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*J Immunol* 2009; 182:547-553; doi: 10.4049/jimmunol.182.1.547
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Toll-Like Receptor-Mediated Production of IL-1Ra Is Negatively Regulated by GSK3 via the MAPK ERK1/2

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IL-1 receptor antagonist (IL-1Ra), a natural inhibitor of IL-1β, has been shown to regulate the progression of a variety of inflammatory diseases. Although experimental studies and clinical trials have demonstrated the importance of IL-1Ra in chronic inflammatory diseases, the cellular mechanisms responsible for regulating the endogenous production of IL-1Ra by innate immune cells are currently unresolved. In the present study, we identify that glycosyn-thase kinase 3 (GSK3) regulates the production of the anti-inflammatory cytokine IL-1Ra via its ability to regulate the MAPK ERK1/2 in TLR-stimulated cells. Elucidation of the cell-signaling pathway by which GSK3 controlled ERK activity demonstrated that GSK3 inhibition resulted in an abrogation in the levels of the inhibitory residue serine 71 on Rac1 and increased the ability of Rac1 to interact with and activate p21-activated protein kinase. siRNA-mediated knockdown of Rac1 attenuated the ability of GSK3 inhibition to augment phosphorylated ERK1/2 levels in LPS-stimulated immune cells. Moreover, inhibiting the ability of GSK3 to augment ERK1/2 activity abrogated enhanced IL-1Ra production by GSK3-inhibited cells. Our findings identify that GSK3 negatively regulates the levels of IL-1Ra produced by LPS-stimulated innate immune cells. The Journal of Immunology, 2009, 182: 547–553.

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Materials and Methods

Reagents

Ultrapure Escherichia coli 0111:B4 LPS was purchased from Invivogen. RPMI 1640 medium was obtained from the American Type Culture Collection (ATCC) and supplemented with 2 mM L-glutamine and 100 IU/ml penicillin–100 µg/ml streptomycin solution (Mediatech) with 10% FBS.
with the MEK1/2 inhibitor U0126 (50 μM) or LiCl (10 mM) for 2 h before the addition of LPS. At the indicated time points, whole-cell lysates obtained from 5 × 10⁶ monocytes were assayed for the levels of phospho-GSK3β (serine 9) using the DuoSet IC human phospho-GSK3β (serine 9) kit from R&D Systems. The Raf1 kinase inhibitor I (26) was purchased from EMD Chemicals. Lipofectamine RNAiMAX reagent was obtained from Invitrogen. The GSK3β and Rac1 SMARTpool siRNA and control siRNA reagents were purchased from Dharmacon. IL-1α and IL-1β cytokine levels were determined in cell-free supernatants by ELISA following protocols recommended by the manufacturer (R&D Systems).

Cell culture

PBMCs were obtained from healthy donors as per protocols approved by the University of Louisville, Institutional Review Board, Human Subjects Protection Program, study number 503.05. Monocytes were isolated by negative selection using the human monocyte isolation kit II from Miltenyi Biotec. The purity of monocytes was >90% as determined by flow cytometry using FITC-labeled anti-CD14. To assess the role of MEK1/2 and GSK3 in IL-1α and IL-1β production, 5 × 10⁶ monocytes were incubated with the MEK1/2 inhibitor U0126 (50 μM) and/or one of the GSK inhibitors SB216763 (10 μM) or LiCl (10 mM) for 2 h in a 96-well plate before stimulation with LPS (1 μg/ml). Experimental controls included an osmolarity control for the MEK inhibitor SB216763 (10 μM) or DMSO (0.1%) as the organic solvent control for the GSK3 inhibitor SB216763 and the MEK1/2 inhibitor U0126. Cell-free supernatants were assayed for IL-1α and IL-1β levels by ELISA 20 h post-stimulation.

Cell transfections

In brief, 2.5 × 10⁶ monocytes were transfected with 6.5 nM GSK3β, Rac1, or control siRNA using the lipofectamine reagent as per the protocol established by Invitrogen. Seventy-two hours post-transfection, cells were seeded in either a 96- or 6-well plate and used for the indicated studies. siRNA-mediated knockdown in Rac1 levels were determined by Western blot. The cellular knockdown in GSK3β levels after siRNA transfection using control or GSK3β-specific siRNA was assessed by flow cytometry.

Western blot analysis

Human monocytes (1.5–3.0 × 10⁶ cells) were seeded in 6-well plates and pretreated with medium, LiCl (10 mM), SB216763 (10 μM), or U0126 (50 μM) for 2 h before the addition of LPS. At the indicated time points, cells were washed with ice-cold PBS and whole-cell lysates were prepared as previously described (27). As determined by the microBCA method (28) (Pierce), equal concentrations of total cellular protein were suspended in lithium dodecyl sulfate buffer, heated for 10 min at 70°C, resolved by lithium dodecyl sulfate-PAGE, and then transferred to polyvinylidene difluoride membranes using the Novex system (Invitrogen). Probing and visualization of immunoreactive bands were performed using the ECL plus kit (Amersham Pharmacia) following the manufacturer’s protocol. Blots were scanned for phosphorylated and total proteins using the Kodak Image Station 400MM system (Eastman Kodak). The molecular imaging software version 4.0.5f12 (Eastman Kodak) was used to determine the mean intensity ratio of phosphorylated proteins to total levels of a loading control.

Flow cytometric analysis

Purified monocytes were plated at 2.5 × 10⁵ cells/well in a 96-well flat-bottom plate. Cells were pretreated with the indicated pharmacological inhibitors or siRNA for 2 h at 37°C. LPS (100 ng/ml) was added to cells that were harvested at the given time point, and transferred to 5 ml poly-styrene round-bottom tubes. Cells were washed twice with 2 ml of FACs buffer (PBS containing 2% FBS and 0.01% sodium azide) and then fixed by adding 500 μl of formaldehyde to a final concentration of 4% in PBS for 10 min at room temperature. Cells were washed once in PBS and resuspended in 500 μl of 90% methanol and incubated on ice for 10 min. Cells were washed in PBS containing 2% FBS, and then resuspended in PBS containing 2% FBS and an anti-phospho-ERK Ab (Alexa Fluor 488 conjugate). Cells were incubated at room temperature for 30 min. Cells were then washed twice in PBS containing 2% FBS and analyzed immediately by flow cytometry. For the detection of intracellular IL-1Ra levels, the levels of IL-1Ra were assessed by adding monensin during the last 8 h of a 20 h stimulation. Cells were fixed in 4% paraformaldehyde, permeabilized using eBioscience Perm buffer, and incubated with anti-human IL-1Ra-FTTC Ab for 30 min. Samples were washed twice with Perm buffer and analyzed immediately by flow cytometry.

Statistical analysis

Statistical significance between groups was evaluated by the ANOVA and multiple comparison test using the InStat program (GraphPad). Differences between groups were considered significant at the level of p values < 0.05.

Results

GSK3 inhibition augments IL-1Ra production by LPS-stimulated monocytes

Molnarfi et al. (19) recently demonstrated that the PI3K pathway differentially regulates IL-1α and IL-1β production by TLR-stimulated immune cells. Taken in conjunction with previous studies demonstrating that the constitutively active serine/threonine kinase, GSK3, is downstream of PI3K and that inhibition of GSK3 results in suppressed IL-1β levels (27), we next investigated whether GSK3 was the downstream kinase within the PI3K pathway responsible for the ability of PI3K to regulate IL-1α production. To initially test this possibility, human monocytes were stimulated with LPS for 30 min and the levels of phospho-GSK3β (Serine 9) were determined by ELISA (Fig. 1A). Monocytes stimulated with LPS exhibited over a 3-fold increase in phospho-GSK3β (Serine 9) levels, as compared with nonstimulated monocytes (Fig. 1A). Moreover, inhibition of PI3K using LY294002 resulted in the reduction of phospho-GSK3β (Serine 9) levels to near those observed in nonstimulated control monocytes (Fig. 1A), as well as a concurrent reduction (***, p < 0.001) in the levels of IL-1α produced by LPS-stimulated monocytes (Fig. 1B). To directly determine whether GSK3 was the downstream kinase within the PI3K pathway that differentially regulated the levels of IL-1α and IL-1β, we next inhibited GSK3 with the aid of the pharmacological inhibitors, LiCl or SB216763, as well as siRNA to knockdown cellular levels of GSK3β (Fig. 1C–G). Inhibition of GSK3 in LPS-stimulated monocytes using either lithium chloride or SB216763 significantly (***, p < 0.001) reduced the levels of IL-1β, whereas the levels of IL-1α were significantly (***, p < 0.001) augmented, as compared with monocytes stimulated with LPS alone (Fig. 1, C and D). Furthermore, as compared with siRNA control cells stimulated with LPS, siRNA-mediated knockdown in the cellular levels of GSK3β (Fig. 1E) significantly (***, p < 0.001) increased the production of IL-1α by LPS-stimulated monocytes (Fig. 1, F and G). Taken together, these data demonstrate that GSK3-β inactivation enhances IL-1Ra levels by LPS-stimulated monocytes while concurrently suppressing the levels of IL-1β.

Ability of GSK3 to regulate IL-1Ra levels by LPS-stimulated cells is dependent upon ERK1/2

Previous studies have shown that the PI3K pathway can regulate the MAPK ERK1/2 (29) and that ERK1/2 is involved in controlling IL-1α production by innate immune cells (30). Because GSK3 can be inactivated by PI3K (Ref. 31, 32 and Fig. 1A), we next wanted to determine whether the ability of GSK3-inhibition to augment IL-1Ra levels was dependent upon ERK1/2, and if so,
did GSK3 inhibition influence the levels of phospho-ERK1/2 in LPS-stimulated cells. For these studies, human monocytes were pretreated with the indicated GSK3 inhibitor in the presence or absence of the ERK1/2 (MEK1/2) inhibitor U0126, and stimulated with LPS. In this regard, the ability of GSK3 to augment IL-1Ra production by LPS-stimulated monocytes was abrogated by the use of the ERK1/2 inhibitor, U0126 (Fig. 1H). Because these data demonstrated that the ability of GSK3 to augment IL-1Ra levels by LPS-stimulated cells was abrogated by inhibiting the direct upstream kinase that can phosphorylate ERK1/2, i.e., MEK1/2, we next assessed whether inhibiting GSK3 increased phospho-MEK1/2 and phospho-ERK1/2 levels in human monocytes. As observed in Fig. 2A and B, GSK3-inhibition using either the GSK3 inhibitor SB216763 (25) or LiCl (24) enhanced both phospho-ERK1/2 and phospho-MEK1/2 (S218/221) levels, as compared with monocytes stimulated with LPS alone. In contrast, blocking the kinase activity of MEK1/2 abrogated the augmented phospho-ERK1/2 levels observed in GSK3-inactivated cells (data not shown).

We next investigated how GSK3 regulates phospho-ERK levels in LPS-stimulated monocytes at the single cell level by flow cytometry. As shown in Fig. 2C, LPS stimulation increased the percent of monocytes expressing phospho-ERK to ~6%, as compared with nonstimulated controls (0.25%). Moreover, GSK3-inhibition increased the frequency of phospho-ERK positive cells to 46%, as compared with monocytes stimulated with LPS alone (6%) (Fig. 2C). Taken together, these data demonstrate that GSK3 negatively regulates the levels of phospho-ERK and that the ability of GSK3 to modulate ERK levels is critical for its ability to regulate IL-1Ra levels by LPS-stimulated monocytes.

In contrast to the observed increases in phospho-ERK levels, inhibition of GSK3 in LPS-stimulated monocytes did not discernibly affect the levels of phospho-p38 or phospho-JNK1/2, as compared with cells stimulated with LPS alone (Fig. 2D). Taken together, these findings show that the ability of GSK3 to increase IL-1Ra levels is mediated via its ability to augment phospho-ERK1/2 levels.

**GSK3 inactivation suppresses phospho-Rac1 (S71) levels and augments the ability of Rac1 to interact with the PAK binding domain of PAK**

We next characterized the cellular mechanism by which GSK3 inhibition regulated ERK1/2 activity in LPS-stimulated cells. Because GSK3 is a constitutively active serine/threonine kinase, it
could be predicted that GSK3 phosphorylates an intermediate kinase within the ERK1/2 pathway that results in the negative regulation of its activity. Using the web-based software Scansite, (www.scansite.mit.edu), it was identified that GSK3 could potentially phosphorylate several kinases that have been reported to negatively regulate ERK1/2 activity, including c-Raf (S259) (33), c-Raf (S621) (34), and Rac1 (S71) (35). As shown in Fig. 3A, GSK3 inhibition did not discernibly affect the phosphorylated levels of c-Raf (S259) or c-Raf (S621) in LPS-stimulated monocytes, as compared with monocytes stimulated with LPS alone. In contrast, could be predicted that GSK3 phosphorylates an intermediate kinase within the ERK1/2 pathway that results in the negative regulation of its activity. Using the web-based software Scansite, (www.scansite.mit.edu), it was identified that GSK3 could potentially phosphorylate several kinases that have been reported to negatively regulate ERK1/2 activity, including c-Raf (S259) (33), c-Raf (S621) (34), and Rac1 (S71) (35). As shown in Fig. 3A, GSK3 inhibition did not discernibly affect the phosphorylated levels of c-Raf (S259) or c-Raf (S621) in LPS-stimulated monocytes, as compared with monocytes stimulated with LPS alone. In contrast,
FIGURE 4. GSK3 inactivation increases the association of active Rac1 to the p21-binding domain (PBD) of PAK. A, Active Rac1 in cellular lysates was pulled down by agarose-coupled PAK1-PBD. Equal protein concentrations (20 μg of total cellular protein) were resolved by SDS-PAGE and probed for active Rac1 levels by Western blot. B, Monocytes were incubated in the presence or absence of the GSK3 inhibitor LiCl (10 mM) or SB216763 (10 μM) for 2 h followed by stimulation with LPS (1 μg/ml). Whole-cell lysates were obtained from monocytes at the indicated time points and probed for phospho-PAK (S199/204) levels by Western blot. Immunoblots were reprobed for total β-actin to ensure equivalent loading. Data are representative of three experiments.

GSK3 inhibition reduced the levels of phosphorylated Rac1 (S71) in LPS-stimulated monocytes, as compared with cells stimulated with LPS alone (Fig. 3B). To next examine the functional role of Rac1 in GSK3-mediated ERK activation, we used the use of siRNA targeting Rac1. As shown in Fig. 3C, inhibition of GSK3 increased phospho-ERK1/2 levels in LPS-stimulated monocytes, as compared with monocytes transfected with control siRNA and stimulated with LPS. In contrast, the ability of GSK3 to augment phospho-ERK1/2 levels in LPS-stimulated monocytes was markedly reduced in cells exhibiting a knockdown in Rac1 levels (Fig. 3C). Taken together, these data demonstrate an essential role for Rac1 in the ability of GSK3 to mediate ERK phosphorylation.

The PAK is a serine/threonine-associated kinase that is a target of Rac-mediated phosphorylation (36). Moreover, the ability of PAK to interact with Rac1 has been shown to be negatively regulated by the phosphorylation of Rac1 (S71) (37). Thus, we next determined if reductions in phospho-Rac1 (S71) levels observed in GSK3-inactivated cells augmented Rac1-PAK interactions. For this, monocytes were stimulated with LPS in the presence or absence of GSK3 inhibition, and the association of PAK with Rac1 was monitored by coimmunoprecipitation (Fig. 4A). As demonstrated in Fig. 4A, GSK3-inhibition enhanced the levels of Rac1 binding to the PAK binding domain of PAK1, as compared with monocytes stimulated with LPS alone (Fig. 4A). To further examine how GSK3 inhibition affects PAK1 activity, cells were stimulated with LPS in the presence or absence of the GSK inhibitor and probed by Western blot for the activation of PAK by monitoring phospho-PAK1 (Ser199/204) levels. GSK3-inhibition increased phospho-PAK1 (S199/204) levels, as compared with monocytes stimulated with LPS alone (Fig. 4B). These findings demonstrate that GSK3 inactivation increases the association of Rac1 to PAK1 and augment the levels of phospho-PAK1 (S199/204).

PAK has previously been shown to phosphorylate several downstream kinases, including c-Raf (S338) (38), a known MEK activator. Based on our data demonstrating that GSK3 regulates PAK activity, we next examined whether GSK3 inhibition affected the activation of the downstream target of PAK, c-Raf, by monitoring phosphorylated levels of c-Raf (S338). As shown in Fig. 5A, GSK3 inhibition using either LiCl or SB216763 enhanced c-Raf (S338) levels, as compared with the levels of c-Raf (S338) in monocytes stimulated with only LPS. To define whether the enhanced phospho-c-Raf (S338) levels played a role in GSK3’s ability to control ERK1/2 activation, we used the use of a specific c-Raf inhibitor (26). As demonstrated in Fig. 5B, inhibition of c-Raf reduced the levels of phospho-ERK induced by LPS-stimulated monocytes. Moreover, c-Raf inhibition abrogated the ability of GSK3 inactivation to increase phospho-MEK and phospho-ERK levels by LPS-stimulated monocytes (Fig. 5B). These data demonstrate that the increased c-Raf activity observed in GSK3-inactivated cells is critical for the ability of GSK3 to augment phospho-ERK levels in LPS-stimulated cells.

Discussion

Due to the biological importance of IL-1Ra in counteracting the inflammatory properties of IL-1β (5, 9, 19, 39), as well as the findings by several laboratories demonstrating that the PI3K pathway was involved in the differential regulation of IL-1Ra (19, 21, 22) and IL-1β (19, 20), a major aim of the current study was to identify the downstream signaling component within the PI3K pathway that was involved in regulating IL-1Ra production. Our results demonstrate that the downstream kinase within the PI3K pathway, GSK3, regulates the production of the anti-inflammatory cytokine IL-1Ra by LPS-stimulated innate immune cells due to its ability to modulate the activity of the MAPK ERK1/2 (Fig. 6).

The PI3K pathway has been shown to regulate the production of the cytokine IL-1β (19, 20) and IL-1Ra (19, 21, 22). Studies by Learn et al. (21) demonstrated that inhibition of PI3K in septic...
monocytes. Because the relative levels of IL-1Ra to that of IL-1 negatively regulates IL-1Ra levels by LPS-stimulated human innate immune cells (19). Our laboratory previously demonstrated that inhibition of PI3K negatively regulates IL-1Ra levels by LPS-stimulated monocytes. These findings agree with those of Wang et al. (43) that identified GSK3 negatively regulates ERK activity in the human cell lines HT29 and Caco-2. However, in contrast to our present findings that GSK3 regulated ERK1/2 activity in a Rac1-dependent manner, the study by Wang et al. (43) showed that the ability of GSK3 to regulate ERK1/2 activity was via a PKCβ-dependent mechanism. Thus, although GSK3 can negatively affect ERK1/2 activation in different cell types, the cell-signaling pathway by which this occurs is likely dependent upon the cell-type and/or cellular stimulus.

Past studies have identified that the activity of the MAPK ERK1/2 is critical for the production of IL-1Ra by LPS-stimulated cells (19, 30). A study by Rabehi et al. (30) demonstrated that inhibition of MEK1/2, the kinase directly upstream of ERK1/2, abrogated the levels of IL-1Ra produced by monocytes stimulated with LPS isolated from Neisseria meningitides. Subsequent studies by others have also highlighted the importance of ERK1/2 activity in positively regulating IL-1Ra by human monocytes stimulated with E. coli LPS (19). Our current data are in agreement with these findings demonstrating that ERK activity is needed to augment IL-1Ra production by LPS-stimulated monocytes or PBMCs. The identification that GSK3 inhibition increased the levels of phospho-ERK and the levels of IL-1Ra, which could be blocked by inhibiting ERK activity, further confirms the importance of ERK in the regulation of IL-1Ra by LPS-stimulated human monocytes. However, although the present study, in conjunction with previously published findings (19, 27), identified that GSK3 inhibition negatively and positively regulates IL-1β and IL-1Ra levels, respectively, it is unlikely that the ability of GSK3 to control ERK activation is responsible for directly controlling both of these cytokines. In support of this, studies assessing IL-1Ra and IL-1β production by human monocytes have demonstrated that a loss of ERK activity results in the reduction of both IL-1Ra and IL-1β (19). Thus, the ability of GSK3 to suppress IL-1β levels by LPS-stimulated monocytes must involve an additional regulatory pathway. In this regard, the attenuation of IL-1β levels upon GSK3 inhibition in LPS-stimulated monocytes has been shown to be due to a loss of NF-κB p65 transcriptional activity (44). In contrast to the ability of NF-κB p65 to regulate IL-1β production, several studies have shown that IL-1Ra production does not appear to be dependent upon NF-κB activity (44, 45). Therefore, the ability of GSK3 to augment ERK activity while concurrently suppressing NF-κB activation is likely responsible for its ability to differentially regulate IL-1β and IL-1Ra levels.

The anti-inflammatory cytokine IL-1Ra has been demonstrated to be important in regulating the progression and severity of several inflammatory diseases (5). The present study elucidated how IL-1Ra production is regulated by LPS-stimulated cells. Our identification that GSK3 is a central kinase involved in mediating IL-1Ra production may help elucidate novel therapeutic targets in the treatment of inflammatory diseases where increased IL-1Ra levels are beneficial.

**Acknowledgments**

We thank Dr. M. Benakanakere for assistance with manuscript preparation, Dr. P. Stathopoulou for technical assistance, and Dr. D. Scott for critical reading of the manuscript.

**Disclosures**

The authors have no financial conflict of interest.