoptosis through a caspases-dependent apoptotic pathway via death receptor signaling and G3/M arrest induced by inhibition of JNK activation, but independent of GSK3β (41). However, the exact regulatory mechanisms of lithium in such signaling remain unknown. In the present study, we show that lithium inhibits TBK1 kinase activity. Besides GSK3, lithium can also displace native Mg2+ and act as a potent inhibitor for other enzymes (42, 43). rTBK1 substantially activated MBP in an Mg2+/ATP containing environment (Fig. 4B, 4C). More importantly, incubation with LiCl inhibited TBK1-mediated phosphorylation of MBP (Fig. 4B, 4C), demonstrating that lithium could directly attenuate TBK1 kinase activity by competing with Mg2+ and inhibit phosphorylation of substrate by TBK1.

High concentrations of lithium in blood can cause side or toxic effects. The dosage used in the present study has previously been demonstrated to result in serum lithium levels within therapeutic concentrations (44). For animal experiments, lithium was commonly administered to mice in the diet to achieve serum levels equivalent to those attained therapeutically in human patients (44–47), and two injections of LiCl (100 mg/kg) i.v. on the first and second days could increase lithium levels more rapidly than can be attained by dietary administration alone (47). In the present study, we gave two injections of LiCl (100 mg/kg) i.v. for 2 d and the viral pathogenesis experiments were performed within 2 d. For cell experiments, the concentration (10–20 mM) of lithium has been widely used (13, 44–46). It has been reported that treatment with LiCl at concentrations of 0.1–30 mM had no effect on the cell viability, whereas 100 mM LiCl greatly reduced cell viability (13). In the present study, LiCl was used at the concentrations of 10 mM for cell experiments.

In the present study, we show that lithium negatively affects IFN-β production and host antiviral responses in a GSK3-independent manner. This inhibitory effect might be caused by inhibition of TBK1 kinase activity. Therefore, the use of lithium to study the function of GSK3 and explanations of the results require caution. Lithium has enormous potential as a therapeutic for bipolar disorder, diabetes, experimental autoimmune encephalomyelitis, Alzheimer disease, and several other neurologic disorders (5, 8, 47). In this study, we show that lithium attenuates IFN-β production and host defense to virus infection by targeting TBK1. Thus, more attention should be focused on using lithium-related drugs in clinical applications. Besides its antiviral activity, IFN-β has pleiotropic effects in the immune system. Excessive IFN-β production has been manifested in diverse pathogenic
autoimmune diseases (48). Thus, our results provide a strategy to downregulate IFN-β production and suggest that lithium may have therapeutic potential for the intervention of autoimmune diseases with uncontrolled IFN-β production.

**Disclosures**

The authors have no financial conflicts of interest.

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