Staphylococcus aureus Induces Microglial Inflammation via a Glycogen Synthase Kinase 3β-Regulated Pathway

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A proinflammatory role for glycogen synthase kinase 3β (GSK-3β) has been demonstrated. Here, we addressed its roles on heat-inactivated Staphylococcus aureus-induced microglial inflammation. Heat-inactivated S. aureus induced tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) production, at least in part, via a Toll-like receptor 2-regulated pathway. Neutralization of TNF-α largely blocked heat-inactivated S. aureus-induced NO. Heat-inactivated S. aureus activated GSK-3β, and inhibiting GSK-3β reduced TNF-α production as well as inducible NO synthase (iNOS)/NO biosynthesis. While activation of NF-κB was essential for heat-inactivated S. aureus-induced TNF-α and NO, inhibiting GSK-3β blocked heat-inactivated S. aureus-induced NF-κB p65 nuclear translocation. Additionally, inhibiting GSK-3β enhanced heat-inactivated S. aureus-induced interleukin-10 (IL-10) production (IL-10 is an anti-inflammatory cytokine which inhibits TNF-α production). Neutralization of IL-10 reduced TNF-α downregulation caused by GSK-3β inhibition. These results suggest that GSK-3β regulates heat-inactivated S. aureus-induced TNF-α and NO production in microglia mainly by activating NF-κB and probably by inhibiting IL-10.

Staphylococcus aureus, a gram-positive bacterium, causes a variety of diseases, such as bacteremia, peritonitis, subcutaneous and brain abscess, and life-threatening staphylococcal septic shock (15). The mechanisms that lead to staphylococcal septic shock are multifactorial but involve especially immune responses, including cytokines (TNF-α, IL-1, IL-6, IL-12, IL-18, and IL-10 itself), chemokines (IL-8, MCP-1, and RANTES), and iNOS/NO (4, 30, 43). IL-10 knockout mice display high mortality and are more susceptible to S. aureus-induced brain abscess (48). Exogenous IL-10 inhibits lethal sepsis, hepatic injury, and TNF-α production induced by staphylococcal enterotoxin B in mice (46, 48).

Inhibiting glycogen synthase kinase 3 (GSK-3) biosynthesis in S. aureus infection (49). The deregulated generation of NO contributes to S. aureus-induced circulatory failure and liver injury (34). IL-10, a potent anti-inflammatory cytokine, inhibits the synthesis of the proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, IL-18, and IL-10 itself), chemokines (IL-8, MCP-1, and RANTES), and iNOS/NO (4, 30, 43). IL-10 knockout mice display high mortality and are more susceptible to S. aureus-induced brain abscess (48). Exogenous IL-10 inhibits lethal sepsis, hepatic injury, and TNF-α production induced by staphylococcal enterotoxin B in mice (46, 48).

Inhibiting glycogen synthase kinase 3 (GSK-3) downregulates TLR-mediated inflammatory responses but increases IL-10 production (41, 53). Since NF-κB is important for inflammatory activation, GSK-3β is also involved in activating NF-κB in response to inflammatory stimuli (17–21, 29, 44, 50, 52). Therefore, GSK-3β inhibitors have been used to confer anti-inflammation against TNF-α administration, endotoxemia, experimental colitis, type II collagen-induced arthritis, ovalbumin-induced asthma, and experimental autoimmune encephalomyelitis (5, 12–14, 18, 20, 31, 41, 50, 52). Notably, current studies also show the effects of GSK-3β inhibition in reducing gram-negative cocobacillus Franciscella-induced inflammation (55). GSK-3β inhibitors have also been widely used to reduce microglial inflammation and neurotoxicity (31, 54).

In search of strategies against S. aureus-induced microglial inflammation, we investigated the possible effects of GSK-3β inhibition. In the present study, we report that inhibiting GSK-3β blocks NF-κB activation, TNF-α production, and iNOS/NO biosynthesis, but increases IL-10 production in heat-inactivated S. aureus-stimulated microglia.
RESULTS

Heat-inactivated S. aureus induces TLR2-regulated TNF-α and NO production in BV-2 mouse microglia. Using ELISA and Griess reagent assay, we found that BV-2 mouse microglial cells treated with heat-inactivated S. aureus showed a significant (P < 0.05) increase in TNF-α and NO production, compared to untreated cells, both at the time periods of 12 and 24 h of treatment (Fig. 1A). To further characterize signaling of TLR2, a receptor of gram-positive bacterial peptidoglycan, for heat-inactivated S. aureus, neutralizing IgG specific for TLR2 was used. Results showed that anti-TLR2 neutralizing IgG, but not isotype-matched antibody control, significantly (P < 0.05) blocked the production of heat-inactivated S. aureus-induced TNF-α and NO (Fig. 1B). TNF-α plays a critical role for proinflammatory responses induced by S. aureus infection (36). Using anti-TNF-α neutralizing IgG, we confirmed that heat-inactivated S. aureus induced NO production in a TNF-α-regulated manner (Fig. 1C). These results indicate that heat-inactivated S. aureus induces NO production, at least in part, via TLR2-mediated and TNF-α-regulated pathways.

Heat-inactivated S. aureus activates GSK-3β in BV-2 mouse microglia. To investigate the activation of GSK-3β in heat-inactivated S. aureus-stimulated BV-2 mouse microglial cells, we used Western blotting to determine the phosphorylation of GSK-3β and its substrate, GS. GSK-3β activity is positively regulated by dephosphorylation of GSK-3β at Ser9 and phosphorylation of GSK-3β at Tyr216 (11, 27, 28, 33). Results showed that heat-inactivated S. aureus did not induce GSK-3β dephosphorylation at Ser9 but significantly induced phosphorylation of GSK-3β (Tyr216) and GS (Ser641) (Fig. 2). Furthermore, GSK-3β inhibitors BIO and LiCl potently blocked these effects (data not shown). These results indicate that heat-inactivated S. aureus induces GSK-3β activation.

Inhibiting GSK-3β blocks the production of heat-inactivated S. aureus-induced TNF-α as well as iNOS/NO biosynthesis in microglia. Inhibiting GSK-3β decreases TLR-mediated inflammation (31, 41). We then tested the effects of GSK-3β inhibition on heat-inactivated S. aureus-induced inflammation. We found, using ELISA, that inhibiting GSK-3β with BIO significantly (P < 0.05) reduced the production of heat-inactivated S. aureus-induced TNF-α (Fig. 3A) and, using Griess reagent, that inhibiting GSK-3β with BIO or LiCl blocked NO production (Fig. 3B) in BV-2 mouse microglial cells as well as in primary rat microglia-enriched cultures. iNOS biosynthesis usually regulates NO production in microglia. To verify that inhibiting GSK-3β causes NO reduction, we further studied iNOS protein expression in heat-inactivated S. aureus-stimulated BV-2 mouse microglial cells. We found, using Western blotting, that BIO and LiCl inhibited iNOS protein expression (Fig. 3B). We also confirmed that GSK-3β regulated NO production in BV-2 cells stimulated with the
different diseases of *S. aureus* infection, including muscle abscess (S2-1065), folliculitis (S2-1509), and bacteremia (S-20) (data not shown). These results provide evidence that inhibiting GSK-3β downregulates TNF-α and iNOS/NO in heat-inactivated *S. aureus*-stimulated microglia.

Inhibiting GSK-3β blocks TNF-α and NO production by downregulating heat-inactivated *S. aureus*-activated NF-κB in BV-2 mouse microglia. GSK-3β may act upstream of NF-κB, an important transcription factor of iNOS and TNF-α (17–21, 29, 44, 50, 52). To confirm the essential role of NF-κB, we evaluated the effects of selective NF-κB inhibitors, including PDTC, CAPE, and BAY. All NF-κB inhibitors significantly (*P < 0.05*) reduced the production of heat-inactivated *S. aureus*-induced TNF-α and NO in BV-2 mouse microglial cells (Fig. 4A). To further investigate the effect of GSK-3β on NF-κB, we used immunocytochemistry to determine the nuclear translocation of NF-κB p65. We found that inhibiting GSK-3β blocked heat-inactivated *S. aureus*-induced NF-κB p65 nuclear translocation (Fig. 4B). These results indicate that inhibiting GSK-3β blocks heat-inactivated *S. aureus*-induced TNF-α and NO production by inactivating NF-κB.

Inhibiting GSK-3β increases the production of heat-inactivated *S. aureus*-induced IL-10 in BV-2 mouse microglia. TNF-α is critical for the pathogenesis of *S. aureus* infection, and IL-10 is essential for diminishing *S. aureus*-induced inflammation (48). Using ELISA, we determined the time kinetics and dose response of heat-inactivated *S. aureus* on TNF-α and IL-10 production. We found that TNF-α was time-dependently expressed following the early expression of IL-10 (Fig. 5A). To further characterize the effects of IL-10 on the production of heat-inactivated *S. aureus*-induced TNF-α, neutralizing IgG specific for IL-10 was used. Results showed that anti-IL-10 neutralizing IgG, but not isotype-matched antibody control, significantly (*P < 0.05*) increased the production of heat-inactivated *S. aureus*-induced TNF-α (Fig. 5B) as well as NO (data not shown). Early reports showed that inhibiting GSK-3β increases IL-10 in TLR signaling (31, 41). We next investigated the effects of inhibiting GSK-3β on IL-10. Treatment of either BIO or LiCl significantly (*P < 0.05*) increased the production of heat-inactivated *S. aureus*-induced IL-10 (Fig. 5C). Our previous study (31) demonstrated that IL-10 is required for inhibiting GSK-3β-regulated anti-inflammation in lipopolysaccharide (LPS)-activated microglia. To evaluate the protective role of upregulated IL-10 by inhibiting GSK-3β, neutralization experiments showed that anti-IL-10 neutralizing IgG, but not isotype-matched antibody control, significantly (*P < 0.05*) blocked inhibiting GSK-3β-induced downregulation of heat-inactivated *S. aureus*-induced TNF-α (Fig. 5D) as well as NO.
These results indicate that inhibiting GSK-3 regulates IL-10 production and IL-10 mediates TNF-α downregulation in heat-inactivated S. aureus-stimulated microglia.

DISCUSSION

Severe microglial inflammation is pathogenic for S. aureus-induced brain abscess (23, 26, 36, 37, 51). S. aureus infection causes TLR2- and NF-κB-regulated microglial inflammation, especially on TNF-α-related inflammatory responses. In search of strategies against S. aureus-induced inflammation in microglia, here we investigated the potential effects of GSK-3 inhibition according to the following rationales. First, GSK-3 has been demonstrated to be a regulator in TLR-regulated NF-κB activation and inflammation (17–21, 29, 44, 50, 52). Second, IL-10 is critical for reducing the progression of S. aureus-induced neuroinflammation (46, 48), and IL-10 can be upregulated by inhibiting GSK-3β in TLR signaling (31, 41, 55). In the present study, we provide evidence that GSK-3β regulates the production of heat-inactivated S. aureus-induced TNF-α and NO by activating NF-κB in microglia. Furthermore, GSK-3β negatively regulates IL-10 production, and IL-10 production may confer protection against heat-inactivated S. aureus-induced microglial inflammation.

Consistent with previous studies (23, 26, 36, 37, 51), we showed that TLR2 is necessary but not sufficient for the production of heat-inactivated S. aureus-induced TNF-α as well as NO in BV-2 mouse microglial cells. In addition to TLR2, other pattern recognition receptors may also be involved in recognition of live or heat-inactivated S. aureus and activation of infected cells. Furthermore, our results showed that proinflammatory TNF-α acts upstream of NO production, thereby im-
plicating TNF-α as the cause of heat-inactivated S. aureus-induced NO. Dysregulation of TNF-α and NO production are contributing factors to S. aureus-induced circulatory failure and organ injury (34, 49). Decreasing TNF-α and NO may be useful strategies against S. aureus-induced severe inflammation.

Although GSK-3β is involved in TLR-induced inflammation, the mechanisms of TLR-mediated GSK-3β activation are still unclear. We (31) and others (55) have demonstrated that TLR caused GSK-3β activation. In general, protein phosphatase and Akt are the positive and negative regulators, respectively, of GSK-3β activation by controlling phosphorylation of GSK-3β at Ser9 (11, 27, 28, 33). Furthermore, phosphorylation of GSK-3β at Tyr216 positively regulates its enzymatic activity. Our data show that inhibiting GSK-3β blocks the production of heat-inactivated S. aureus stimulation; however, the mechanisms need further investigation.

Inhibiting GSK-3β confers neuronal protection against LPS-induced microglial inflammation in neurodegenerative disorders (9, 31, 42, 54). Additionally, systemic inflammation is also regulated by GSK-3β (5, 12–14, 18, 20, 31, 41, 50, 52). Here, we show that inhibiting GSK-3β blocks the production of heat-inactivated S. aureus-induced TNF-α in BV-2 mouse microglial cells. Consistent with previous studies (5, 12, 13, 17–21, 29, 31, 41, 44, 50, 52), we found that GSK-3β can be regulated by a variety of inflammatory stimuli to cause the production of proinflammatory cytokines, including TNF-α, gamma interferon, IL-1, IL-6, IL-12p40, and COX-2, and chemokines, including RANTES and MCP-1. Furthermore, inhibiting GSK-3β blocked LPS- (31, 54) and heat-inactivated S. aureus-induced iNOS/NO biosynthesis in BV-2 mouse microglial cells. In addition to TLR4, stimulation of the natural ligands of TLR2, including peptidoglycan and lipoteichoic acid, and gram-positive Streptococcus pyogenes also caused GSK-3β-regulated NO production (data not shown). According to our results, iNOS/NO biosynthesis is downregulated due to the diminished expression of TNF-α following GSK-3β inhibition.

GSK-3β is an important regulator for a variety of transcription factors, including activated protein 1, cyclic AMP response element binding protein, heat shock factor 1, nuclear factor of activated T cells, Myc, β-catenin, CCAAT/enhancer binding protein, and NF-κB (3, 11, 27, 28, 33). A mechanistic study showed that NF-κB is inactivated in GSK-3β-deficient mice (29). However, the regulation on NF-κB signaling by GSK-3β remains unclear. Schwabe and Brenner (47) found that NF-κB p65 contains four potential GSK-3β phosphorylation sites within its COOH-terminal domain. This process causes up-regulation of NF-κB transactivation in TNF-α stimulation. In the present study, we show that heat-inactivated S. aureus-induced TNF-α and NO production is NF-κB dependent. Furthermore, we demonstrate that inhibiting GSK-3β blocks heat-inactivated S. aureus-induced NF-κB p65 nuclear translocation.
Consistent with previous studies, we show that GSK-3β modulates NF-κB activation in LPS-, TNF-α-, type II collagen-, zymosan-, and ovalbumin-induced inflammatory responses (5, 17–21, 44, 50, 52). Thus, we hypothesize that GSK-3β may contribute to NF-κB transactivation and NF-κB-regulated TNF-α and NO production. The molecular mechanisms need further investigation.

Exogenous treatment and transgenic expression of IL-10 have been used to reduce a variety of inflammatory diseases (4, 8, 16, 22, 38). In S. aureus infection, IL-10 plays a key role in reducing severe inflammation (46, 48). In the present study, we confirm that inhibiting IL-10 increases the production of heat-inactivated S. aureus-induced TNF-α. We further provide evidence that inhibiting GSK-3β also enhances the production of heat-inactivated S. aureus-induced IL-10. Nevertheless, the mechanisms need further investigation. Consistent with previous studies (41, 53), we show that inhibiting GSK-3β is able to increase the production of TLR-induced IL-10. Its biological effects by increased IL-10 are still unclear, however. IL-10 acts as an anti-inflammatory cytokine by triggering the expression of the suppressor of cytokine signaling 3 (4, 43). We previously showed that IL-10 was required for the downregulation of TNF-α and RANTES by GSK-3β inhibition in LPS-activated microglia (31). In this study, we also demonstrate that IL-10 is partly essential for inhibiting GSK-3β-conferred protection against S. aureus-induced microglial inflammation. In conclusion, activation of GSK-3β is essential for S. aureus-induced microglial inflammation. Inhibiting GSK-3β raises anti-inflammatory activities against heat-inactivated S. aureus-induced TNF-α and NO production in microglia mainly by inactivating NF-κB and probably by upregulating IL-10.

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REFERENCES

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