Published in final edited form as:

Pulm Pharmacol Ther. 2013 December ; 26(6): . doi:10.1016/j.pupt.2013.06.001.

Inhibition of GSK3 α/β promotes increased pulmonary endothelial permeability to albumin by reactive oxygen/nitrogen species

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Abstract

Glycogen synthase kinase 3 / (GSK3 /) is a serine/threonine kinase that participates in numerous processes in many cell types. Importantly, the role of GSK3 / in homeostatic maintenance of the pulmonary endothelial cell barrier to protein is not known. We tested the hypothesis that GSK3 / regulates endothelial barrier function by measuring the permeability to albumin of a rat pulmonary microvessel endothelial cell monolayer (PMECM) treated with and without the selective GSK3 / inhibitor SB 216763 (1.0, 5.0 and 10 μ M) for 1 hour. The treatment with the inhibitor SB 216763 caused a dose dependent decrease in phospho- -catenin-Ser^{33/37} levels indicating effective suppression of GSK3 / . SB 216763 caused an increase in both permeability to albumin and DCFDA (6-Carboxy-2,7-Dichlorodihydrofluorescein Diacetate, Di(Acetoxyethyl Ester)) oxidation that were prevented by co-treatment with the anti-oxidant tiron or the nitric oxide synthase inhibitor L-NAME (N -nitro-L-arginine-methyl ester). In separate studies PMECMs were treated with the Akt inhibitor triciribine (12.5 μ M) for 1 hour to unmask Akt dependent constitutive suppression of GSK3 / . Triciribine decreased phospho-GSK3 / -Ser^{21/9} (i.e., the product of Akt) which was associated with an increase in phospho- -catenin-Ser^{33/37} (i.e., the product of GSK3 /) indicating constitutive activity of Akt for GSK3 / -Ser^{21/9}. The data indicates GSK3 / inhibition causes increased endothelial monolayer protein permeability which is mediated by reactive oxygen/nitrogen species.

Keywords

endothelial permeability; Inflammation; -catenin; Lung injury

Introduction

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that exists in two isoforms which are GSK3 α and GSK3 β [1]. GSK3 / has constitutive activity for various substrates such as glycogen synthase [1], Tau [1] and -catenin [2–4]. GSK3 / is inactivated by the phosphorylation of serine 21 of GSK3 α or serine 9 of GSK3 β by Akt [5, 6] and/or PKC (e.g., , ,) [1, 2, 7, 8]. GSK3 / has been shown to regulate pathways that are pertinent to

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inflammation such as the decreased expression of occludin, claudin-1 and E-cadherin in intestinal and kidney epithelial cell lines following inhibition of GSK3 / [9]. In a variety of epithelial cell lines, inhibition of GSK3 / increases inducible nitric oxide synthase (iNOS) expression and ^oNO generation [10]. Conversely, GSK3 / inhibition has been shown to suppress lung vascular inflammation in response to a variety of conditions such as hemorrhage and resuscitation [11], asthma [12], carrageenan [13], tumor necrosis factor [14] and experimental spinal cord trauma [15]. The pulmonary inflammatory response *in vivo* is characterized, in part, by increased vascular permeability to protein which is prevented by inhibitors of GSK3 / [3, 12, 13]. In addition, we showed that reactive oxygen/nitrogen species increase albumin permeability of lung endothelial monolayers and pulmonary vascular permeability [14, 16, 17]. Yet, despite the protective effect of GSK3 / inhibition on the vasculature *in vivo*, the effect of GSK3 / inhibition on lung vascular permeability and the generation of reactive oxygen/nitrogen species in endothelium is not clear.

The GSK3 / inhibitor SB 216763 [3, 14] blocks the binding site for ATP of GSK3 / and is a commonly used pharmacologic agent to assess the role of GSK3 / inhibition in vascular biology. Yet, the effect of inhibition of GSK3 / activity on lung microvessel endothelial cell pathways pertinent to lung inflammation have never been studied; therefore, the present study examines the effect of altered GSK3 / activity, induced by SB 216763, on albumin permeability and reactive oxygen-nitrogen species generation of a pulmonary microvessel endothelial cell monolayer (PMECM).

Materials and Methods

Pulmonary Microvessel Endothelial Cell Culture

Rat pulmonary microvessel endothelial cell monolayers (PMECM) were studied using our previously published methods [17]. In brief, rat lung microvessel endothelial cells (RLMVEC) were obtained at 4th passage (Vec Technologies, Rensselaer, NY). The preparations were identified by Vec Technologies as pure populations by: 1) the characteristic “cobblestone” appearance as assessed by phase contrast microscopy, 2) the presence of factor VIII-related antigen (indirect immunofluorescence), 3) the uptake of acylated low-density lipoproteins, and 4) the absence of smooth muscle actin (indirect immunofluorescence). For all studies, RLMVEC were cultured from 4 to 10 passages in culture medium consisting of MCDB-131 complete media (VEC Technologies) supplemented with 20% fetal bovine serum (FBS) (Hyclone; Hyclone Laboratories, Logan, UT). The cells were maintained in 5% CO₂ plus humidified air at 37°C. A confluent PMECM was reached within two to three population doublings, which took 3–4 days.

Reagents

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Triciribine, 1,5-Dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazacenaphthylen-3-amine, (API-2, Tocris, Ellisville, MO) was used to specifically inhibit Akt-1, 2 and 3 [5]. SB 216763, 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (BIOMOL, Plymouth Meeting, PA) blocks the binding site for ATP and was utilized as a selective inhibitor of GSK3 / [3, 14]. Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt), a cell permeable superoxide scavenger [18], and L-NAME (N^o-nitro-L-arginine-methyl ester), a substrate antagonist of nitric oxide synthase (NOS) [19] were used to elucidate reactive oxygen-nitrogen species generation.

Treatments

Treatment medium—For all studies, PMECM were incubated with reagents in phenol-free DMEM (pf-DMEM) (GIBCO-BRL), supplemented with 10% FBS, to avoid a potential

antioxidant effect of phenol. PMECM were treated for 60 minutes with Triciribine (12.5 μ M) [20] or SB 216763 (1, 5 and 10 μ M) prior to the assays.

Assay of Endothelial Permeability

The assay of endothelial cell monolayer permeability was adapted from our previously described technique [17]. Transwells (6.5 mm diameter, 8 μ m pore size; Corning Costar, Corning, NY) were coated with Rat Tail Collagen Type 1 (BD Biosciences, Bedford, MA). Then RLMVEC (0.1×10^6) in MCDB-131 were plated in the Transwells according to manufacturer's instructions and allowed to reach confluence within 3–5 days (37°C, 5% CO₂). The experimental apparatus for the study of transendothelial transport in the absence of hydrostatic and oncotic pressure gradients have been described [17]. In brief, the system consists of two compartments separated by a microporous polycarbonate membrane lined with the endothelial cell monolayer as described above. The luminal (upper) compartment (0.1 ml) was suspended in the abluminal (lower) compartment (0.6 ml). The entire system was kept in a CO₂ incubator at a constant temperature of 37°C. The fluid height in both compartments was the same to eliminate convective flux. Endothelial permeability was characterized by the clearance rate of Evans Blue-labeled albumin. A buffer solution containing Hanks' balanced salt solution (GIBCO-BRL) containing 0.5% bovine serum albumin (BSA) and 20 mM HEPES buffer were used on both sides of the monolayer. The luminal compartment buffer was labeled with a final concentration of 0.057% Evans blue dye in a volume of 100 μ l. The absorbance of free Evans blue in the luminal and abluminal compartments was always <1% of the total absorbance of Evans blue in the buffer. At the beginning of each study a luminal compartment sample was diluted 1:100 to determine the initial absorbance of that compartment. Abluminal compartment samples (100 μ l) were taken every 10 minutes for 1 hour. The absorbance of the samples were measured in a BioTek Synergy 2 microplate spectrophotometer (BioTek, Winooski, VT) at 620 nm. The clearance rate of Evans blue-labeled albumin was determined by least-squares linear regression between 10 and 60 minutes for the control and experimental groups.

Immunoblot Analysis

Preparation of PMECM Lysate Fractions—RLMVEC were seeded into 12-well plastic culture plates and incubated for 3–4 days until confluent. After interventions, the PMECM were washed on ice two times with ice-cold PBS without ions. Cells were then scraped with 60 μ l/well ice-cold extraction buffer (Tris HCl: 10 mM-pH 7.5; SDS: 0.1%; Triton X-100: 0.5%; Sodium Deoxycholate: 0.5%; DTT: 0.1 mM) supplemented with 1x mammalian protease inhibitor cocktail and 1x phosphatase inhibitor cocktails 1 and 2. Lysates were cleared by centrifugation at $18,500 \times g$ for 30 minutes at 4°C. Cell lysate protein concentrations were determined by BCA assay (Pierce Biotechnology, Inc., Rockford, IL) against BSA protein standards. All samples were normalized for protein content, diluted 4:1 in 5x Laemmli buffer, heated 5 min at 95°C, and stored at –80°C.

Western Blot—The lysate proteins were separated by SDS-PAGE on 8.75%, 1.5 mm thick, 15-lane Mini-Protean III gels using standard procedures (Bio-Rad, Hercules, CA). All lanes were loaded such that each lane contained 16 μ g of total protein. The gels were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) at 125 volts for 1 hour with Towbin's transfer buffer. The membranes were then blocked with 5% blotto with phosphatase inhibitors (BPI) (5g/100ml nonfat dry milk in TTBS [Tween 20: 0.05%; Tris HCl: 10 mM, pH 7.5; NaCl: 100 mM]; NaF: 50 mM; Na₃VO₄: 1.0 mM) for 30 minutes at room temperature (RT).

Immunoprobings—Rabbit polyclonal anti-phospho- β -catenin-Ser^{33/37}, anti-Akt, anti-phospho-Akt-Ser⁴⁷³ and anti-phospho-GSK3 β (Ser^{21/9}) were obtained from Cell

Signaling Technology (Danvers, MA). Mouse monoclonal anti-phospho-GSK3 / (Tyr^{279/216}), clone 5G-2F, was from Millipore (Billerica, MA). Rabbit polyclonal anti-GSK3 (H-76) and anti- β -Catenin (H-102), and goat polyclonal anti-GSK3 (R-20) and anti-VE-Cadherin (C-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were incubated overnight at 4°C; all primary antibodies were diluted 1:2000 in either TTBS with 5% BSA or BPI according to manufacturers instructions. Secondary antibody blot incubation was 1 hour at room temperature (RT) with either bovine anti-rabbit IgG or goat anti-mouse IgG HRP conjugates (Santa Cruz) diluted 1:5000 in BPI. Goat anti-biotin-HRP, 1:5000, was included in the secondary antibody incubation to detect biotin-labeled molecular weight markers (Cell Signaling) on the blots. Unbound material was removed from the blots when required by washing five times for 5 minutes each with TTBS at RT. Signal was generated with a 1:1 dilution of SuperSignal West Dura- and Super Signal West Pico- Chemiluminescent Substrates (Thermo Scientific, Rockford, IL). All blots were stripped between each sequential reprobe with Restore PLUS Western Blot Stripping Buffer (Thermo) for 15 minutes at RT.

Detection and Analysis—Western blot images were acquired with a Chemidoc XRS (Bio-Rad) and net band intensity units were measured with Image Lab image analysis software (Bio-Rad). All blots contained equal numbers of samples of each experimental treatment. The mean band intensity of all samples on an individual blot was normalized across all blots to an arbitrarily chosen value to compensate for interblot variability and individual sample band values were then adjusted proportionally to reflect that normalized mean.

Fluorescence detection of Reactive Nitrogen Species in PMECM lysate

RLMVEC (2.5×10^4 /200 μ l of culture medium) were plated in Costar 96-well black clear-bottom culture plates (Corning, Corning, NY, #3603) and grown to confluence. After treatment, PMECM were washed 1X with PBS(–) (Fisher Scientific, Waltham, MA) and incubated with 10 μ M 6-Carboxy-2',7'-Dichlorodihydrofluorescein Diacetate, Di(Acetoxymethyl Ester) (DCFDA) (Life Technologies, Grand Island, NY) in treatment media (75 μ l/well) at 37°C for 30 minutes. The probe was then removed, PMECM were washed 1X with PBS(–), and 50 μ l/well HBSS was added prior to fluorescence measurement. Appropriate blanks, to which no DCFDA probe was added, were used to correct for background autofluorescence of control or treated (i.e. SB 216763) groups. Fluorescence was measured with a BioTek Synergy2 plate reader (BioTek Instruments) using excitation and emission wavelengths of 485nm and 620nm (DHE) or 485 and 528 nm (DCFDA), respectively. Fluorescence is presented as percentage of control by the formula $[F_{\text{exp}}/F_{\text{control}}]$, where F_{exp} = fluorescence at any time after treatment in a given lysate and F_{control} = mean fluorescence of the respective untreated control replicates.

Statistics

A two-tailed Student's T-Test was used for comparing two groups of data. Otherwise, a one-way analysis of variance 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 test to determine significant differences among the groups. Each PMECM well and flask represents a single experiment. All data were reported as means $\pm$ S.E.M. Significance is at $P < 0.05$.

Results

Akt is activated by phosphorylation of Thr³⁰⁸ in the activation loop by PDK1 [21] and by phosphorylation of Ser⁴⁷³ within the carboxy terminus by mTOR (mammalian target of

rapamycin) [21–23]. The Akt inhibitor triciribine, a tricyclic nucleoside, prevents phosphorylation and hence activation of Akt. Western blot data from the Akt activation site at Ser⁴⁷³ and total Akt in PMECM lysate following a 1.0 hour incubation with the Akt inhibitor triciribine are shown in Figure 1. Representative Western blots are shown in Panel A. As expected, there is a substantial decrease (~70%) in phospho-Akt-Ser⁴⁷³ in the triciribine group compared to the control (Panel B). There is also a slight, but significant (~5%), increase in the total Akt level in the triciribine group compared to that of the control (Panel C). Overall Akt activity, represented by the ratio of Ser⁴⁷³ phosphorylation to total Akt is shown in Panel D. The data of Figure 1 supports the idea that there is a high constitutive level of phospho-Akt-Ser⁴⁷³/Akt activity and that triciribine suppresses phospho-Akt-Ser⁴⁷³/Akt activity as well as possibly altering Akt metabolism.

GSK3 β has been shown to be inactivated by the phosphorylation of Ser²¹ of GSK3 β or Ser⁹ of GSK3 β by Akt [5, 6] and/or several PKCs [1, 2, 7, 8]. Figure 2 shows representative Western blots (Panel A), of the relative phosphorylation levels of the GSK3 β activation site Tyr²⁷⁹, inhibition site Ser²¹, and total GSK3 β after 1 hour Akt inhibition with triciribine. The phosphorylation level of the activation site (Panel B) remained relatively unchanged whereas the inhibition site (Panel C) significantly decreased in the triciribine group compared to the control group. The total GSK3 β values (Panel D) were similar. GSK3 β activity expressed as the ratio of active site phosphorylation over total GSK3 β (Panel E) indicates no net change. GSK3 β inhibition expressed as the ratio of inhibitory site phosphorylation over total GSK3 β (Panel F) indicates a net decrease following 1 hour triciribine treatment. Finally, the ratio of the active site phosphorylation over inhibitory site phosphorylation (Panel G) indicates a significant increase in GSK3 β activity (~40%) following 1 hour triciribine treatment. The data of Figure 2 supports the idea that there is constitutive Akt-dependent mediation of GSK3 β activity.

Figure 3 shows representative Western blots (Panel A), of the relative phosphorylation levels of the GSK3 α activation site Tyr²¹⁶, inhibition site Ser⁹, and total GSK3 α after 1 hour incubation with triciribine. Phosphorylation levels of both the activation (Panel B) and inhibition (Panel C) sites of GSK3 α decreased following 1 hour Akt inhibition. The total GSK3 α values (Panel D) were unchanged following triciribine inhibition of Akt. GSK3 α activity expressed as the ratio of active site phosphorylation over total GSK3 α (Panel E) indicates a significant decrease following Akt inhibition compared to control. GSK3 α inhibition expressed as the ratio of inhibitory site phosphorylation over total GSK3 α (Panel F) also indicates a net decrease following 1 hour triciribine inhibition of Akt. GSK3 α activity expressed as the ratio of active over inhibition site phosphorylation indicates a significant increase in activity (~40%) following 1 hour triciribine treatment (Panel G), similar to that seen with GSK3 β . The data of Figure 3 supports the notion that there is constitutive Akt-dependent mediation of GSK3 α activity.

β -catenin is an integral component of stable adherence junctions between endothelial cells as well as a transcriptional co-transactivator and ubiquitin-proteosomal degradation of β -catenin is mediated primarily by GSK3 β phosphorylation of β -catenin at Ser^{33/37} and Thr⁴¹ [1, 2, 4]. Figure 4 shows representative Western blots (Panel A) of the relative phosphorylation levels of phospho- β -catenin-Ser^{33/37} and total β -catenin after 1 hour incubation with the GSK3 inhibitor SB 216763 (1, 5 and 10 μ M) or the Akt inhibitor triciribine. The phospho- β -catenin-Ser^{33/37} level dose dependently decreases in the SB 216763 group and is increased in the triciribine group relative to the control group (Panel B). There is a slight but significant drop in the level of total β -catenin following 1 hour treatment with triciribine but no significant change from control with increasing concentration of SB 216763 (Panel C). The data of Figure 4 shows that SB 216763 is an effective inhibitor of GSK3 β and that the constitutive level of phospho- β -catenin-Ser^{33/37} is

mediated by the degree of GSK3 β activity. The data from Figures 1–4 supports the notion that there is constitutive Akt-dependent-GSK3 β activity in PMECM, which is involved, in part, in maintaining tight control of β -catenin phosphorylation.

Du et al, showed β -catenin-dependent expression of inducible nitric oxide synthase and nitric oxide production in cancer and embryonic kidney cell lines. Furthermore, their data reveal an early (1 hour), pre-expression increase in nitric oxide following inhibition of GSK3 β with LiCl [10]. Therefore, the effect of the specific GSK3 inhibitor SB 216763 on oxidant production in PMECMs was examined at the one hour time point. Figure 5 shows the DCFDA oxidation after 1.0 hour incubation in the control and SB 216763 groups with and without the superoxide scavenger tiron or the NOS inhibitor L-NAME. DCFDA oxidation was significantly greater in the SB 216763 group compared to the control and this effect was eliminated in the presence of tiron and attenuated with L-NAME. The data from Figure 5 suggests that constitutive GSK3 activity is essential to maintaining oxidant balance in PMECM.

It has been shown that reactive oxygen/nitrogen species increase albumin permeability of lung endothelial monolayers [17]. To further verify the significance of the GSK3 inhibition-induced production of oxidants, the effect of GSK3 inhibition on endothelial barrier integrity was examined. Figure 6 shows the albumin clearance rate in PMECMs after 1.0 hr incubation in control and SB 216763 treated groups in the presence or absence of tiron or L-NAME. SB 216763 caused a significant increase in albumin clearance compared to control which was eliminated in the presence of either tiron or L-NAME. The effect of triciribine on both oxidant production and permeability was not examined because the multitude of additional downstream targets of Akt would have rendered interpretation of changes difficult with respect to GSK3 β activity alone. The data from Figures 5 and 6 support the idea that GSK3 inhibition promotes endothelial barrier dysfunction mediated by reactive oxygen/nitrogen species.

Discussion

The literature indicates that GSK3 β is closely associated with vascular endothelial barrier function. In human and bovine pulmonary artery endothelial monolayers the serine-9 phosphorylation of GSK3 β directly correlated with the electrical resistance increasing effect of hepatocyte growth factor (HGF); however, a frank role of GSK3 β in endothelial barrier function was not examined [24]. Conversely in bovine retinal endothelium, the vascular endothelial growth factor (VEGF) induced decrease in electrical resistance was directly correlated to the serine 9 phosphorylation of GSK3 β [25]. Interestingly, the protective effect (i.e., increased electrical resistance) of pigment epithelium-derived factor (PEDF) was inversely proportional to phospho-GSK3 β -Ser⁹ but a role for GSK3 β in the barrier function was not examined [25]. Finally, Severson et al showed in intestinal and renal epithelial monolayers that reduction of GSK3 β with siRNA or inhibition with SB415286 decrease electrical resistance which was associated with increased flux of 4kD FITC-dextran and 70 kD rhodamine [9]. In addition, the altered barrier function correlated with the decreased protein expression of transmembrane proteins occludin, claudin-1 and E-cadherin [9].

The present study shows that in rat lung microvessel endothelial cells, triciribine [5] successfully targeted Akt because there was a decrease in phospho-Akt-Ser⁴⁷³, a noted response indicative of repressed activity of Akt [5, 6, 26]. Akt is activated both by PDK-1 [5, 6, 21, 26], by mTOR [22, 23] and, in part, by autophosphorylation at the Ser⁴⁷³ hydrophobic site [26]. The Akt inhibitor triciribine induced a decrease in phosphorylation of the inhibition sites of GSK3 α and GSK3 β and a decrease in the phosphorylation of the

GSK3^β activation site. However, if activity is defined as the ratio of activation site phosphorylation /inhibition site phosphorylation, ratios which were similar between GSK3^α and GSK3^β, triciribine induced a similar increase in activity of GSK3^α and GSK3^β. This is similar to what is usually reported in the literature wherein a decrease in the phosphorylation of GSK3^α / -Ser^{21/9} inhibition sites would enhance the enzyme activity of GSK3^α / [1, 4].

The increase in GSK3^β activity in the triciribine group was evidenced by the increase in phospho- β -catenin-Ser^{33/37} associated with a decrease in total β -catenin. This decrease in total β -catenin supports the concept that Ser^{33/37}-phosphorylated β -catenin is targeted for degradation by the ubiquitin-proteasome pathway. β -catenin-Ser^{33/37} is a classic target for GSK3^α / [1, 4, 27] as noted by the dose dependent decrease in phospho- β -catenin-Ser^{33/37} in the SB 216763 group. There was a clear dose response of SB 216763 (between 1 μ M and 10 μ M) vs phospho- β -catenin-Ser^{33/37}; thus, to insure specificity the experiments described used 1 μ M SB 216763.

The inhibition of GSK3^α / is associated with altered activity of a myriad of signaling molecules in various cell types which could result in altered endothelial barrier function such as: gene expression via NF κ B [28] and TCF [29] TRAIL mediated apoptosis [30], iNOS/NO biosynthesis [10, 27], NOX1 expression [31] and occludin, claudin-1 and E-cadherin expression [9]. The present data indicates that GSK3^α / inhibition promoted reactive oxygen/nitrogen species mediated endothelial barrier dysfunction because inhibition of GSK3^α / with SB 216763 increased albumin clearance and reactive oxygen/nitrogen species generation of the PMECM. Moreover, the increase in albumin clearance was prevented by the anti-reactive oxygen/nitrogen species agents tiron and L-NAME. Tiron is a superoxide dismutase mimetic that directly scavenges $^{\circ}\text{O}_2$ [18]. L-NAME is a substrate antagonist of NOS [19] which suggests the effect of GSK3^α / inhibition is via ONOO⁻ by the reaction $^{\circ}\text{NO} + ^{\circ}\text{O}_2 \rightarrow \text{ONOO}^-$. L-NAME alone did not decrease DCF fluorescence indicating minimal constitutive $^{\circ}\text{NO}$ generation. Du et. al. showed in a variety of non-endothelial cell lines that GSK3^α /-inhibition and β -catenin increase inducible nitric oxide synthase (iNOS) promoter activity via the transcription factors TBE1 and TBE2 which increased iNOS expression and $^{\circ}\text{NO}$ [10]. We, however, detected no iNOS in endothelial cells that were treated with SB 216763 (1 μ M) for up to 24 hours (data not shown). Kim et. al. showed that GSK3^α / inhibition and β -catenin increase Nox1 expression in macrophages [31]. We showed that reactive oxygen/nitrogen species increase albumin permeability of a lung endothelial monolayer and isolated lung [14, 17, 19]. In the present study, it is possible that mechanisms exist in endothelial cells during SB 216763-induced GSK3^α / inhibition, such as increased eNOS activity, which contribute to the increase in reactive oxygen/nitrogen species and endothelial barrier dysfunction, which will be a topic for our future investigation.

Severson et al showed a decrease in expression of occludin, claudin-1 and E-cadherin in response to GSK3^α / inhibition in epithelium [9]. Vines et al revealed increased GSK3^α activity downregulates cytokine expression following LPS challenge [32]. In preliminary studies (data not shown) the inhibition of GSK3^α / decreases the expression of VE-cadherin promoter activity by 4 hours. The promoter region of the mouse VE-cadherin gene contains multiple sites that could bind Tcf-4 complexed with β -catenin with resultant suppression of the VE-cadherin gene [33–35]. VE-cadherin is vital for maintenance of endothelial cell adherence junctions [35]; thus, a decrease in VE-cadherin protein expression would likely compromise endothelial barrier function. Conversely, Eto et al showed, using human aortic and umbilical vein endothelial cells, different from the pulmonary microvessel endothelium used in the present study, that inhibition of GSK3 with LiCl and TDZD-8 prevents the TNF-induced increase in VCAM-1 and tissue factor in human umbilical vein and aortic endothelium [36]. Thus, the GSK3^α / mediation of the inflammatory response may be

dependent on cell type. In the present study the acute inhibition of pulmonary GSK3 / activity may exacerbate the inflammatory response with respect to endothelial barrier integrity both directly (e.g., increased oxidant production) and indirectly (e.g., gene regulation).

In summary, the data indicates a constitutive degree of GSK3 / inhibition, as shown by the inhibition of GSK3 / phosphorylation in the presence of the Akt inhibitor triciribine. In addition, an outcome of SB 216763 -induced inhibition of GSK3 / is decreased endothelial barrier function to protein flux. The increased endothelial monolayer permeability is mediated by reactive nitrogen/oxygen species.

Acknowledgments

Research Supported by NIH R01 HL059901

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Figure 1. Triciribine reduces constitutive active site phosphorylation of Akt in PMECM
A. Representative Western blots of Akt activation site phosphorylation at Ser⁴⁷³ (upper bands) and total Akt (lower bands) in PMECM lysates following a 1.0 hour incubation of PMECM with 12.5 μ M triciribine (Trb), an Akt inhibitor. **B.** The phospho-Akt-Ser⁴⁷³ band densities and **C.**, the total Akt band densities for all blots, expressed in Relative Density Units (RDU). **D.** The ratio of phospho-Akt-Ser⁴⁷³ / total Akt band densities. Values are mean $\pm$ S.E.M., N = 10, * = significantly different from control.

Figure 2. Akt inhibition with triciribine does not alter net GSKα activity in PMECM

A. Representative Western blots of GSKα activation site phosphorylation at Tyr²⁷⁹ (upper bands), inhibition site phosphorylation at Ser²¹ (center bands), and total GSKα (lower bands) in PMECM lysates following a 1.0 hour incubation of PMECM with 12.5 μM triciribine (Trb), an Akt inhibitor. B. The phospho-GSKα-Tyr²⁷⁹ band densities, C. the phospho-GSKα-Ser²¹ band densities, and D. the total GSKα band densities for all blots, expressed in Relative Density Units (RDU). E. The ratio of phospho-GSKα-Tyr²⁷⁹ / total GSKα band densities and F. the ratio of phospho-GSKα-Ser²¹ / total GSKα band densities. G. The ratio of phospho-GSKα-Tyr²⁷⁹ / phospho-GSKα-Ser²¹ band densities, the active-to-inhibition site ratio, as a measure of net activity. Values are mean ± S.E.M., N = 9, * = significantly different from control.

Figure 3. Akt inhibition with triciribine increases net GSK activity in PMECM

A. Representative Western blots of GSK activation site phosphorylation at Tyr²¹⁶ (upper bands), inhibition site phosphorylation at Ser⁹ (center bands), and total GSK (lower bands) in PMECM lysates following a 1.0 hour incubation of PMECM with 12.5 μ M triciribine (Trb), an Akt inhibitor. B. The phospho- GSK -Tyr²¹⁶ band densities, C. the phospho- GSK -Ser⁹ band densities, and D. the total GSK band densities for all blots, expressed in Relative Density Units (RDU). E. The ratio of phospho- GSK -Tyr²¹⁶ / total GSK band densities and F. the ratio of phospho- GSK -Ser⁹ / total GSK band densities. G. The ratio of phospho- GSK -Tyr²¹⁶ / phospho- GSK -Ser⁹ band densities, the active-to-inhibition site ratio, as a measure of net activity. Values are mean $\pm$ S.E.M., N = 10, * = significantly different from control.

Figure 4. Baseline phospho- β -Catenin-Ser^{33/37} is maintained by GSK3 in PMECM

A. Representative Western blots of phospho- β -Catenin-Ser^{33/37} (upper bands) and total β -Catenin (lower bands) of lysate of rat PMECM treated for 1 hour with media alone (Con), the GSK3 inhibitor SB 216763 (1, 5, and 10 μM), or with 12.5 μM triciribine (Trb), an Akt inhibitor. **B.** The phospho- β -Catenin-Ser^{33/37} band densities and **C.** the total β -Catenin band densities for all blots, expressed in Relative Density Units (RDU). Values are mean $\pm$ S.E.M., N = 4, * = significantly different from control.

Figure 5. GSK3 activity mediates oxidant balance in PMECM

PMECM grown in 96-well plates were assessed for reactive oxygen/nitrogen species with the oxidant probe DCFDA. Cells were treated for 1 hour with either media or 1 μ M of the GSK3 inhibitor SB 216763 (SB2) in the absence or presence of the NOS inhibitor L-NAME (100 μ M) or the superoxide scavenger tiron (5mM). Wells treated with media alone or 1 μ M SB 216763 without DCFDA probe added were subtracted as blanks from their respective treatment groups to account for background as well as SB 216763 auto-fluorescence. The data is expressed as the mean $\pm$ S.E.M. of all experiments of the proportional difference of treatment wells from the average control value within an individual experiment, N = 11. * = significantly different from control, # = significantly different from SB 216763 treatment alone.

Figure 6. GSK3 inhibition promotes endothelial barrier dysfunction mediated by oxidant production

Rat PMECM were prepared and analyzed for endothelial permeability as described in Methods. PMECM were treated for 1 hour with either media or 1μM of the GSK3 inhibitor SB 216763 (SB2) in the absence or presence of the NOS inhibitor L-NAME (100μM) or the superoxide scavenger tiron (5mM). The samples were assessed for protein flux with Evans blue-albumin. The data is expressed as clearance rate (μl/min), mean ± S.E.M., N = 7. * = significantly different from control, # = significantly different from SB 216763 treatment alone.