TNF-induced activation of pulmonary microvessel endothelial cells: a role for GSK3β

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Johnson A. TNF-induced activation of pulmonary microvessel endothelial cells: a role for GSK3β. Am J Physiol Lung Cell Mol Physiol 296: L700–L709, 2009. First published February 13, 2009; doi:10.1152/ajplung.90566.2008.—The hypothesis tested was PKCα mediates the phosphorylation of glycogen synthetase kinase 3β (GSK3β) and that the GSK3β inhibition modulates the response to tumor necrosis factor-α (TNF) in rat pulmonary microvessel endothelial cells (PMEC). PMEC were treated with TNF for 4.0 h (100 ng/ml) or vehicle. First, to assess the role of PKCα in the phosphorylation of GSK3β (i.e., an indicator of GSK3β inhibition), PMEC were pretreated with 1) nonsense-RNA-PKCα, 2) siRNA-PKCα, and 3) the PKC inhibitor Gö6983. In the nonsense RNA-PKCα+TNF and TNF groups, there was increased phosphorylated GSK3β-Ser9 that did not occur in the Gö6983+TNF group. In the TNF groups, there was a significant correlation between PKCα protein and phosphorylated GSK3β-Ser9 that did not occur in the groups without TNF. Second, to assess the role of GSK3β in β-catenin activity, PMEC were pretreated with 1) wild-type (w) GSK3β plasmid to enhance GSK3β activity, 2) kinase dead (kd)-GSK3β plasmid, and 3) the GSK3β inhibitor SB-216763. In the TNF group, there was increased unphosphorylated β-catenin-Ser37/33 compared with the control group. In the GSK3β-inhibited groups (i.e., SB-216763 and kdGSK3β) ± TNF, the unphosphorylated β-catenin-Ser37/33 was similar to the TNF group. In the GSK3β-enhanced group ± TNF, the unphosphorylated β-catenin-Ser37/33 was similar to the control. Finally, PMEC were also treated with TOPFlash, a β-catenin-dependent promoter luciferase reporter, or the mutant construct FOPFlash, 2 days before treatment with TNF. In the TNF group, there was an increased TOPflash/FOPflash activity ratio compared with the control group. In the GSK3β-inhibited groups (i.e., SB-216763 and kdGSK3β) ± TNF, the TOPflash/FOPflash activity ratio was similar to the TNF group. In the GSK3β-enhanced group ± TNF, the TOPFlash/FOPFlash activity ratio was similar to the control. The data indicate that TNF induces endothelial activation that is modulated by a PKCα-dependent inhibition of GSK3β.

β-catenin is constitutively phosphorylated by GSK3α/β (1, 3, 22). Phosphorylation of β-catenin (Ser45, Ser37, Ser33, and Thr41) occurs within a multiprotein destruction complex composed of GSK3β, axin, adenomatous polyposis coli (APC), and other putative partners (1, 3, 22). Phosphorylated β-catenin is targeted for ubiquitination by β-transducin repeat containing protein (βTrCP) and degraded by the proteasome (1, 3, 22). Conversely, the inhibition of GSK3α/β is associated with suppressed phosphorylation and increased nuclear translocation of β-catenin.

TNFα is a mediator of sepsis syndrome and acute respiratory distress syndrome (9, 25, 31). In pulmonary microvessel endothelial cells, this laboratory demonstrated TNF causes a PKCα-mediated barrier dysfunction associated with dislocation of β-catenin from β-actin within the zonular adherence (10, 12, 18, 23). Thus, the dislocation of β-catenin may result in genmic activity of β-catenin that can be modulated by GSK3β activity (1, 4, 5, 15, 25). A role for GSK3β in modulating the nuclear activity of β-catenin in response to TNF in pulmonary microvessel endothelium is not known. Thus, we tested the hypothesis that PKCα activation causes inhibition of GSK3β, which modulates the nuclear activity of β-catenin in response to TNF.

METHODS

Cell Culture

Rat (RLMVEC) and bovine (BLMVEC) lung microvessel endothelial cells were obtained at 4th passage (Vec Technologies, Rensselaer, NY) (6). Preliminary studies showed that the TNF-induced responses of rat and bovine endothelium were similar using the protocols indicated below; thus, the data derived from the rat and bovine endothelium were combined. The preparations were identified by Vec Technologies as pure populations by 1) the characteristic “cobblestone” appearance as assessed by phase-contrast microscopy, 2) the absence of smooth muscle actin.

Address for reprint requests and other correspondence: A. Johnson, Dept. of Pharmaceutical Science, Albany College of Pharmacy and Health Sciences, Room 104D BRB, Albany, NY 12208 (e-mail: arnie.johnson@acphs.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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phenol-free DMEM (pD-MEM, Gibco-BRL) to avoid any antioxidant effect of phenol. The pD-MEM was supplemented with 10% FBS.

Reagents

All reagents were supplied by Sigma Chemical (St. Louis, MO) unless otherwise noted.

Highly purified recombinant human TNFα from Escherichia coli (endotoxin <0.1 ng/µg) was obtained from American Research Products (Belmont, MA). PMEM were treated with TNF at 100 ng/ml (from a stock solution of 10 µg/ml), a dose that induces a consistent permeability increase (13, 23). G66976, a selective inhibitor of cPKC (26), and SB-216763, a selective inhibitor of GSK3β that blocks the GSK3β binding site, were provided by Dr. Woodgett (28), were obtained from BIOMOL International (Plymouth Meeting, PA). LiCl2 was used as a positive control for chemical inhibition of GSK3β. PMEM were cotreated with TNF with G66976 (50 nM), SB-216763 (1 nM), or LiCl2 (30 mM) control for chemical inhibition of GSK3β.

Plasmids, siRNAs, and Transfections

Plasmids. Hemoagglutinin (HA)-tagged plasmids, human HA-GSK3β (wild type: wGSK3β) and HA-GSK3β K85A (kinase dead: kδGSK3β), ligated to CMV-driven pcDNA3 vectors (Addgene plasmids 14753 and 14755, developed by Dr. Woodgett, ref. 28), were provided by Dr. Woodgett and were used to elucidate the role of GSK3β in the response to TNF (17, 28). The kinase dead-GSK3β isoform is derived by the mutation within the catalytic site Lys85 and Lys86 to Meth85 and Ala86.

siRNAs. Double-stranded predesigned HPLC-grade siRNA oligos were obtained from Eurofins MWG Operon (Huntsville, AL). The following siRNA oligos were used: rat PKCα 1 AGAAGGCAACAGUG-GAACUCtt, rat PKCα 2 GGAGCCAAAGAGAUUACtt, and non-specific control AGGUAGUUGAUAGCGGUUtt.

siRNA transfection. Transfection of siRNAs was accomplished using the Targefect-siRNA transfection kit (Targeting Systems). Transfection efficiency was measured as follows: six-well plates were transfected with 1 ml of complex/well for 2 h, after which the complex and media were removed and replaced with normal growth medium, and the cells were incubated for 24 h until confluent.

Immunoblotting

Protein identification using PAGE-Western blot was done with adaptations of previously described techniques from this laboratory (12, 13, 23). Equal quantities of cell lysates, 12–16 µg/lane, were separated on either 8–16% gradient or 7.5% single density 1.5-mm-thick polyacrylamide minigels. Equal loading of proteins was confirmed in all blots following the final immunoblot by stripping the blots, incubating with Bradford protein reagent, destaining with successive washes of a 50% methanol 1% acetic acid solution, and then comparing total protein densities between lanes as previously described (23). The gels were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA), rinsed in TBS (10 mM Tris-HCl, pH 7.5, 100 mM NaCl), blocked in bilto plus phosphate inhibitors (BPI) (TTBS: (TBS: 0.05% Tween 20), 5% wt/vol nonfat dry milk, 50 mM NaF, 0.1 mM activated Na3VO4, and) and probed in a 1:2,000 dilution of rabbit polyclonal anti-phospho-GSK3α/β-Ser21/9 (Cell Signaling) according to the manufacturer’s directions. Following five 5-min washes in TTBS, the blots were then incubated for 1.5 h at room temperature in BPI containing a mixture of goat anti-rabbit (Santa Cruz) and goat anti-biotin (Cell Signaling) horseradish peroxidase (HRP) conjugates diluted 1:5,000 each. After imaging, the blots were stripped with Restore Western Blot Stripping Buffer (Pierce), reblocked in BPI, washed, and incubated for 2 h at room temperature in TTBS + 5% BSA containing mouse monoclonal anti-phospho-
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GSK3α/β-Tyr279/216 (clone 5G-2F, Millipore) diluted 1:2,500, followed by 1 h in BPI with the appropriate secondary antibodies. The blots were again stripped after imaging, reblocked, and reprobed in BPI with either a 1:2,000 dilution of rabbit polyclonal anti-β-catenin (Santa Cruz) or a 1:5,000 dilution of mouse monoclonal anti-non-phosphorylated-β-catenin (clone 8E4, Millipore), which binds only β-catenin with non-phosphorylated Ser33 and Ser37. The primary and secondary incubations for these immunobots were both at 37°C for 45 min each. For confirmation of transfact expression, blots were probed with either goat polyclonal anti-HA-HRP conjugate (Bethyl Laboratories, Montgomery, TX) or mouse monoclonal anti-luciferase (clone LUC-1, Sigma), diluted 1:5,000 each in BPI for 1 h at room temperature. Cruz Marker (Santa Cruz Biotechnology, Santa Cruz, CA) molecular weight standards and/or Biotinylated Protein Ladder (Cell Signaling Technology, Danvers, MA) were used for in-image molecular weight reference. Imaging substrates were used West Dura Extended Duration Substrate (Pierce; GSK3, luciferase, hemagglutinin) or Supersignal West Pico Chemiluminescent Substrate (Pierce; GSK3, luciferase, hemagglutinin) or extended duration substrate (Pierce). Images were acquired on a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY) and analyzed for net band intensity using the ROI threshold method with Kodak 1D software.

Luciferase Assay

TOPflash and FOPflash transfected cells in six-well plates were harvested after treatments in 100 μl/well Lysis Solution (Tropix PE Biosystems, Bedford, MA) containing anti-protease (1×) and phosphatase-1 inhibitor cocktails and 0.5 mM DTT. Lysates cleared by centrifugation at 18,500 g for 30 min at 4°C were analyzed for protein content and normalized to 0.7 μg/μl. Luciferase activity was measured in 7 μg lysate/well against 0.5–50 pg/well recombinant luciferase (Promega, Madison, WI) in 96-well white microplates (no. 3917; Corning Costar, Cambridge, MA) with Luciferase Assay Reagent (Promega) on a Wallac 1420 Victor2 multilabel counter (EG&G Wallac, Turku, Finland). Results were calculated to yield luciferase (pg)/total protein (μg) and are reported as a ratio of TOPflash/FOPflash for a given treatment.

Statistics

A one-way ANOVA was used to compare values among the treatments. If significance among treatments was noted, a post hoc multiple comparison test was done with a Bonferroni (parametric–equal variance) test to determine significant differences among the groups (29). A Student’s t-test was performed when appropriate. Each PMEM well and flask represents a single experiment. All data are reported as means ± SE. Significance was at P < 0.05. There are 5–10 samples per group in all studies.

RESULTS

TNF Causes the Phosphorylation of GSK3α/β

The literature implicates GSK3α/β in the lung microveessle endothelial response to TNF (31, 32); therefore, the following experiments will demonstrate if TNF induces a change in the phosphorylation of GSK3α/β. Figure 1A (n = 5) is a representative Western blot of phospho-Ser21/Ser9-GSK3α/β using lysate from the control and TNF group. Inactive GSK3α/β is detected using anti-phospho-Ser21/Ser9-GSK3α/β (i.e., GSK3α-Ser21 and GSK3β-Ser9). The blot shows phospho-GSK3α-Ser21 and phospho-GSK3β-Ser9 at the expected molecular weight of the modified isoforms.

Figure 1B (n = 5) demonstrates mean lysate phospho-GSK3α-Ser21 and phospho-GSK3α-Ser9 for the control and TNF group. In the TNF-4.0-h group, there was an insignificant increase in the phospho-GSK3α-Ser21 but a significant increase in phospho-GSK3β-Ser9 compared with the control group. In addition, the same blots were stripped and reprobed for phospho-GSK3α-Tyr279 and phospho-GSK3β-Tyr216 indicating insignificant decreases in the TNF group compared with the control group (Table 1). Finally, the same blots were stripped and reprobed for native GSK3β indicating no significant affect on native GSK3β expression between the control and TNF group (control 83,467 ± 6,624 relative density units vs. TNF 69,348 ± 7,539 relative density units). In separate studies, the PMEM were treated with TNF-0.5 h, which showed no significant change in the phospho-GSK3β-Ser9, compared with the control group (control 7,857 ± 687 relative density units vs. TNF 7,689 ± 367 relative density units).

Figure 1C (n = 5) demonstrates mean compartmentalized phospho-GSK3β-Ser9 for the control and TNF group. In the TNF-4.0-h group, the increased phospho-GSK3β-Ser9 is in the cytoplasm, and, interestingly, in the nuclear compartment of the PMEM. The data of Fig. 1 show that TNF induces the phosphorylation of GSK3β-Ser9, an indication of decreased activity of GSK3β in response to TNF.

PKCα Causes the Phosphorylation of GSK3β

PKCα modulates GSK3β phosphorylation in different cell types; however, the role of PKCα in TNF-induced GSK3β phosphorylation in lung microvesSEL endothelial cells is not known (4). The following experiments will test the idea that PKCα modulates the phosphorylation of GSK3β. Figure 2A (n = 5) demonstrates mean lysate phospho-GSK3β-Ser9 for the control and G66976 ± TNF groups. In the TNF-4.0-h group, there is an increase in the phospho-GSK3β-Ser9 compared with the control group. In the G66976 + TNF group, there is no change in the phospho-GSK3β-Ser9 compared with the G66976 group.

Table 2 shows the PKCα protein levels in the control, nonsense-RNA, and siRNA-PKCα groups with and without TNF. In the siRNA-PKCα ± TNF groups, there is a significant depletion of PKCα protein compared with the respective control and nonsense-RNA groups. In the TNF and nonsense-RNA + TNF groups, the PKCα protein was less than in the respective control and nonsense-RNA groups. Figure 2B (n = 5) demonstrates mean lysate phospho-GSK3β-Ser9 vs. the PKCα protein levels in the control, nonsense-RNA, and siRNA-PKCα groups with and without TNF. In the control, nonsense-RNA, and siRNA-PKCα groups, there is no correlation between phospho-GSK3β-Ser9 vs. the PKCα protein levels. In the TNF, nonsense-RNA + TNF, and siRNA-PKCα + TNF groups, the increase in phospho-GSK3β-Ser9 positively correlates y = 10.204(x)/25.912 + x (P = 0.0126) with increasing PKCα protein. The data of Fig. 2 support the idea that TNF induces the phosphorylation of GSK3β-Ser9, which is modulated by PKCα.

TNF Induces Increased Unphospho-β-Catenin-Ser33/Ser37 That Is Mediated By GSK3β

β-catenin is a primary target for GSK3β-mediated phosphorylation; therefore, the purpose of the next series of experiments is to prove that the TNF-induced inhibition of GSK3β is associated with increased unphospho-β-catenin-Ser33/Ser37 in the pulmonary microvesSEL endothelium.
Figure 3 \((n \geq 5)\) demonstrates mean total lysate unphospho-
\(\beta\)-catenin-Ser33/Ser37 and the associated nuclear/cytosol ratio in the control and TNF group. In the TNF-4.0-h group, there is increased total and nuclear/cytosol ratio of unphospho-\(\beta\)-catenin-Ser33/Ser37 compared with the control group. The data indicate that TNF causes an increase in the nuclear translocation of unphospho-\(\beta\)-catenin-Ser33/Ser37.

Table 1. \(p\)-GSK3\(\alpha\)-Tyr279 and \(p\)-GSK3\(\beta\)-Tyr216

<table>
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<th>Control</th>
<th>TNF</th>
<th>Control</th>
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<tr>
<td>Relative Density Units</td>
<td>44,926±2,06</td>
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Figure 4A \((n \geq 5)\) demonstrates mean total lysate unphospho-
\(\beta\)-catenin-Ser33/Ser37 in the control and SB-216763 groups with and without treatment with TNF. In the TNF and SB-216763 group, there is increased unphospho-\(\beta\)-catenin-Ser33/Ser37 compared with the control group. In the SB-216763 group, the unphospho-\(\beta\)-catenin-Ser33/Ser37 was similar to the value in the SB-216763 + TNF and TNF groups. In the lithium-treated group, there was increased unphospho-\(\beta\)-catenin-Ser33/Ser37 compared with the control group, which verified GSK3\(\beta\) dependence for unphospho-\(\beta\)-catenin-Ser33/Ser37.

Figure 4B \((n \geq 5)\) is a representative Western blot for detection of HA using lysate from the control, wGSKK3\(\beta\), and kd-GSK3\(\beta\) groups with and without TNF treatment. The blot shows similar increases in the HA, based on the expected molecular weight, in the GSK3\(\beta\) groups with and without TNF.
treatment. The same blot was stripped and reprobed for GSK3β indicating that transfection had no affect on the native GSK3β expression and equally increased the wGSK3β and kd-GSK3β to 5× the native GSK3β level (data not shown).

Figure 4C demonstrates, in separate studies, mean total lysate unphospho-β-catenin-Ser33/Ser37 in the wGSK3β (i.e., activity repletion) and kd-GSK3β (i.e., activity inhibition) groups with and without treatment with TNF. In the wGSK3β group, there is no change in unphospho-β-catenin-Ser33/Ser37 compared with the wGSK3β group. In the kdGSK3β group, there is increased unphospho-β-catenin-Ser33/Ser37 compared with the wGSK3β group. In the kdGSK3β group, the unphospho-β-catenin-Ser33/Ser37 was similar compared with the kdGSK3β + TNF group, and greater than the value in the wGSK3β + TNF group. The data indicate that TNF causes an increase in unphospho-β-catenin-Ser33/Ser37 that is mediated by inhibition of GSK3β.

The purpose of the following protocols is to test the idea that the increase in unphospho-β-catenin-Ser33/Ser37 is due to a TNF-induced increase in the total β-catenin. Figure 5A demonstrates mean total lysate β-catenin (i.e., using an antibody against an idiotype sequence distal to the β-catenin phosphorylation sites Ser33/Ser37) and the associated nuclear/cytosol ratio in the control and TNF groups. In the TNF-4.0-h group, there is no change total β-catenin compared with the control group; however, there is an increased total β-catenin nuclear/cytosol ratio compared with the control group.

Figure 5B shows, in separate studies, the β-catenin in the control and SB-216763 + TNF groups. In the control + TNF and SB-216763 + TNF groups, there is no change in β-catenin compared with the respective control and SB-216763 groups. Figure 5C shows (n ≥ 4), in separate studies, total lysate β-catenin in the wGSK3β and kdGSK3β groups with and without TNF treatment. In the wGSK3β + TNF and kdGSK3β + TNF groups, the transfection had no affect on the native GSK3β expression and equally increased the wGSK3β and kd-GSK3β to 5× the native GSK3β level (data not shown). Figure 2. PKCα mediates phosphorylation of GSK3β-Ser9. A: mean data of phospho-GSK3β-Ser9 in the control and Go6976 groups with and without TNF. B: data of phospho-GSK3β-Ser9 vs. PKCα protein in the control, nonsense-RNA, siRNA groups with and without TNF. *Different (P < 0.05) from the control group using an unpaired t-test.

Table 2. PKCα

<table>
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<th>siRNA</th>
<th>siRNA + TNF</th>
<th>nonRNA</th>
<th>nonRNA + TNF</th>
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<td>Units</td>
<td>47.662±1.717</td>
<td>36.351±1.074*</td>
<td>20.259±1.082#</td>
<td>25.594±3.086#</td>
<td>48.831±3.411</td>
<td>39.564±2.877*</td>
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*Different from respective group without TNF; #different from all other groups without siRNA.
there is no change in total β-catenin compared with the respective wGSK3β and kdGSK3β groups. The data support the idea that the TNF-induced increase in unphospho-β-catenin-Ser33/37 is because of a decrease in the phosphorylation of an unchanged total pool of β-catenin, rather than due to the constitutive phosphorylation of an increased total pool of β-catenin.

**TNF Induces Increased β-Catenin-Dependent Promoter Activity Mediated By GSK3β**

The purpose of the following experiments is to show that an outcome of TNF-induced phosphorylation of GSK3β and increased unphospho-β-catenin-Ser33/37 is altered activity of β-catenin-dependent promoter activity. Figure 6A (n ≥ 5) is a representative Western blot demonstrating the luciferase protein for the control and TNF group. The blot shows TOPflash plasmid transfection causes expression of luciferase protein based on the expected molecular weight.

Figure 6B (n ≥ 5) demonstrates the TOPflash/FOPflash luciferase activity ratio in the control, SB-216763, wGSK3β, and kdGSK3β groups with and without treatment with TNF. FOPflash (i.e., mutant consensus sequence for β-catenin-dependent activity) is used to correct for β-catenin-independent expression and transfection efficiency. In the TNF and SB-216763 group, there is increased TOPflash/FOPflash luciferase activity ratio compared with the control group. In the SB-216763 group, the TOPflash/FOPflash luciferase activity ratio is similar to the value in the SB-216763 + TNF and TNF groups. In separate studies, Fig. 6B demonstrates the mean TOPflash/FOPflash luciferase activity ratio in the wGSK3β and kdGSK3β groups with and without treatment with TNF. In the wGSK3β + TNF group, the TOPflash/FOPflash luciferase activity ratio is similar to the value in the wGSK3β group. In the kdGSK3β group, there is increased TOPflash/FOPflash luciferase activity ratio compared with the wGSK3β group. In the kdGSK3β group, the TOPflash/FOPflash luciferase activity ratio is similar to the value in the kdGSK3β + TNF group and greater than the value in the wGSK3β + TNF group. The data of Fig. 6 support the notion that an outcome of TNF-induced phosphorylation of GSK3β and increased unphospho-β-catenin-Ser33/37 is altered activity of β-catenin-dependent promoter activity.

**DISCUSSION**

The present data indicate the novel finding, in pulmonary microvessel endothelium, that TNF causes phosphorylation of
GSK3β-Ser9, an indicator of GSK3β inhibition, which is modulated by PKCα. In addition, the present study supports the theory that TNF induces an increase in unphospho-β-catenin-Ser33/37, which is modulated by inhibition of GSK3β because of the following facts. First, the TNF-induced increase in the GSK3β-Ser9 directly correlates with the enhanced unphospho-β-catenin-Ser33/37. Second, in the kdGSK3β and SB-216763 groups, there is increased unphospho-β-catenin-Ser33/37 compared with the respective wGSK3β and control groups. In the kdGSK3β plasmid, the mutation of Lys85 and Lys86 to Meth85 and Ala86 inhibits the catalytic site, thereby preventing GSK3β-dependent phosphorylation (28). Third, in the GSK3β-inhibited groups (i.e., kdGSK3β and SB-216763), the level of unphospho-β-catenin-Ser33/37 was similar to the TNF group. Fourth, in the GSK3β-inhibited groups, there was no further TNF-induced increase in unphospho-β-catenin-Ser33/37. Fifth, in the wGSK3β + TNF group, there was no increase in unphospho-β-catenin-Ser33/37 compared with the wGSK3β group, which was less than the values in the TNF, kdGSK3β + TNF, and SB-216763 + TNF groups. The repletion of GSK3β activity using wGSK3β assumes that the phosphorylation-mediated inhibition of GSK3β is substrate limited, reaching an asymptote within the concentration range of the expressed wGSK3β. The dose of 1.5 µg/ml for wGSK3β was chosen based on preliminary studies indicating that a lower dose of 1.0 µg/ml for wGSK3β did not prevent the TNF-induced increase in unphosphorylated β-catenin. Importantly, the present study shows that the dose of 1.5 µg/ml for wGSK3β consistently prevented the increase in unphosphorylated β-catenin. Finally, the positive control lithium chloride, a classic inhibitor of GSK3β, caused an increase in the unphospho-β-catenin-Ser33/37 (3). The effect of GSK3β plasmid transfection on the response to TNF is specific to the GSK3β isoform, rather than transfection, because of the different

Fig. 5. TNF induces an increase in nuclear translocation of total β-catenin. A: mean (n = 5) total lysate β-catenin and associated nucleus/cytoplasm ratio, derived from the immunoblots, for the control and TNF group. B: mean total lysate β-catenin for the SB-216763 + TNF group. C: mean (n = 5) total lysate β-catenin for the GSK3β-plasmid isotypes with and without TNF. *Different (P < 0.05) from the control group using an unpaired t-test.
TNF-mediated outcomes between the similarly constructed wild-type and inactive GSK3β plasmids. Interestingly, TNF induced an insignificant increase in GSK3α-Ser21 and an insignificant decrease in phosphorylation of GSK3α-Tyr279 and GSK3β-Tyr216, an indication of complex and possibly time-dependent mechanisms for further reductions in GSK3α/β activity.

In the present study, the TNF-induced increase in phosphorylation of GSK3β-Ser9 was inhibited by Gö6976, an inhibitor of the classic isoforms PKCα, PKCβ, and PKCγ (26). It is known that PKC can alter the phosphorylation of GSK3β in other cell types (1, 4, 22). This laboratory previously showed that antisense-PKCα prevents the TNF-induced alterations in endothelial barrier function (12). A limitation with the use of antisense/siRNA is the inability to completely deplete the target protein without inducing untoward events. We could not completely deplete PKCα, which is similar to our previous study, but discovered the important direct correlation of phospho-GSK3β-Ser9 (i.e., dependent variable) with PKCα protein levels (i.e., independent variable). The correlation occurred only among the TNF groups and not in the similarly treated groups without the TNF. The use of this regression $y = 10.204(x)/[25.912 + x]$ is the result of determining the best fit for the significance of the relationship between PKCα and phospho-GSK3β-Ser9, which infers that the relationship of PKCα with phospho-GSK3β-Ser9 is characteristic of ligand binding. The formula indicates that in the range of PKCα protein (i.e., 10,000–60,000 relative density units) there is a TNF-induced functional association between PKCα and GSK3β-Ser9, which supports the previous data that TNF activates PKCα in bovine lung arterial endothelium (12). PKCα may directly phosphorylate GSK3β or modulate other events including control of Akt activity, which can phosphorylate GSK3β (3, 11, 22, 25, 28).

Presently, in the rat lung microvessel endothelium, TNF causes a decrease in PKCα protein that contrasts with our previous data in bovine lung microvessel endothelium showing an increase in PKCα protein at TNF-4.0 h (27). Activated PKCα is degraded by calpains, a noted mechanism for a decrease in PKCα (27). Importantly, the PKCα inhibitors siRNA-PKCα and Gö6976 prevent the effect of TNF, despite the differential affect of TNF on the PKCα protein. We previously showed that PKC and/or PKCα mediates TNF-induced increases in superoxide, peroxynitrite, lung vasoconstriction, edema, and endothelial permeability, events that can be linked to GSK3β (25, 27).

In the present study, TNF induced an increase in the β-catenin-dependent TOPflash/FOP flash activity ratio indicating enhanced genomic activity of β-catenin (17). Similarly, other inflammatory mediators, such as histamine and thrombin, increase the nuclear translocation of β-catenin that is correlated with the increased activity of the β-catenin-sensitive promoter-report construct TOPflash (2, 16). The TNF-induced increase in the TOPflash activity is dependent on the suppression of GSK3β activity because in the kdGSK3β and SB-216763 groups 1) there was increased TOPflash/FOP flash activity ratio compared with the control group, 2) the TOPflash/FOP flash ratio was similar to the TNF group, and 3) TNF did not induce a further increase in the TOPflash/FOP flash ratio. The efficacy of SB-216763 and kdGSK3β for decreasing GSK3β activity, as noted above, is supported by the increased unphosphorylated β-catenin-Ser33/37 in the SB-216763 and kdGSK3β groups. In addition, in the wGSK3β + TNF group, the competitive inhibition of the TNF-induced increase in the TOPflash/FOP flash ratio can be explained by the prevention of the increase in unphosphorylated β-catenin as indicated above. The TOPflash/FOP flash ratio represents β-catenin-dependent TCF-transcriptional activity because the FOP flash vector, by not having active β-catenin/TCF-consensus binding sites, corrects for nonspecific transfection events (11, 17).

Nuclear β-catenin, via the interaction with transcription factors TCF, LEF-1, and other putative partners, can alter activity of the promoter region of genes such as cyclooxygenase, iNOS, and VE-cadherin (7, 8, 14, 16, 20, 24, 30). Importantly, inflammatory mediators such as cyclooxygenase, iNOS, and VE-cadherin are within the paradigm for TNF-induced vascular injury; therefore, β-catenin-dependent transcription activity may indeed impact on pulmonary vascular injury (7, 8, 10-204(25,912)/10,204(25,912)


