Mechanical Stretch Inhibits Lipopolysaccharide-induced Keratinocyte-derived Chemokine and Tissue Factor Expression While Increasing Procoagulant Activity in Murine Lung Epithelial Cells*

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Background: The influence of mechanical stretch on LPS-induced alveolar epithelial cell inflammatory (keratinocyte-derived chemokine (KC)) and coagulant (tissue factor (TF)) responses is unclear.

Results: Stretch down-regulates LPS-mediated KC and TF expression while enhancing TF activity.

Conclusion: Different signaling pathways regulate stretch- and LPS-induced TF and KC.

Significance: Lung stretch during mechanical ventilation may alter the innate immune response of the lung epithelium.

Acute respiratory distress syndrome (ARDS)2 and acute lung injury (ALI) are devastating illnesses caused by direct (pneumonia, aspiration, inhalational injury) or indirect (sepsis, trauma) insults (1). These syndromes are characterized by diffuse alveolar damage, accumulation of protein-rich pulmonary edema fluid, an influx of neutrophils into the lung, and an increase in alveolar fibrin deposition, indicating a shift in the balance of procoagulant and fibrinolytic pathways (2, 3). Although there have been significant advances made in the past few decades, the mortality rate associated with ALI/ARDS remains high, and improved therapeutic strategies are needed.

Currently, the therapeutic strategy for patients with ALI/ARDS is the use of mechanical ventilation (MV) (4–7). However, at high tidal volumes, MV activates procoagulant and pro-inflammatory pathways, leading to ventilator-associated lung injury (8, 9). In addition, MV also potentiates pre-existing ALI induced by other inflammatory stimuli such as LPS (4), a component of the cell wall of Gram-negative bacteria shown to be elevated early on in the blood of patients with ALI, indicating that the innate immune stimulant plays a role in potentiation of ventilator-associated lung injury (10–12). Despite this evidence, little is known about the cellular and molecular mechanisms that are responsible for the harmful effects of MV. Previous studies investigating the effects of MV on LPS-induced responses have largely utilized endothelial and immune cells, with few in vitro studies focused on alveolar epithelial cell-derived procoagulant and inflammatory responses (13–17). In addition, prior reports have focused on inflammatory mediator susceptibility of the lung to bacterial infections in the setting of mechanical ventilation.

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2 The abbreviations used are: ARDS, acute respiratory distress syndrome; ALI, acute lung injury; MV, mechanical ventilation; MS, mechanical stretch; 1,2-bis(2-aminophenoxy)ethane-N,N′,N″-tetraacetic acid tetrakis(acetoxymethyl ester); DN, dominant-negative; TF, tissue factor; KC, keratinocyte-derived cytokine; TLR, Toll-like receptor.
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production and not coagulant responses; the effects of MV on coagulant responses in the lung are essentially unknown.

We aimed to test the hypothesis that mechanical stretch (MS) increases proinflammatory cytokine production and procoagulant activity in alveolar epithelial cells, thereby potentiating ventilator-induced lung injury and contributing to the development of ALI/ARDS. In contrast to previous studies (15, 16, 18), we applied MS to a murine lung type II-like epithelial cell line, MLE-12, with or without LPS to determine whether there was a synergistic effect on cytokine and coagulant responses in combination with MS.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine lung epithelial type II-like cell line MLE-12 was cultured for 24 h on type I collagen-coated, 6-well silicone elastomer-bottomed culture plates (Flexcell International Corp., Hillsborough, NC) in DMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C in a humidified 5% CO2 atmosphere. Restriction of the cell seeding area was necessary to prevent cells from attaching to the periphery of the substrate surface beyond the dimensions of the mechanical indenter to provide a uniform strain to all cells. Therefore, cell seeding was confined to an area of 4.8 cm2 in the center of each well using a wash.

Cell Treatment—After the growth period, cells were washed with Hanks’ balanced salt solution and placed in serum-free DMEM. After 24 h of incubation, cells were once again washed with Hanks’ balanced salt solution, placed in serum-free DMEM, and then incubated with or without 0.4% (w/v) EtOH (Fisher) for 10 min or 20 μM BAPTA-AM (Sigma) for 30 min before exposure to 10 μg/ml LPS (Escherichia coli 055:B5, catalog no. L2880, Sigma) for 4 h. For BAPTA-AM studies, MLE-12 cells were treated with 0.01% Me2SO as a vehicle control. After 4 h, cells were stretched in BioFlex plates equibiaxially at 0.1 Hz (5 s of deformation alternating with 5 s of relaxation, half-sine curve shape) for 30 min or 2 h (unless otherwise stated) using a computer-controlled vacuum strain apparatus (Flexcell strain unit, Flexcell International Corp.) with a vacuum pressure sufficient to generate 20% strain. The cell supernatant was removed, spun at 1200 × g for 6 min at 4 °C to remove debris, and then frozen. Cells were washed with Hanks’ balanced salt solution, lysed in radioimmunoprecipitation assay buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.1% Nonidet P-40) with one Complete mini-protease inhibitor mixture tablet (one tablet/10 ml; Roche Applied Science), snap-frozen in liquid nitrogen, and stored at −80 °C until analysis.

Plasmids—The IκBα dominant-negative (IκBα-DN) (19) and NF-κB-driven luciferase reporter (20) plasmids were kindly provided by Dr. Timothy Blackwell (Vanderbilt University). The Renilla luciferase reporter plasmid was kindly provided by Dr. Roger Cone (Vanderbilt University).

Transient Transfection and Luciferase Assay—MLE-12 cells were grown in DMEM with 10% FBS and transfected 48 h before experiments with LipoD293 (SignaGen Laboratories, Rockville, MD), which was used as recommended by the manufacturer. For luciferase assays, cells were cotransfected with the plasmid encoding the IκBα-DN cDNA and with the plasmid encoding the NF-κB-driven luciferase. As a control for transfection efficiency and cell viability, separate cells were cotransfected with the plasmid encoding the Renilla luciferase and the plasmid encoding the IκBα-DN cDNA. 24 h later, cells were serum-starved overnight before exposure to 10 μg/ml LPS or control medium for 6 h. Cells were then incubated with media containing substrate as recommended by the manufacturer (NanoLight Technology, Pinetop, AZ): for NF-κB-driven luciferase reporter activity, one-step luciferase assay (catalog no. 318), and for Renilla luciferase activity, coelenterazine h (catalog no. 301). The luminescence was recorded on a SpectraMax M5 plate reader (100-ms integration; Molecular Devices). For all other assays, cells were transfected with the plasmid encoding the IκBα-DN cDNA or an empty vector (pBSII-KS/Asc) 48 h before exposure to 10 μg/ml LPS or control medium for 4 h. After 4 h, cells were stretched as described above.

Cell Viability—Cell death was assessed by trypan blue exclusion assay or using a CellTiter AQeous cell proliferation kit (Promega, Madison, WI) according to the manufacturer’s instructions.

Total Protein Measurement—Total protein was determined by bicinchoninic acid assay (Pierce).

Clot Time Measurement—Clot time was measured in duplicate in cell supernatants using a mechanical clot detection system (STart4 coagulometer, Diagnostica Stago S.A.S., Asnières-sur-Seine, France). Briefly, 25 μl of cell culture-conditioned medium was warmed for 15 min at 37 °C. Samples were then incubated with 25 μl of pooled normal citrated murine plasma (Bioreclamation, LLC, Hicksville, NY) for 2 min. Clot time was determined as recalcification time following the addition of 25 μl of 25 mM calcium chloride.

Tissue Factor Activity Assay—Following treatment, MLE-12 cells were washed twice with serum-free DMEM and then incubated at 37 °C for 60 min with 40 nM human factor VIIa and 1 μM human factor X. A human factor Xa (human factors VIIa, X, and Xa; Enzyme Research Laboratories, South Bend, IN) standard curve was prepared in a new 96-well plate. 100 μl from each sample well was transferred to the new plate. After transfer, 5 μl of 100 mM EDTA was added to each well to stop the enzymatic reaction. Factor Xa was quantified by adding 100 μl of the chromogenic substrate S-2222 (Chromogenix, Milan, Italy) to each well and incubating at room temperature for 15 min, followed by measurement of absorbance at 405 nm. Control experiments were performed without the addition of factor VIIa and/or factor X. No tissue factor (TF) activity was measurable in the absence of added human factor VIIa (data not shown).

Quantitation of Keratinocyte-derived Cytokine—Utilizing a sandwich ELISA (R&D Systems, Minneapolis, MN), keratinocyte-derived cytokine (KC) was quantified in duplicate in conditioned medium.

Western Blotting for Total Cellular TF—Cells were washed with Hanks’ balanced salt solution and lysed in radioimmunoprecipitation assay buffer with one Complete mini-protease inhibitor mixture tablet (one tablet/10 ml). Supernatants were then collected, and loading buffer with 100 mm dithiothreitol was added, boiled for 5 min, and centrifuged. The proteins were
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separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with a primary antibody against mouse TF (1:1000; catalog no. AF3178, R&D Systems) or α/β-tubulin (1:1000; catalog no. 2148, Cell Signaling Technology, Inc., Boston, MA), followed by incubation with the appropriate HRP-conjugated anti-goat (1:1000; catalog no. HAF017, R&D Systems) or anti-rabbit (catalog no. sc-2004, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) IgG secondary antibody. The membranes were visualized using an ECL system and developed on Hyperfilm (Amersham Biosciences). Immunoreactive proteins were visualized using an ECL Western blot detection kit (Pierce). Blots were scanned and analyzed using ImageJ software.

Fixed Cell ELISAs for Surface TF and Surface and Total TLR4—To measure cell surface proteins, a modified ELISA protocol was applied to fixed adherent cells as described previously (21). MLE-12 cells were rinsed with PBS, fixed for 10 min with 4% paraformaldehyde (Thermo Scientific, Rockford, IL), and washed again with PBS for 5 min. Cells were blocked for 20 min with 5% nonfat dry milk either in PBS with 0.1% Triton X-100 (Sigma) or in PBS alone to detect total TLR4 or surface TLR4 and TF, respectively. Cells were then incubated with PBS alone or with 0.1% Triton X-100 containing 5% nonfat dry milk and goat anti-mouse TF (0.1 μg/ml) or rabbit anti-mouse TLR4 (1:1000; catalog no. sc-30002, Santa Cruz Biotechnology, Inc.) antibody. Cells were washed and incubated with PBS alone or with 0.1% Triton X-100 containing 5% nonfat dry milk and HRP-linked rabbit anti-goat or goat anti-rabbit secondary antibody (1:1000) for 45 min, washed, and incubated for 5–20 min with 3,3′,5,5′-tetramethylbenzidine (Sigma). The reaction was terminated with 5% sulfuric acid, 150 μl was transferred to a 96-well plate, and the absorbance was read at 450 nm on a Synergy HT spectrophotometer (BioTek, Winooski, VT). To control for specificity of the anti-TLR4 and anti-TF antibodies, cells were treated with secondary antibody alone, and all values were normalized to the background signal.

Isolation and Analysis of TF, TLR4, and KC mRNAs—RNA was extracted following the commercial protocol with an Invitrogen PureLink RNA minikit and quantified by absorbance at 260 nm. mRNA (0.25–0.5 μg) was used to synthesize first-strand cDNA with a SuperScript Vilo cDNA kit (Invitrogen). Quantitative real-time PCR was performed using Assays-on-Demand primer/MGB probes (TaqMan probe/primer sets, Applied Biosystems) for murine TF (Mm00438853_m1), murine KC (Mm00433859_m1), murine TLR4 (Mm00445273_m1), and GAPDH (Mm99999915_g1). Standard curves were made with serial dilutions of a mixed pool of all cDNAs assayed. All values were normalized to the GAPDH mRNA content of each sample.

Analysis of NF-κB Activation—The DNA-binding activity of NF-κB was quantified by ELISA using a TransAM™ NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA) in MLE-12 cells with an NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific). Nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide (5′-AGTTGAGGGGACTTTCCAGGC-3′) containing a consensus binding site (5′-GGGACTTTCC-3′) for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p65 (Active Motif), visualized by HRP-conjugated anti-IgG antibody and developing solution, and quantified by spectrophotometry at 450 nm with a reference wavelength of 655 nm.

Immunofluorescence Imaging—To detect F-actin, fixed and permeabilized cells were stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes) diluted 1:40 in PBS containing 1% BSA (Sigma) for 30 min at room temperature in the dark. Nuclei were stained further with DAPI (Molecular Probes). Finally, coverslips were washed with PBS and mounted on glass microscope slides in ProLong Gold antifade reagent (Molecular Probes). Fluorescent images were produced using a Zeiss Axio Imager Z1 microscope with AxioVision version 4.5 software.

Statistics—All values represent two to three test wells from each of three to four independent experiments and are expressed as means ± S.E. Differences among three or more experimental groups were analyzed by one-way analysis of variance and Student-Newman-Keuls post hoc analysis using GraphPad Prism software. Differences were considered significant when p < 0.05. For calculations of percent change, treatment group values are expressed as percent difference compared with controls.

RESULTS

MS Down-regulates LPS-induced TF Expression—MLE-12 cells express TF constitutively as determined by Western blotting (Fig. 1A). MS alone significantly reduced total cell-associated TF protein, whereas exposure to increasing concentrations (5–25 μg/ml; 25 μg/ml data not shown) of LPS increased TF levels. However, at the highest concentration (25 μg/ml), cell viability as measured by trypan blue exclusion assay or with a cell proliferation kit after 6 h of exposure to either LPS alone or LPS in the presence of stretch was notably decreased compared with control cells (data not shown). 10 μg/ml had no detectable effect on cell viability; thus, this concentration was used for subsequent experiments. LPS-mediated up-regulation of total cell-associated TF was attenuated in the presence of MS (Fig. 1, A and B). LPS-induced cell surface expression of TF (measured by direct ELISA) was also significantly reduced in the presence of MS (Fig. 1C).

To determine whether changes in cell surface and total cell-associated TF are regulated transcriptionally, TF mRNA levels were measured by quantitative real-time RT-PCR. MS alone did not modulate TF mRNA expression, whereas exposure to LPS significantly increased TF mRNA expression (Fig. 1D). MS reduced the LPS-mediated increase in TF mRNA levels, indicating that the stretch-induced decrease in LPS-mediated surface TF expression is due, at least in part, to transcriptional down-regulation.

MS Decreases LPS-mediated KC—Because LPS-induced TF mRNA and protein expression was down-regulated in the presence of MS, we next tested whether MS also modulates inflammatory responses in MLE-12 cells. LPS alone significantly augmented KC release measured in culture supernatants compared with control medium-treated cells (Fig. 2A). MS alone had no effect on KC levels. However, LPS-induced KC release was significantly reduced after MS compared with LPS treatment alone (Fig. 2A). To determine whether changes in KC
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release are transcriptionally regulated, mRNA levels were measured. LPS significantly increased KC mRNA expression, an effect that was inhibited by MS (Fig. 2B).

Effects of MS on TLR4 Expression—Next, we investigated the mechanisms behind MS-mediated reductions in LPS-induced TF and KC. To determine whether MS modulates expression of the cognate receptor for LPS (TLR4), TLR4 mRNA levels were measured. As shown in Fig. 3A, MLE-12 cells constitutively express TLR4 mRNA, and exposure of cells to any treatment did not significantly change expression compared with untreated cells at 6 h (Fig. 3A, dark gray bars) or at the earlier time point (4.5 h) (light gray bars). To determine whether stretch was affecting responses to LPS through altered cellular localization of the TLR4 receptor, we measured total and cell surface TLR4. As shown in Fig. 3B, there were no significant differences in total TLR4 expression by any treatment group. However, cell surface expression of TLR4 was significantly increased with either cell stretch or LPS alone compared with static-treated cells (Fig. 3C). By contrast, LPS exposure followed by MS significantly down-regulated TLR4 on the cell surface, suggesting that MS alters LPS-induced changes in cellular localization of TLR4.

MS Decreases LPS-induced NF-κB Activation—To determine whether MS-mediated changes in LPS-induced surface TLR4 result in reduced cell responsiveness to LPS, the activation state of NF-κB was measured from nuclear lysates isolated at various time points by a p65-specific activity assay. Exposure of cells to LPS alone for 30 min did not alter NF-κB activation

FIGURE 1. LPS-induced total and cell surface TF expression and mRNA levels are reduced by cell stretch. MLE-12 cells were treated with 10 μg/ml LPS for 4 h prior to stimulation with equibiaxial strain (20%) for 2 h. Cells were lysed, and proteins were resolved by SDS-PAGE. Blots were probed with anti-TF antibody (A), and then quantitative analysis of total cell-associated TF was performed as described under "Experimental Procedures" (B). In other experiments, surface expression of TF was measured by ELISA in non-permeabilized cells (C), whereas detection of TF mRNA from mRNA extracts was analyzed by RT-PCR (D). Results are means ± S.E. (n = three to four individual experiments (three wells/n)). *, p < 0.05 (versus control medium-treated cells); #, p < 0.05 (LPS-treated versus (LPS + stretch)-treated cells).

FIGURE 2. Cell stretch decreases LPS-mediated KC secretion and mRNA levels. MLE-12 cells were treated with 10 μg/ml LPS for 4 h prior to stimulation with equibiaxial strain (20%) for 2 h (black bars). A, cell culture supernatants were then assayed for KC by sandwich ELISA. B, RNA isolation from cell lysates was also analyzed for KC as described under "Experimental Procedures." Results are means ± S.E. (n = three to four individual experiments (three wells/n)). *, p < 0.05 (compared with control medium-treated cells); #, p < 0.05 (LPS-treated versus (LPS + stretch)-treated cells).
compared with untreated cells (data not shown); however, LPS stimulation for 4.5 h significantly increased activation (Fig. 3D).

Interestingly, MS alone during the 30 min in the 4.5-h time point also significantly increased NF-\(\kappa B\)/N9260B activation compared with static-treated cells (Fig. 3D). LPS-mediated NF-\(\kappa B\) activation in the presence of stretch was significantly reduced compared with that in LPS-treated cells.

**Stretch Reduces LPS-induced NF-\(\kappa B\) Activation, Which Is Required for LPS-mediated TF and KC Expression**—MLE-12 cells that were cotransfected with the plasmid encoding IxkBa-DN (19) and an NF-\(\kappa B\)-driven luciferase reporter plasmid (20) and that were exposed to LPS for 6 h had significantly decreased NF-\(\kappa B\) activation compared with LPS alone-treated cells (data not shown). Assays using the plasmid encoding the Renilla luciferase gene showed that these effects were not due to enhanced cell death (data not shown). Therefore, to further confirm the role of NF-\(\kappa B\) activation in LPS-mediated TF and KC expression, MLE-12 cells were transiently transfected with the plasmid encoding the IxkBa-DN cDNA or were mock-transfected with the empty vector. Similarly to previous experiments (Figs. 1C and 2A), mock-transfected LPS-treated cells exposed to MS also had significantly reduced TF and KC mRNA expression (Fig. 4, A and B). In addition, LPS-mediated KC release was also significantly reduced in the presence of the dominant-negative inhibitor (Fig. 4C). The effect of stretch on LPS-mediated KC release was not different between mock- and inhibitor-transfected cells (Fig. 4C).

**Effect of MS on Actin Organization**—To determine the mechanisms whereby cell surface TLR4 localization and NF-\(\kappa B\) are modulated by MS, we next analyzed actin organization. In control cells, phalloidin staining of stress fibers, which are predominantly composed of F-actin, showed clearly defined and linear fibers (Fig. 5A). This pattern of staining was unchanged by LPS treatment (Fig. 5B). However, these fibers appeared shorter and thicker in cells stretched for 2 h (Fig. 5C). Cells exposed to both LPS and cyclic MS displayed a less dense, more diffuse staining of stress fibers (Fig. 5D), suggesting that stretch-mediated reorganization of actin in the presence of LPS is modulated differentially compared with stretch alone.

**Ethanol Inhibits LPS-induced Up-regulation of Surface TLR4 and KC Release**—Recently, studies have shown that EtOH interferes with LPS-mediated cell responses. Mechanistic studies showed that EtOH-mediated effects involved reduction in surface TLR4 expression dependent on modulation of actin filaments (22, 23). To determine whether EtOH modulates surface TLR4 expression and thus cell responsiveness to LPS similar to MS, MLE-12 cells were exposed to 0.4% (w/v) EtOH for 10 min before LPS exposure for 4.5 h. Analysis of cell viability demonstrated no significant differences between any treatment group (data not shown). As shown in Fig. 6A, exposure of cells...
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to LPS alone increased surface TLR4, whereas, similar to MS, EtOH inhibited LPS-mediated up-regulation of cell surface TLR4. Further analysis of the downstream effects of EtOH (Fig. 6B) demonstrated that the LPS-mediated increase in KC secretion was also significantly reduced.

Effects of MS on LPS-induced Procoagulant Activity—LPS exposure significantly increased cell surface TF procoagulant activity (Fig. 7A). Exposure of LPS-treated cells to MS increased TF activity further, suggesting that cell surface TF procoagulant activity is differentially regulated by stretch compared with TF mRNA or protein levels. To determine whether MS modifies LPS-induced release of TF into the medium, supernatants from cells were analyzed for TF procoagulant activity using a plasma recalcification assay. As shown in Fig. 7B, LPS significantly increased procoagulant activity in the media (reduced clot time) at all concentrations. MS alone significantly increased procoagulant activity in the media compared with static-treated cells. However, MS did not modulate LPS-mediated TF release compared with LPS alone.

Stretch-mediated Increase in LPS-induced TF Activity Is Not Mediated by Calcium Signaling—To investigate the potential mechanisms behind stretch-induced effects on LPS-mediated cell surface TF procoagulant activity, we pretreated MLE-12 cells with 20 μM BAPTA-AM, a cell-permeable calcium chelator, for 10 min prior to LPS exposure and subsequent MS. BAPTA-AM did cause measureable cytotoxicity; therefore, the results are shown as TF activity normalized to cell viability. As shown in Fig. 8, MLE-12 cells exposed to BAPTA-AM did not significantly alter TF activity, indicating that calcium signaling is not involved in stretch-induced increases in TF activity mediated by LPS.

DISCUSSION

MV can potentiate ALI caused by infection or other insults and the mechanisms that underlie this ventilator-associated lung injury are incompletely understood. The objective of this study was to test the hypothesis that MS accentuates the innate
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immune response triggered by LPS exposure in alveolar epithelial cells as measured by both procoagulant (TF) and proinflammatory (KC) responses. Contrary to our hypothesis, MS of lung epithelial cells reduced LPS-induced total and cell surface protein expression of TF, the key activator of the extrinsic coagulation cascade, and reduced secretion of the proinflammatory chemokine KC. MS-mediated changes in both LPS-induced TF and KC levels were transcriptionally regulated and involved reduction of LPS-induced NF-κB activation. However, despite the prevention of induction of LPS-mediated TF mRNA and protein expression by stretch, cell surface TF activity was significantly increased, suggesting that different pathways regulate TF expression and activity in this model system.

Our findings can be compared with prior studies of the effect of MS on TLRs, a family of innate immune receptors that recognize conserved molecular motifs from microorganisms and play a central role in the initiation of inflammatory responses in numerous diseases (24–26). Mild cyclic stretch (20% elongation at 20 times/min for 24 h) of human lung epithelial cells (A549) up-regulated TLR2 mRNA and protein levels (17). Exposure to the synthetic bacterial lipopeptide Pam3CSK4 24 h after treatment with mild MS significantly increased inflammatory mediator production compared with Pam3CSK4 alone-treated cells (17). Similarly, our data show that exposure to either LPS or mild cyclic MS for short periods increased surface TLR4 expression. However, enhanced surface expression was not explained by increased transcription, as mRNA levels did not change, suggesting that LPS- and MS-mediated effects are regulated post-transcriptionally. In contrast to the TLR2 findings, however, combined exposure to LPS and stretch significantly reduced membrane-bound TLR4 in MLE-12 cells, which

FIGURE 6. EtOH inhibits LPS-mediated surface TLR4 and KC release. MLE-12 cells were pretreated for 10 min with 0.4% (w/v) EtOH before exposure to 10 μg/ml LPS for 4.5 h. A, surface expression of TLR4 was examined on fixed non-permeabilized cells. B, cell culture supernatants were assayed for KC by sandwich ELISA. *, p < 0.05 (compared with control medium-treated cells or LPS-treated cells (brackets)).

FIGURE 7. Cell stretch augments surface TF activity without modifying LPS-mediated clot time. MLE-12 cells were treated with 10 μg/ml LPS for 4 h prior to stimulation with equibiaxial strain (20%) for 2 h (black bars). Cell lysates were then assayed for surface TF activity (A), whereas culture supernatants were assayed for clot time (B) as described under “Experimental Procedures.” Results are means ± S.E. (n = three to four individual experiments (three wells/n)). *, p < 0.05 (compared with control medium-treated cells); #, p < 0.05 (LPS-treated versus (LPS + stretch)-treated cells).

FIGURE 8. MS-induced increase in LPS-induced cell surface TF activity is not mediated through calcium signaling. MLE-12 cells were pretreated with 20 μM BAPTA-AM for 30 min prior to stimulation with 10 μg/ml LPS for 4 h. Cells were then stretched at 20% for 2 h. As a vehicle control, cells were incubated with 0.01% Me2SO. Cells were then assayed for TF activity. Results are means ± S.E. (n = three individual experiments (three wells/n)).
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again appeared to be post-transcriptionally regulated and not a result of increased degradation because total cell-associated TLR4 was not altered by any treatment (Fig. 3B). Thus, surface TLR4 expression is modulated differentially by stretch superimposed on LPS exposure than by LPS exposure alone. Whether our findings differ from other reports because of a difference in time course, differences between cell lines, or differences in regulation of the different TLRs is a topic for future study.

Similar to other cell types (27–29), exposure of MLE-12 cells to LPS induced activation of the transcription factor NF-κB (Fig. 3D). Furthermore, NF-κB activation was required for LPS-induced KC and TF mRNA expression as well as KC release (Fig. 4, A–C). However, in contrast to a prior study that demonstrated that co-expression of macrophages to MS and LPS induced NF-κB activation (29), we found that MS of MLE-12 cells reduced LPS-mediated NF-κB nuclear localization (Fig. 3D). Interestingly, the down-regulation of TF and KC mRNA expression by MS in the presence of LPS was not significantly reduced by transfection with a dominant-negative inhibitor that blocks NF-κB activation (Fig. 4, A and B) (19).

Investigation into potential mechanisms regulating stretch-mediated inhibition of LPS-induced surface TLR4 localization demonstrated a potential role for actin filament reorganization. MLE-12 cells subjected to MS in the presence of LPS showed diffuse cell-spanning stress fibers compared with LPS-treated cells (Fig. 5, B and D). These changes in actin filament organization coincided with stretch-mediated reduction of LPS-induced TF and KC expression, suggesting a possible role for actin in these processes. These findings are consistent with prior reports that actin plays a critical role in the translation of mechanical stimuli into intracellular biochemical processes (30–32). For example, in human trabecular meshwork cells, unstretched cells had a defined linear microfilament organization, whereas cells subjected to 10% MS for 15 min displayed frequent formations of polygonal microfilament networks consisting of short thick filament bundles radiating from common vertices (33). This correlated with a rapid reduction in MAPK activity and the c-fos pathway while maintaining JNK signal transduction, indicating that stretch-induced modulation of signaling pathways is regulated differentially in human trabecular meshwork cells (33).

EtOH can modulate LPS-mediated cell signaling through actin reorganization (23, 34, 35). In a study by Dai and Pruett (23), exposure of the human macrophage cell line RA W264.7 to LPS in the presence of EtOH altered the distribution of TLR4 and its coreceptor, CD14, such that LPS-induced TNF-α was reduced. EtOH-induced modulation of TLR4 localization was found to be dependent on actin organization, thus indicating a role for actin filaments (23). In another study, exposure of rat intestinal epithelial cells to increasing concentrations of EtOH led to the collapse of the actin cytoskeleton as depicted by extensive disorganization and fragmentation (36). In our experiments, exposure of MLE-12 cells to 0.4% EtOH in the presence of LPS significantly reduced surface TLR4 expression similar to MS-mediated effects (Fig. 6A). This was concomitant with a reduction in LPS-mediated KC release (Fig. 6B). These results suggest that EtOH, a known modulator of actin filament organization, decreases MLE-12 cell responses to LPS potentially through similar mechanisms compared with stretch.

Both the expression and activity of the key initiator of the extrinsic coagulation cascade, TF, are increased in pulmonary edema fluid and plasma from patients with ALI/ARDS, and TF-dependent procoagulant activity is believed to be a major contributor to fibrin deposition in the airspace (37). Although macrophages are known to contribute to fibrin deposition in the airspace during lung disease, alveolar epithelial cells are also a potential source of intra-alveolar TF (38, 39). TF is up-regulated in human alveolar epithelial cells (A549) in response to proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ), indicating that the alveolar epithelium may play a role in the pathogenesis of ALI/ARDS by modulating intra-alveolar coagulation and fibrin deposition (37). However, despite increased understanding of TF regulation in the lung, there are still many unanswered questions. The explanation for enhanced LPS-mediated cell surface TF activity in the presence of MS despite the reduction in total cellular TF expression is not yet clear but is consistent with other studies that have shown discrepancies between TF expression and activity. For example, in human smooth muscle cells isolated from human saphenous veins and aortas, exposure to the immunosuppressant drug sirolimus increased TF expression without modifying activity (40). In another study, exposure of human monocytes to increasing concentrations of LPS increased TF activity without modulation of expression, an effect that was attributed to enhanced cell death (41). To ensure that reduced TF expression in our studies was not a result of decreased viability, we measured cell death by both trypan blue exclusion assay and cell proliferation assay (data not shown). In both instances, there was no difference in viability compared with control cells. Finally, although TF activity was not studied, investigation of the expression patterns of TF and cytoskeletal proteins in various epithelial cell lines showed colocalization of TF with cell membrane areas rich in actin filaments (42), suggesting that modification of cytoskeletal protein organization alters TF localization and/or activity. These studies suggest that different signaling pathways regulate TF expression and activity potentially through actin filament reorganization.

Calcium flux is an important mediator of cell surface TF activity and release of procoagulant microparticles (43–45). Given the critical role for calcium signaling in TF activation and the fact that studies in other experimental models have shown that MV/MS can alter calcium flux (46–48), we tested whether stretch-mediated augmentation of cell surface TF activity is calcium-mediated. However, our experiments did not show a role for calcium signaling at the time point studied. Another possibility is that there is a role for MAPK activation in modulating stretch-mediated LPS-induced TF activity; this is an avenue for future study. With regard to activation of cell surface TF, because the transcription factor activity was reduced in the presence of stretch and LPS, the data suggest that the NF-κB pathway most likely does not contribute to stretch-mediated increases in TF activity.

There are some limitations to this study. As stated above, this study focused on acute stretch-mediated changes to LPS-induced inflammatory and coagulant pathways. Alveolar epithe-
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REFERENCES

1. Ware, L. B., Bastarache, J. A., and Wang, L. (2005) Coagulation and fibrinolysis in human acute lung injury—new therapeutic targets? *Keio J. Med.* **54**, 142–149
2. Sebag, S. C., Bastarache, J. A., and Ware, L. B. (2011) Therapeutic modulation of coagulation and fibrinolysis in acute lung injury and the acute respiratory distress syndrome. *Curr. Pharm. Biotechnol.* **12**, 1481–1496
3. Bhadade, R. R., de Souza, R. A., Harde, M. J., and Khot, A. (2011) Clinical characteristics and outcomes of patients with acute lung injury and ARDS. *J. Postgrad. Med.* **57**, 286–290
4. Altemeier, W. A., Matute-Bello, G., Frevert, C. W., Kawata, Y., Kajikawa, O., Martin, T. R., and Glenny, R. W. (2004) Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L533–L542
5. Altemeier, W. A., Matute-Bello, G., Gharib, S. A., Glenny, R. W., Martin, T. R., and Liles, W. C. (2005) Modulation of lipopolysaccharide-induced gene transcription and promotion of lung injury by mechanical ventilation. *J. Immunol.* **175**, 3369–3376
6. Brégeon, F., Delpierre, S., Chetaille, B., Kajikawa, O., Martin, T. R., and Glenny, R. W. (2004) Mechanical ventilation affects lung function and cytokine production in an experimental model of endotoxiaemia. *Anesthesiology* **102**, 331–339
7. Girard, T. D., and Bernard, G. R. (2007) Mechanical ventilation in ARDS: a state-of-the-art review. *Chest* **131**, 921–929
8. Chang, C. H., Chuang, C. H., Liu, S. L., Lee, T. S., Ko, Y. R., and Zhang, H. (2011) Apocynin attenuates ventilator-induced lung injury in an isolated and perfused rat lung model. *Intensive Care Med.* **37**, 1360–1367
9. Schultz, M. J., Millo, J., Levi, M., Hack, C. E., Weaverling, G. J., Garrard, C. S., and van der Poll, T. (2004) Local activation of coagulation and inhibition of fibrinolysis in the lung during ventilator-associated pneumonia. *Thorax* **59**, 130–135
10. Siore, A. M., Parker, R. E., Steenken, A. A., Cuppels, C., McKeen, M., Christman, B. W., Cruz-Gervis, R., and Brigham, K. L. (2005) Endotoxin-induced acute lung injury requires interaction with the liver. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **289**, L769–L776
11. Jeyaseelan, S., Chu, H. W., Young, S. K., and Worthy, G. S. (2004) Transcriptional profiling of lipopolysaccharide-induced acute lung injury. *Infect. Immun.* **72**, 7247–7256
12. Brigham, K. L., and Meyrick, B. (1986) Endotoxin and lung injury. *Am. Rev. Respir. Dis.* **133**, 913–927
13. Silverman, M. D., Manolopoulos, V. G., Unsworth, B. R., and Lelkes, P. I. (1996) Tissue factor expression is differentially modulated by cyclic mechanical strain in various human endothelial cells. *Blood Coagul. Fibrinolysis* **7**, 281–288
14. Sumpio, B. E. (1991) Hemodynamic forces and the biology of the endothelium: signal transduction pathways in endothelial cells subjected to physical forces in vitro. *J. Vasc. Surg.* **13**, 744–746
15. Chien, S., Li, S., and Shy, Y. J. (1998) Effects of mechanical forces on signal transduction and gene expression in endothelial cells. *Hypertension* **31**, 162–169
16. Chu, E. K., Cheng, J., Foley, J. S., Meccham, B. H., Owen, C. A., Haley, K. J., Mariani, T. J., Kohane, I. S., Tschumperlin, D. J., and Dzenz, J. M. (2006) Induction of the plasminogen activator system by mechanical stimulation of human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **35**, 628–638
17. Charles, P. E., Tissières, P., Barbar, S. D., Croisier, D., Dufour, J., Dunn-Siegrist, L., Chavanet, P., and Pugin, J. (2011) Mild-stretch mechanical ventilation up-regulates toll-like receptor 2 and sensitizes the lung to bacterial lipopeptide. *Crit. Care* **15**, R181
18. Bourgeon, E., Isowa, N., Keshavye, S., Zhang, X., Slutsky, A. S., and Liu, M. (2000) Mechanical stretch stimulates macrophage inflammatory protein-2 secretion from fetal rat lung cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279**, L699–L706
19. Cheng, D. S., Han, W., Chen, S. M., Sherrill, T. P., Chont, M., Park, G. Y., Shelker, J. R., Polosukhin, V. V., Christman, J. W., Yull, F. E., and Blackwell, T. S. (2007) Airway epithelium controls lung inflammation and injury through the NF-κB pathway. *J. Immunol.* **178**, 6504–6513
20. Everhart, M. B., Han, W., Sherrill, T. P., Arutjunov, M., Polosukhin, V. V., Burke, J. R., Sadikot, R. T., Christman, J. W., Yull, F. E., and Blackwell, T. S. (2006) Duration and intensity of NF-κB activity determine the severity of endotoxin-induced acute lung injury. *J. Immunol.* **176**, 4995–5005
21. Sebag, J. A., and Hinkle, P. M. (2010) Regulation of G protein-coupled receptor signaling: specific dominant-negative effects of melanocortin 2 receptor accessory protein 2. *Sci. Signal.* **3**, ra28
22. Dolganjic, A., Bakis, G., Kodya, K., Mandrekar, P., and Szabo, G. (2006) Acute ethanol treatment modulates Toll-like receptor-4 association with lipid rafts. *Alcohol. Clin. Exp. Res.* **30**, 76–85
23. Dai, Q., and Pruett, S. B. (2006) Ethanol suppresses LPS-induced Toll-like receptor 4 clustering, reorganization of the actin cytoskeleton, and associated TNF-α production. *Alcohol. Clin. Exp. Res.* **30**, 1436–1444
24. Akira, S., Takeda, K., and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**, 675–680
25. Beutler, B. (2004) Toll-like receptors and their place in immunology. Where does the immune response to infection begin? *Nat. Rev. Immunol.* **4**, 498
26. Shin, H. S., Xu, F., Bagchi, A., Herrup, E., Prakash, A., Valentine, C., Kulkarni, H., Wilmheilsen, K., Warren, S., and Hellman, J. I. (2011) Bacterial lipoprotein TLR2 agonists broadly modulate endothelial function and coagulation pathways in vitro and in vivo. *J. Immunol.* **186**, 1119–1130
27. Tiruppathi, C., Shimizu, I., Miyawaki-Shimizu, K., Vogel, S. M., Bair, A. M., Minshall, R. D., Predescu, D., and Malik, A. B. (2008) Role of NF-κB-dependent caveolin-1 expression in the mechanism of increased endothelial permeability induced by lipopolysaccharide. *J. Biol. Chem.* **283**, 4210–4218
28. Skerrett, S. I., Liggett, H. D., Hajjar, A. M., Ernst, R. K., Miller, S. I., and Wilson, C. B. (2004) Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L143–L152
29. Pugin, J., Dunn, J., Lolljet, P., Tassaux, D., Magenot, J. L., Nicod, L. P., and Cheurove, J. C. (1998) Activation of human macrophages by mechanical ventilation in vitro. *Am. J. Physiol.* **275**, L1040–L1050
30. Janmey, P. A., and Weitz, D. A. (2004) Dealing with mechanics: mechanics of force transduction in cells. *Trends Biochem. Sci.* **29**, 364–370
31. Chao, B., Yang, R., and Sha, Q. (2006) Mechanical stretch modifies

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the promoter activity of the profibrotic factor CCN2 through increased actin polymerization and NF-κB activation. *J. Biol. Chem.* **281**, 20608–20622

32. Cohen, T. S., DiPaolo, B. C., Lawrence, G. G., and Margulies, S. S. (2012) Sepsis enhances epithelial permeability with stretch in an actin-dependent manner. *PLoS ONE* **7**, e38748

33. Tumminia, S. J., Mitton, K. P., Arora, J., Zelenka, P., Epstein, D. L., and Russell, P. (1998) Mechanical stretch alters the actin cytoskeletal network and signal transduction in human trabecular meshwork cells. *Invest. Ophthalmol. Vis. Sci.* **39**, 1361–1371

34. Loureiro, S. O., Heimfarth, L., Reis, K., Wild, L., Andrade, C., Guma, F. T., Gonçalves, C. A., and Pessoa-Pureur, R. (2011) Acute ethanol exposure disrupts actin cytoskeleton and generates reactive oxygen species in C6 cells. *Toxicol. In Vitro* **25**, 28–36

35. Szabo, G., Dolganiuc, A., Dai, Q., and Pruett, S. B. (2007) TLR4, ethanol, and lipid rafts: a new mechanism of ethanol action with implications for other receptor-mediated effects. *J. Immunol.* **178**, 1243–1249

36. Banan, A., Smith, G. S., Kokoska, E. R., and Miller, T. A. (2000) Role of actin cytoskeleton in prostaglandin-induced protection against ethanol in an intestinal epithelial cell line. *J. Surg. Res.* **88**, 104–113

37. Bastarache, J. A., Wang, L., Geiser, T., Wang, Z., Albertine, K. H., Matthay, M. A., and Ware, L. B. (2007) The alveolar epithelium can initiate the extrinsic coagulation cascade through expression of tissue factor. *Thorax* **62**, 608–616

38. Sitrin, R. G., Kaltreider, H. B., Amsfeld, M. J., and Webster, R. O. (1983) Procoagulant activity of rabbit alveolar macrophages. *Am. Rev. Respir. Dis.* **128**, 282–287

39. Gross, T. J., Simon, R. H., and Sitrin, R. G. (1992) Tissue factor procoagulant expression by rat alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **6**, 397–403

40. Zhu, S., Viswambharan, H., Gajanayake, T., Ming, X. F., and Yang, Z. (2005) Sirolimus increases tissue factor expression but not activity in cultured human vascular smooth muscle cells. *BMC Cardiovasc. Disord.* **5**, 22

41. Henriksson, C. E., Klingenberg, O., Ovstebo, R., Joo, G. B., Westvik, A. B., and Kierulf, P. (2005) Discrepancy between tissue factor activity and tissue factor expression in endotoxin-induced monocytes is associated with apoptosis and necrosis. *Thromb. Haemost.* **94**, 1236–1244

42. Müller, M., Albrecht, S., Gölöfft, F., Hofer, A., Funk, R. H., Magdolen, V., Flössel, C., and Luther, T. (1999) Localization of tissue factor in actin-filament-rich membrane areas of epithelial cells. *Exp. Cell Res.* **248**, 136–147

43. Bach, R., and Rifkin, D. B. (1990) Expression of tissue factor procoagulant activity: regulation by cytosolic calcium. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6995–6999

44. Bach, R. R. (1998) Mechanism of tissue factor activation on cells. *Blood Coagul. Fibrinolysis* **9**, S37–S43

45. Carson, S. D., Perry, G. A., and Pirruccello, S. J. (1994) Fibroblast tissue factor: calcium and ionophore induce shape changes, release of membrane vesicles, and redistribution of tissue factor antigen in addition to increased procoagulant activity. *Blood* **84**, 526–534

46. Wirtz, H. R., and Dobbs, L. G. (1990) Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* **250**, 1266–1269

47. Kato, T., Ishiguro, N., Iwata, H., Kojima, T., Ito, T., and Naruse, K. (1998) Up-regulation of COX2 expression by uni-axial cyclic stretch in human lung fibroblast cells. *Biochem. Biophys. Res. Commun.* **244**, 615–619

48. Parker, J. C. (2000) Inhibitors of myosin light chain kinase and phosphodiesterase reduce ventilator-induced lung injury. *J. Appl. Physiol.* **89**, 2241–2248