Novel Anti-inflammatory Role for Glycogen Synthase Kinase-3β in the Inhibition of Tumor Necrosis Factor-α- and Interleukin-1β-induced Inflammatory Gene Expression*§

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Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase with a broad array of cellular targets, such as cytoskeletal proteins and transcription factors. Recent studies with GSK-3β-null mice showed impaired NFκB-mediated survival responses. Because NFκB serves a dual role as a key regulator of cytokine-induced inflammatory gene expression and apoptosis, we investigated whether modulation of GSK-3β expression affects cytokine-induced and NFκB-mediated inflammatory gene expression. We observed that tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) treatment of primary cultures of human microvascular cells reduced net endogenous active GSK-3β protein levels while inducing inflammatory cytokine (IL-6 and monocyte chemoattractant protein-1 [MCP-1]) expression. Interestingly, inhibition of GSK-3β by antisense oligonucleotides or pharmacological agent (10 mM lithium) potentiated TNF-induced expression of IL-6 and MCP-1 by 2–6-fold suggesting that inhibition of GSK-3β under inflammatory conditions (exposure to TNF-α and IL-1β) may contribute to enhanced cytokine expression. Overexpression of GSK-3β in endothelial cells, in contrast, significantly inhibited (by 70%, p < 0.01) both TNF-α and IL-1β-induced expression of IL-6, MCP-1, and vascular cell adhesion molecule-1. Using adenoviruses in lipopolysaccharide-stimulated mice, overexpression of GSK-3β significantly decreased TNF-α expression in lung and heart tissues (38 and 15%, respectively), further confirming the anti-inflammatory role of GSK-3β. Overexpression of GSK-3β did not affect the TNF-α-induced nuclear translocation of NFκB but reduced the nuclear half-life of TNF-α-induced NFκB considerably (by as much as 9 h) and enhanced phosphorylation (by as much as 33%). Interestingly, neither endothelial cell survival nor NFκB-mediated expression of anti-apoptotic genes was affected by GSK-3β overexpression. We conclude that GSK-3β selectively regulates NFκB-mediated inflammatory gene expression by controlling the flow of NFκB activity between transcription of inflammatory and survival genes.

GSK-3β was originally identified in 1980 as a serine/threonine kinase involved in glycogen metabolism (1–7). Two isomers, GSK-3α and -3β, sharing an 85% overall sequence homology have been isolated in mammals. The isomers have been implicated in multiple biological processes including cell proliferation, cell differentiation, embryonic development, cell motility, cell cycle, transcription, and metabolism. Unique to GSK-3β is its reported involvement in NFκB-mediated cell survival (8). GSK-3β is also responsible for the phosphorylation of a variety of proteins, including transcription factors (c-Jun, c-Myc, and CCAAT/enhancer-binding protein), translation eukaryotic initiation factor 2, and cytoskeletal proteins. GSK-3β activity is down-regulated by phosphorylation.

In vivo, the function of GSK-3β is not clear. Data from knock-out mice studies show GSK-3β to be necessary for cell survival and NFκB activity (8). Knock-out mice deficient in components involved in the NFκB signaling pathway, such as p65 and IkB kinase-β (9–12), are observed to share a similar phenotype to the GSK-3β knock-out mice, including massive liver degeneration and TNF-α-mediated hepatocyte apoptosis. These results point to an unexpected role of GSK-3β in the suppression of TNF-α-mediated apoptosis.

NFκB is known to regulate both inflammatory genes (e.g., genes encoding IL-6, MCP-1, and VCAM-1) as well as genes related to cell survival (e.g., genes encoding Bcl-xl, IAP1, and TRAF2). Previous studies have shown data that GSK-3β affects NFκB activity in hepatocytes and fibroblasts (8, 13). However, it is not clear if GSK-3β differentially regulates NFκB-dependent inflammatory and survival genes (12, 14). Here we show that in endothelial cells, GSK-3β selectively regulates NFκB activity and inflammatory gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—Stock preparations of primary lung cell cultures from human lung microvascular endothelial cells (HMVEC) were maintained according to the manufacturer’s instructions (Clonetics), and passages no higher than 10 were used in experiments. Cells were stimulated overnight in basal microvascular medium supplemented with 0.2% bovine serum albumin. Biological reagents that were used to stimulate tissue culture cells included TNF-α and IL-1β (R&D Systems) and lithium chloride and dexamethasone (Sigma).

Western Blot Analysis and ELISAs—Extracts for Western blots were prepared using NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce). Protein concentrations were determined with BCA protein assay reagent (Pierce). Both nuclear and cytoplasmic extracts were fractionated by SDS-polyacrylamide gel electrophoresis (4–12% acrylamide) and then electroblotted onto polyvinylidene difluoride membranes. The membranes were probed with the appropriate primary antibody. The following antibodies were used: polyclonal rabbit antibodies against phospho-GSK-3β (Ser-9), phospho-NFκB p65 (Ser-536) (either Cell Signaling Technology or Santa Cruz Biotechnology), and NFκB p65 (Zymed Laboratories Inc.). Monoclonal antibodies against GSK-3β and Bcl-xl were obtained from BD Transduction Laboratories and Oncogene.
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Science, respectively. Protein detection was carried out with the appropriate species-specific horseradish peroxidase conjugated secondary antibodies (Zymed Laboratories Inc.) and enhanced chemiluminescence detection system (Amersham Biosciences). To demonstrate equivalent protein loading, membranes were probed with anti-actin antibodies and with the appropriate secondary antibodies (Zymed Laboratories Inc.) as described above. Densitometry analysis was carried out on all Western-blotted membranes using TotalLab software (Non-linear Dynamics). For total tissue TNF-α concentration, protein was extracted with T-PER tissue protein extraction reagent (Pierce) at a ratio of 20 μg/mg of tissue, and TNF-α was measured by ELISA. ELISAs were performed on tissue culture supernatants (for IL-6 or MCP-1), methanol-fixed tissue culture cells (for VCAM-1, ICAM-1), and total tissue extracts (for TNF-α). ELISA reagents including cytokine specific antibodies for IL-6, MCP-1, VCAM-1, ICAM-1, and TNF-α were obtained from RD Systems, and all protocols were carried out according to the manufacturer’s instructions. Cytokine expression data were collected at 3 h of transfection, the mixture was replaced with fresh basal treatment medium supplemented with 1% bovine serum albumin and TNF-α (10 ng/ml). After overnight treatment, the supernatants were saved for ELISA analysis, and the cells were lysed using 100 μl/well lysing solution containing 50 mM Tris–Cl (pH 6.8), 2% SDS, and 20% glycerol. The cellular lysates were assayed to determine protein concentrations using BCA protein assay reagent (Pierce). Equivalent amounts of each sample were subjected to Western blotting as described above to evaluate the effects of silencing the GSK-3β gene in stimulated microvascular cells.

NFκB Gene Arrays—Commercially available human NFκB and apoptosis arrays were obtained from SuperArray (Human PathwayFinder GEArray Q series, version 1 and Human Apoptosis GEArray Q series, version 1, respectively). Each array had 96 marker genes associated with the NFκB signal transduction pathway or apoptosis, which also included five housekeeping genes on each array. The arrays were processed according to the manufacturer’s instructions. Briefly, HMVEC were infected with Ad295ΔE1 or AdGSK-3β virus for 3 h at a multiplicity of infection of 250. Viruses were removed, and cells were allowed to recover in HMVEC growth medium (Clonetics) for 36 h. Cells were then washed and subjected to a treatment medium (basal growth medium plus 0.2% bovine serum albumin and TNF-α (10 ng/ml)) for 24 h. Total RNA was isolated by using Ambion’s RNAqueous kit, and the concentrations were determined spectrophotometrically. One microgram of RNA from each Ad295ΔE1 and AdGSK-3β-infected HMVEC was reversely transcribed into biotin-16-deoxy-UTP-labeled single-strand cDNA by using Moloney murine leukemia virus reverse transcriptase. After prehybridization, membranes were hybridized with biotin-labeled cDNA and incubated with alkaline phosphatase-conjugated streptavidin. Chemiluminescence was visualized by autoradiography. The five housekeeping genes on each array were used to confirm the integrity of RNA and the correct loading of different samples. Because the NFκB array included inflammation and only a few survival genes, we included the NFκB apoptosis array so that a representative number of survival genes would also be evaluated. By comparing the hybridization profiles in RNA samples from AdGSK-3β- and Ad295ΔE1-infected cells, we determined, for example, the expression profile for the Rel/NFκB/ixB family, which are the genes that are responsive to NFκB signaling (such as genes encoding ICAM-1–5, E-selectin, and VCAM-1) as well as other genes involved in the NFκB signal transduction pathway. The arrays were scored solely on the presence or absence of the gene expression. Array results were verified, in part, by Western blot and reverse transcription-PCR.

Plasmid and Adenovirus Constructs—PCR was done with forward primer 5′-TCCCTTTGGAATCTGCCCAGC-3′ and reverse primer 5′-GTCGAGAAGCTTATGTCAAAAGG-3′ to prescreen several cDNA libraries for GSK-3β gene amplification. Gel electrophoresis produced a 403-bp PCR amplicon that was highly expressed in a human skeletal muscle retroviral library (Clonetics). Five clones were positive for having GSK-3β cDNA. Sequence analysis showed that the clones contained GSK-3β cDNA (1263 bp) and some untranslated regions at the 5′ (145 bp) and 3′ (~300 bp) ends. A 2-kb region containing the GSK-3β expression cassette was released from pLIB retroviral vector (Clontech), using Sal and XbaI and ligated in the shuttle vector pCI-neo plasmid (Promega) for the construction of the pCI-GSK-3β encoding GSK-3β. An intermediate vector called pAdGSK-3β (7.5 kb) was then constructed. pAdGSK-3β was generated from a 3.5-kb BglII-FspI fragment from pCI-GSK-3β containing a cytomegalovirus-GSK-3β promoter and SV40 poly(A) signal. pAdGSK-3β was inserted into pZap2.2 (optical density of 260) that contained the E1 and E3 regions of the adenovirus 5 genome, between the BglII and EcoRV sites and then cloned into Escherichia coli top 1 (Invitrogen). Briefly, the expression cassette was inserted in place of the E1 region (the E1 deletion was 3151 bp long and corresponds to the region 354–3503 in the Ad5 genome). The two SfiI sites that are naturally present in wild type/Ad5 DNA were mutated, introducing silent mutations in the adenovirus PVII and DNA-binding protein coding sequences. A control plasmid, pAd295Δ, was constructed similarly, except that GSK-3β gene was omitted. Adenoviruses AdGSK-3β and Ad295ΔE1 (experimental and control, respectively) were both constructed using the Adenoma™ cloning system (AdenoZap). Virus amplification and titration were done in human embryonic kidney 293 cells, which were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Animal Experiments—One-month-old C57BL/6 mice were infected intravenously with adenovirus AdGSK-3β or Ad295ΔE1 at 10^9 plaque-forming units of virus per mouse. Three days later, the mice were treated with lipopolysaccharide (LPS) (50 μg/kg) and sacrificed 3 h later. Pre- and post-LPS plasma were collected and tested for TNF-α, IL-6, and MCP-1 expression by ELISA. Tissues including liver, lung, and heart were isolated and snap-frozen. Cellular lysates were prepared using T-PER tissue protein extraction reagent (Pierce) according to the manufacturer’s instructions and analyzed by Western blotting.

RESULTS

GSK-3β Is Down-regulated by Inflammatory Cytokines—To determine the levels of GSK-3β in the presence of inflammatory agents, we used TNF-α and IL-1β, which are proinflammatory agents and dexamethasone, an anti-inflammatory agent. Fig. 1A confirms that TNF-α and IL-1β induce a robust inflammatory response evidenced by an increased production of IL-6. Dexamethasone, as expected, did not change the basal IL-6 levels. Fig. 1B shows the effects of these agents on GSK-3β and pGSK-3β proteins (Western blot data from three independent experiments). We observed a trend for GSK-3β expression to be downregulated by proinflammatory stimuli and not anti-inflammatory agents regularly, including dexamethasone. Interestingly, phospho-GSK-3β
revealed a 75% reduction in active GSK in antisense-treated cells compared with control cells. C, effects of lithium (a pharmacological inhibitor of GSK-3β; 10 μM) on TNF-α-induced expression of IL-6. Pretreatment of HMVEC with lithium significantly enhanced TNF-α-induced IL-6 expression (8-fold).

FIGURE 2. Inflammatory response exacerbated by inhibition of GSK-3β activity. HMVEC were transfected with sense or antisense GSK-3β oligonucleotides and then stimulated with TNF-α (1 ng/ml) for 24 h. IL-6 levels in culture supernatants were determined by ELISA. Cellular lysates were analyzed for GSK-3β and pGSK-3β by Western blotting. β-Actin was used to normalize expression of GSK-3β and pGSK-3β. The histograms represent the average volume times the intensity of the bands (taken from three independent Western blot experiments). A, GSK-3β antisense-transfected cells showed a greater than 3-fold increase in IL-6 levels over the “sense” negative controls (average of three independent experiments: p < 0.01). B, analysis of GSK-3β and pGSK-3β revealed a 75% reduction in active GSK in antisense-treated cells compared with control cells. C, effects of lithium (a pharmacological inhibitor of GSK-3β; 10 μM) on TNF-α-induced expression of IL-6. Pretreatment of HMVEC with lithium significantly enhanced TNF-α-induced IL-6 expression (8-fold).

For further reading, please refer to the full-length article in JBC.
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Anti-inflammatory Effects of GSK-3β in Vivo—Next, the in vivo anti-inflammatory effects of GSK-3β were determined. We used mice infected with AdGSK-3β or control vectors and challenged them with LPS for 3 days postinfection. As expected, most of GSK-3β expressions were found in the liver. Plasma levels of TNF-α in these animals were unaffected (data not shown). The tissue expression of TNF-α, however, was significantly decreased in the lung and heart (38%, p < 0.05 and 15%, p = 0.059, respectively) (Fig. 4). Plasma IL-6 levels were also significantly decreased (p < 0.05), when GSK was overexpressed (Fig. 4C). Our in vivo and in vitro data suggest an anti-inflammatory role for GSK-3β.

Overexpression of GSK-3β Does Not Affect Nuclear Translocation of NFκB or Survival Genes—NFκB is a key regulatory molecule in inflammation. NFκB is responsible for the transcription of over 150 known genes including inflammatory genes such as those coding for IL-6, MCP-1, and VCAM-1 (12). The NFκB translocation to the nucleus is a critical event in the regulation of the NFκB-dependent genes. Therefore, we investigated whether overexpression of GSK-3β had an effect on NFκB function. Interestingly, Western blot analysis showed that overexpression of GSK-3β did not show a significant effect on nuclear NFκB levels (Fig. 5, A and C). Thus, nuclear translocation of NFκB was not interrupted by excess GSK-3β. In addition, NFκB-dependent expression of Bclw (gene associated with cell survival) was not affected by GSK-3β overexpression (Fig. 5, B and C). We further evaluated the effect of GSK overexpression on different NFκB-dependent genes using the cDNA GE array containing 96 NFκB target genes (supplemental Table 1). Total RNA from control and GSK overexpressing cells was hybridized to membranes containing probes for different NFκB genes. The expression levels were normalized to housekeeping genes on each membrane. Only 17 of 96 genes were affected by GSK-3β overexpression. The majority of those affected was inflammatory-related and showed a decrease in gene expression. Apart from cytokines, proteins related to NFκB transcription and those involved in TNF-α and toll-like receptor signaling (myd88, TIL4, and TLR3) were affected. The expression of proteins involved in apoptosis (e.g. inhibitor of apoptosis, 0.05) in cells that were infected with AdGSK-3β (Fig. 3B). Similar results were found with VCAM-1 and ICAM-1 (data not shown). Therefore, these data suggest that overexpression of GSK-3β down-regulates inflammatory gene expression.

FIGURE 3. Adenovirus-mediated overexpression of GSK-3β down-regulates cytokine expression. HMVEC were infected with recombinant adenoviruses encoding GSK-3β vector or empty vector as described under “Experimental Procedures.” Forty-eight hours after infection, cells were stimulated with TNF-α (5 ng/ml) or IL-1β (1 ng/ml) for 24 h. IL-6 and MCP-1 concentrations in tissue culture supernatants were determined by ELISA. A, Western blot analysis shows an abundant expression of GSK-3β in GSK-3β vector-infected cells compared with control vector-infected cells. B, IL-6 and MCP-1 expressions are down-regulated significantly (54–90%; *, p < 0.02; **, p < 0.03; or ***, p < 0.005) in cells infected with AdGSK-3β compared with control vector-infected cells.

FIGURE 4. Anti-inflammatory effects of GSK-3β in a mouse model of acute inflammation. Mice were injected with an adenovirus containing GSK-3β gene or empty vector at a multiplicity of infection of 108 as described under “Experimental Procedures.” Seventy-two hours postinfection, mice were injected with LPS (10 μg/kg) and sacrificed 3 h later, and plasma and tissues were collected. Cytokine levels in plasma and tissue lysates were analyzed by ELISA. The tissue expression of TNF-α was significantly decreased in the lung and heart (38%, p < 0.05, and 15%, p = 0.059, respectively) (A and B). Plasma IL-6 levels were also significantly decreased (p < 0.05) when GSK-3β was overexpressed (C).
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Our studies identified a critical role for GSK-3β in the regulation of inflammatory gene expression induced by TNF-α and IL-1β. Several lines of evidence support this observation. First, we found that TNF-α and IL-1β decreased endogenous GSK-3β expression in primary cultures of HMVEC when inflammatory cytokines like IL-6 and MCP-1 were induced (Fig. 1). When GSK-3β was partially inhibited (using antisense oligonucleotides or GSK-3β inhibitors) in TNF-α- and IL-1β-stimulated cells, proinflammatory cytokine expression was significantly increased (Fig. 2). Furthermore, expression of proinflammatory cytokines was significantly down-regulated when GSK-3β was overexpressed in stimulated cells (Fig. 3). Taken together, these data suggest that GSK-3β plays a critical role in modulating inflammatory responses.

Our data also suggest that the anti-inflammatory effects of GSK-3β are downstream of NFκB translocation. NFκB is a critical regulator of caspases, and the Bcl2 family of proteins) was not affected (supplemental Table 1). These data suggest that overexpression of GSK-3β preferentially inhibits NFκB-dependent inflammatory genes without affecting survival genes.

**FIGURE 5. Overexpression of GSK-3β does not affect nuclear translocation of NFκB nor expression of survival genes.** HMVEC were transfected with adenoviruses containing either GSK-3β vector or empty vector. Forty-eight hours later, cells were stimulated with TNF-α (1 ng/ml) for 8 h. Nuclear extracts were analyzed by Western blotting using anti-p65 (NFκB subunit) antibodies to monitor NFκB translocation (A and C; quantified data). Overexpression of GSK-3β did not show a significant effect on nuclear NFκB levels. In separate experiments, 48 h after infection, cells were stimulated with TNF-α (5 ng/ml) for 24 h, and BclxL levels were determined by (an NFκB-driven gene) Western blotting. Overexpression of GSK-3β did not show a significant effect on BclxL levels (B and D).

**DISCUSSION**

Our studies identified a critical role for GSK-3β in the regulation of inflammatory gene expression induced by TNF-α and IL-1β. Several lines of evidence support this observation. First, we found that TNF-α and IL-1β decreased endogenous GSK-3β expression in primary cultures of HMVEC when inflammatory cytokines like IL-6 and MCP-1 were induced (Fig. 1). When GSK-3β was partially inhibited (using antisense oligonucleotides or GSK-3β inhibitors) in TNF-α- and IL-1β-stimulated cells, proinflammatory cytokine expression was significantly increased (Fig. 2). Furthermore, expression of proinflammatory cytokines was significantly down-regulated when GSK-3β was overexpressed in stimulated cells (Fig. 3). Taken together, these data suggest that GSK-3β plays a critical role in modulating inflammatory responses.

Overexpression of GSK-3β reduces the half-life of nuclear p65—Although we found that nuclear translocation of NFκB is not affected by excess GSK-3β (Fig. 5), we examined whether the residence time for NFκB is affected by GSK-3β overexpression. Nuclear p65 (NFκB subunit) levels at different time points (post-TNF-α treatment) were analyzed by Western blotting. At 16 h, although 65–70% of the initial p65 was still present in control cells, it was reduced to 30% in GSK-3β-treated cells (Fig. 6A). Because GSK-3β is known to phosphorylate members of the NFκB superfamily, we tested whether the decreased half-life of NFκB was because of increased phosphorylated p65. To accomplish this, we monitored phosphorylated p65 levels and expressed them as a ratio to total p65. After 4 h, the ratio of phospho-p65/p65 was increased 3-fold in GSK-3β-infected cells and remained high (2-fold) until 8 h (Fig. 6B). Thus, it is conceivable that by phosphorylating p65, GSK-3β reduces the residence time of p65 in the nucleus.

**FIGURE 6. GSK-3β reduces the half-life of nuclear NFκB in TNF-α-induced HMVEC by affecting the phosphorylation of p65.** HMVEC were transfected with adenoviruses containing either GSK-3β vector or empty vector. A, forty-eight hours later, cells were stimulated with TNF-α (1 ng/ml) for 8, 16, and 24 h. Nuclear extracts were prepared at each time point. Nuclear extracts were analyzed by Western blotting using anti-p65 antibodies to monitor NFκB translocation (quantified data, average of triplicates, actin normalized, p < 0.05). Although overexpression of GSK-3β did not show a significant effect on nuclear NFκB levels at 8 h, at 16 h, while 65–70% of the initial p65 was still present in control cells, it was reduced to 30% in GSK-3β-treated cells. B, in parallel experiments, p65 levels were determined at different time points following TNF-α treatment (phospho-p65/p65 ratio quantified). After 4 h, the ratio of phospho-p65/p65 was increased 3-fold in GSK-3β-infected cells and remained high (2-fold) until 8 h.
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Inflammatory genes as well as genes related to cell survival. Several pathways have been shown to regulate NFκB activity (reviewed in Ref. 12), and more recently GSK-3β has been identified as an important regulator of NFκB activity (8). Loss of GSK-3β in mice results in embryonic lethality through hepatocyte apoptosis, and cells from these mice have diminished NFκB activity (8). Further studies on the mechanisms revealed that nuclear accumulation of p65 following TNF-α treatment is unaffected by the loss of GSK-3β. However, NFκB DNA binding activity is reduced in GSK-3β-null cells as well as in cells treated with lithium (8). Based on these studies, we hypothesized that GSK-3β activity is reduced in GSK-3β-null cells as well as in cells treated with lithium (8).

The activity of NFκB is controlled at several levels including the phosphorylation of p65 (RelA) subunit. The overall number of phosphorylation sites, the signaling pathways, and the protein kinases that target p65 NFκB are not completely understood. Buss et al. (17) have shown that GSK-3β regulates phosphorylation of p65 at Ser-468 and thereby controls the basal activity of NFκB. Similarly, Demarchi et al. (18) have shown that GSK-3β regulates the stability of NFκB (p105) subunits through phosphorylation. These data suggest that GSK-3β has the potential to regulate NFκB both directly and indirectly through phosphorylation. Our data support a role for GSK-3β in the modulation of NFκB function through phosphorylation of p65 subunit under stimulated conditions (TNF-α). Our data also suggest that phosphorylation of p65 significantly reduces its residence time in the nucleus, thus leading to the availability of functional NFκB.

The role of GSK-3β in diseases is also controversial. GSK-3β inhibitors have been shown to have beneficial effects in animal models of inflammation. Whittle et al. (19) have shown that GSK-3β inhibitors TDZD-8 and SB 415286 can substantially reduce the systemic inflammation associated with endotoxic shock in vivo and the acute colitis provoked by trinitrobenzene sulfonic acid in the rat. Kinase inhibitors often show nonselective activity, and it is not clear whether the inhibition is entirely because of their effects on GSK-3β. GSK-3β is known to down-regulate inflammatory pathways including JNK and phosphatidylinositol 3-kinase. JNK appears to be a major player in many inflammatory diseases. In our experiments, GSK-3β expression significantly down-regulated cytokine expression in LPS-induced inflammation. We conclude that GSK-3β could be an ideal therapeutic target in inflammation because of its “selective” anti-inflammatory effects, thus averting the global negative effects associated with total disruption of NFκB function.

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