

Mechanical Ventilation Induces Desensitization of Lung Axl Tyrosine Kinase Receptors

Gail Otulakowski, Ph.D., Doreen Engelberts, A.H.T., Martin Post, Ph.D., Claire Masterson, Ph.D., Brian P. Kavanagh, M.B., M.R.C.P.(I), F.R.C.P.(C)

ABSTRACT

Background: Lower tidal volumes are increasingly used in acute respiratory distress syndrome, but mortality has changed little in the last 20 yr. Therefore, in addition to ventilator settings, it is important to target molecular mediators of injury. Sepsis and other inflammatory states increase circulating concentrations of Gas6, a ligand for the antiinflammatory receptor Axl, and of a soluble decoy form of Axl. We investigated the effects of lung stretch on Axl signaling.

Methods: We used a mouse model of early injury from high tidal volume and assessed the effects of inhibiting Axl on *in vivo* lung injury (using an antagonist R428, $n = 4/\text{group}$). We further determined the effects of stretch on Axl activation using *in vitro* lung endothelial cells.

Results: High tidal volume caused mild injury (compliance decreased 6%) as intended, and shedding of the Axl receptor (soluble Axl in bronchoalveolar fluid increased 77%). The Axl antagonist R428 blocked the principal downstream Axl target (suppressor of cytokine signaling 3 [SOCS3]) but did not worsen lung physiology or inflammation. Cyclic stretch *in vitro* caused Axl to become insensitive to activation by its agonist, Gas6. Finally, *in vitro* Axl responses were rescued by blocking stretch-activated calcium channels (using guanidinium chloride [GdCl₃]), and the calcium ionophore ionomycin replicated the effect of stretch.

Conclusions: These data suggest that lung endothelial cell overdistention activates ion channels, and the resultant influx of Ca²⁺ inactivates Axl. Downstream inactivation of Axl by stretch was not anticipated; preventing this would be required to exploit Axl receptors in reducing lung injury. (ANESTHESIOLOGY 2018; 129:143-53)

MECHANICAL ventilation is the main supportive therapy in patients with acute respiratory distress syndrome. However, lung stretch from ventilation can cause—or worsen—lung injury, and contribute to adverse outcomes.¹ There are multiple mechanisms whereby mechanical ventilation contributes to lung injury,²⁻⁴ and a common final result is increased lung inflammation and dysfunction.

While low tidal volume certainly reduces mortality in acute respiratory distress syndrome,⁵ efforts to further decrease mortality by adjusting ventilator parameters have not yielded positive results in randomized controlled trials.⁶ In parallel with this trend, the mortality associated with acute respiratory distress syndrome has been stable (and high, approximately 40%) for the last 20 yr.⁷

Among explanations for this “plateau,” two factors may be important. First, while “global” tidal volume (V_T) is important, there is considerable distension of some parts of the lung, even with low V_T . Due to widespread injury, consolidation, and atelectasis, the aerated lung—sometimes referred to as the “baby” lung⁸—is a small fraction of the total lung volume. Because the baby lung receives all of the V_T (albeit a low V_T), it is usually overdistended and inflamed during inspiration,⁹⁻¹¹ even though such a V_T would cause minimal distension (and no inflammation) if it were distributed across the whole lung.

What We Already Know about This Topic

- Mechanical ventilation is a core strategy in acute respiratory distress syndrome management, yet mechanical stretch can contribute to ventilator-associated lung injury.
- Although low tidal volume ventilation strategies are increasingly used, mortality remains high. Therefore, it is important to understand molecular mediators of injury and protection.
- Axl is a membrane receptor with potential protective effects; its ligand and a soluble decoy form of Axl are both increased in sepsis. The effects of Axl signaling in acute respiratory distress syndrome are unknown.

What This Article Tells Us That Is New

- Overdistention of lung endothelial cells caused an influx of calcium, which rendered Axl insensitive to the activating effects of its ligand.
- The immune regulatory and antiapoptotic effects of Axl in endothelial cells may thus be compromised by injurious mechanical ventilation.

Second, the limited benefit from further ventilator titration suggests that the secondary inflammatory mechanisms should be evaluated as potential targets. The TAM (Tyro3, Axl, Mer) family of tyrosine receptor kinases is one important regulatory group that regulates inflammation in the immune and vascular systems.¹² One of these receptors, Axl, mediates antiinflammatory effects^{13,14} in acute organ injury

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and antiapoptotic¹⁵ effects in cultured endothelial cells, and blocking its actions would seem to be a potentially testable means of reducing lung injury.

Indeed, the main endogenous ligand of Axl, growth arrest-specific 6 (Gas6), activates antiinflammatory pathways, and upregulates downstream antiinflammatory signaling genes called suppressor of cytokine signaling (SOCS)–1 and SOCS3.¹⁶ Consistent with this, Axl stimulation attenuates tissue injury in several nonpulmonary settings,^{13,17} and inhibition of Axl¹³ or impairment of its signaling¹⁸ can worsen injury.

Axl is of interest in acute respiratory distress syndrome because its endogenous agonist Gas6 is increased in sepsis,¹⁹ and sepsis is one of the major risk factors for acute respiratory distress syndrome.²⁰ However, activation of Axl also involves shedding of the receptor's extracellular component, and the shed (soluble) receptors can sequester agonist and function as “decoy” receptors.¹² Therefore, the net impact of receptor activation is difficult to predict.

We explored the role of Axl signaling and the impact of Axl inhibition in an *in vivo* murine model of mild ventilator-induced lung injury. We hypothesized that inhibition of Axl would reduce expression of downstream SOCS1,3 and thereby increase lung injury during injurious mechanical ventilation. Surprisingly, Axl blockade was not accompanied by increased injury. Using isolated pulmonary microvascular endothelial cells, we found that cyclic mechanical stretch desensitizes Axl to the effects of Gas6, and this phenomenon appears to be due to calcium influx *via* stretch-activated calcium channels.

Materials and Methods

The detailed methodology can be found in Supplemental Digital Content 1 (<http://links.lww.com/ALN/B637>).

Ethical Approval

All animal procedures were reviewed and approved by the animal care committee of the Hospital for Sick Children (Toronto, Canada) in accordance with the Guidelines of the Canadian Council on Animal Care (Ottawa, Canada).

Murine Model of Ventilator-induced Lung Injury

C57BL/6J male mice (20 to 25 g, Charles River, Canada) were anesthetized (ketamine 150 mg/kg, xylazine 15 mg/kg, intraperitoneal), and ventilated *via* tracheotomy using a computer-controlled small-animal ventilator (SCIREQ, Flexivent, Canada). Baseline (low-stretch) ventilation was with V_T 10 ml/kg, positive end-expiratory pressure 2.0 cm H₂O, frequency 135/min, and fraction of inspired oxygen 0.21. Lung compliance was measured at baseline and hourly thereafter.

In series 1, animals were randomized to continue baseline ventilation or receive high tidal ventilation (V_T 20 ml/kg, positive end-expiratory pressure 0 cm H₂O, frequency 45/min, fraction of inspired oxygen 0.21) for 4 h ($n = 4$ /group).²¹ In series 2, C57/BL6J male mice were randomized to receive Axl antagonist R428 (100 mg/kg in 1% dimethylsulfoxide,

balance saline) or vehicle ($n = 4$ /group), by intraperitoneal administration¹³ 2 h before mechanical ventilation.

After completing the experiment, mice were euthanized by exsanguination during anesthesia, bronchoalveolar lavage was performed for protein analysis, and lungs were removed and snap frozen. Lung myeloperoxidase activity was measured spectrophotometrically from lung tissue homogenized in 0.5% hexadecyltrimethylammonium bromide and incubated with 0.2 mg/ml *o*-dianisidine and 0.001% H₂O₂.²²

Endothelial Cell Culture, Cyclic Stretch, and Axl Activation by Gas6

Rat pulmonary microvascular endothelial cells were seeded on Bioflex six-well plates (Flexcell International, USA) for stretch experiments, or onto standard six-well tissue culture plates for ionomycin experiments. Confluent monolayers were washed with phosphate-buffered saline and starved for 4 h in serum-free media. Inhibitors (TNF α protease inhibitor 2 [TAPI-2], 20 μ M; Y27632, 5 μ M; GdCl₃, 50 μ M), or vehicle was added 15 to 30 min before initiation of stretch. Cells were subjected to cyclic stretch for 30 min at a setting of 17% change in surface area, equibiaxial strain at 0.5 Hz. Axl agonist Gas6 (5 nM) was added, and cells were incubated at 37°C for 10 min under static conditions before harvest. Ionomycin (50 nM) was added 30 min before Gas6 in experiments using unstretched cells as indicated in the figures.

Detection of Soluble Axl Ectodomain Shedding

To detect Axl ectodomain shedding *in vitro*, rat pulmonary microvascular endothelial cell monolayers on Bioflex plates were serum starved and subjected to stretch as in “Endothelial Cell Culture, Cyclic Stretch, and Axl Activation by Gas6.” Conditioned media was harvested and concentrated using a centrifugal filter device (Amicon Centriprep YM-10; Millipore Ltd, Canada). Conditioned media and matched cell lysates were analyzed by Western blot as described in “Immunoprecipitation and Western Blots” to detect soluble Axl ectodomain and Axl, and shedding expressed as soluble Axl ectodomain in conditioned media divided by total Axl (soluble Axl ectodomain in media + Axl in lysate).

Quantitation of Messenger RNAs

Changes in gene expression were measured using relative quantitative real-time polymerase chain reaction. Gene expression was calculated relative to 18S ribosomal RNA using the comparative cycle threshold ($\Delta\Delta C_t$) method with reverse-transcribed complementary DNA from a nonventilated mouse lung as a calibrator.

Immunoprecipitation and Western Blots

For direct immunoblot analysis, an aliquot of cell lysate or conditioned media was denatured in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. For immunoprecipitation, cell lysate was

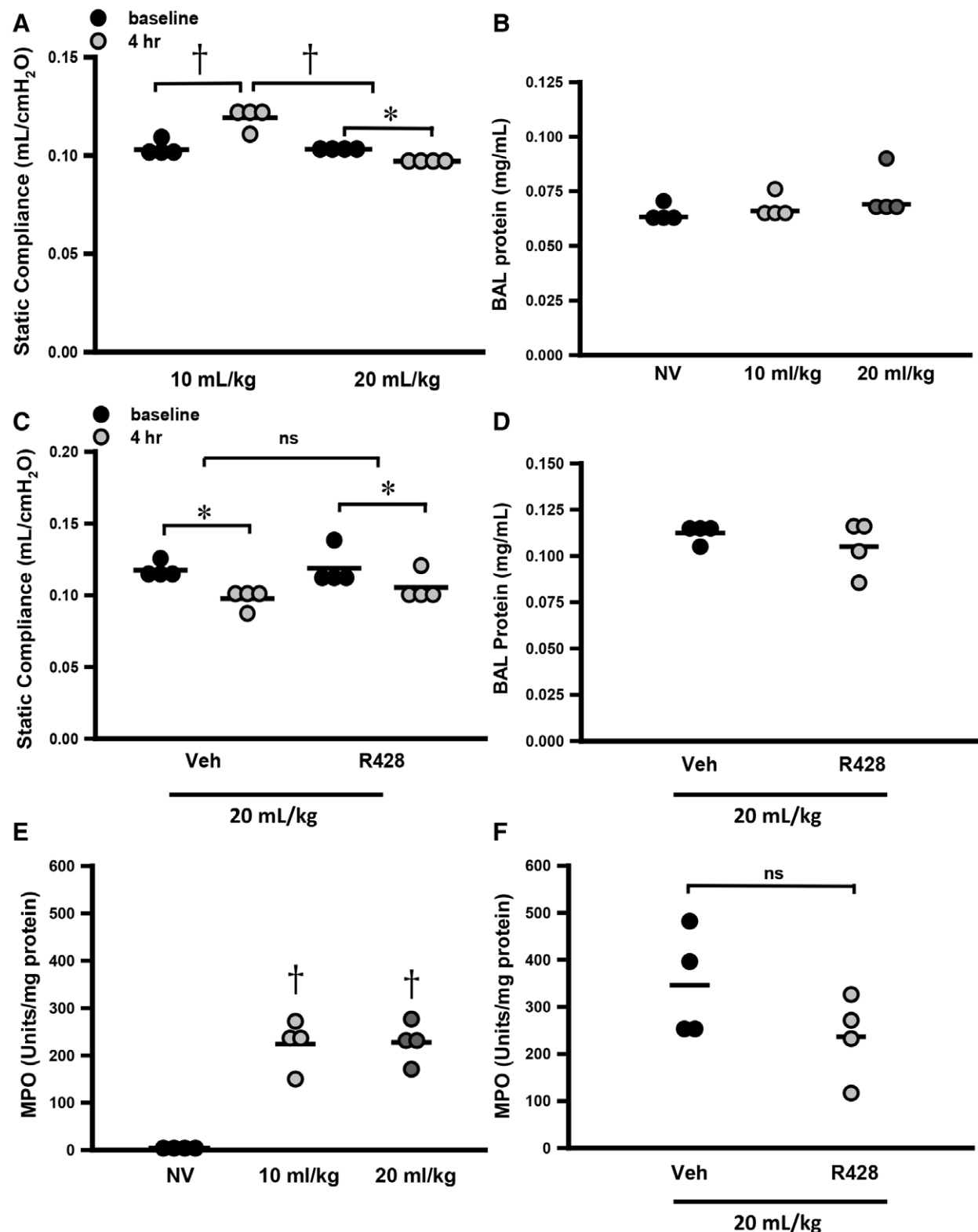


Fig. 1. Inhibition of Axl with R428 did not worsen lung injury in murine ventilator-induced lung injury. Mice ventilated with injurious V_T (20 mL/kg) exhibited decreased static compliance over time (* P < 0.05 vs. baseline) and compared to mice ventilated with protective V_T (10 mL/kg). † P < 0.001 vs. 10 mL/kg (A, two-way repeated measures ANOVA for effects of group and time). Bronchoalveolar lavage (BAL) protein increased by up to 10%, but this finding did not reach significance (B, ANOVA on ranks). In mice ventilated with V_T 20 mL/kg for 4 h, static compliance decreased over time (* P < 0.001 vs. baseline), but there was no difference in static compliance (C, two-way repeated measures ANOVA as above) or BAL protein (D, t test) between vehicle (Veh) and R428 groups. n = 4 per group. Myeloperoxidase (MPO) was increased in ventilated mice. † P < 0.001 versus nonventilated (NV; E, one-way ANOVA), but not significantly affected by R428 (F, t test). ns = nonsignificant.

incubated overnight at 4°C with Axl antibody (1 µg), followed by protein G-agarose for 2 h. After washing, immunoprecipitates were eluted in SDS-PAGE sample loading buffer at 95°C. Protein samples were electrophoresed through polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blocked, and proteins were detected by chemiluminescence after incubation with appropriate antibodies.

Statistical Analysis

A formal sample size calculation was not performed; sample sizes were an estimate based on experience and preliminary data. Experimenters were not blinded during data collection or analysis. No data have been excluded or lost. Data where $n \leq 4$ are presented as dot density plots with lines indicating mean or median depending on whether parametric or nonparametric analysis was performed; where $n > 4$, data are presented as bar graphs of mean \pm SD for normally distributed data, or median and quartiles where nonparametric analyses were used. Statistical differences were calculated with Sigmaplot v12.3 (Systat Software Inc., USA) using ANOVA for multigroup comparisons followed by Student-Newman-Keuls (one-way ANOVA) or Holm-Sidak (two-way ANOVA) *post hoc* tests. Repeated measures ANOVA was used for analysis of changes in static compliance over time; all other analyses studied effects between subjects.

Two-group comparisons used *t* tests for normally distributed data, and Mann-Whitney U tests for nonnormally distributed data. Detailed statistical descriptions can be found in individual figure legends. Differences with a *P* value < 0.05 were considered significant.

Results

Inhibition of Axl Did Not Worsen Injury in Ventilated Mice

We established a model of mild lung injury²¹ to permit testing of our hypothesis that inhibition of Axl with R428 would exacerbate injury. Ventilation with high V_T (V_T 20 ml/kg, for 4 h) led to a 6% decrease in compliance compared to low V_T ventilation (V_T 10 ml/kg; fig. 1A). Median bronchoalveolar lavage protein increased by 10% after 4-h high V_T (compared to nonventilated controls), but this result was not statistically significant (fig. 1B); the rank order of impact of V_T was 20 ml/kg $>$ 10 ml/kg $>$ spontaneous breathing. In high V_T mice (V_T 20 ml/kg, for 4 h), there was no difference between the vehicle or R428 groups in terms of respiratory system compliance (fig. 1C), protein concentration in the bronchoalveolar lavage fluid (fig. 1D), or oxygenation (Supplemental Digital Content 2, fig. S1, <http://links.lww.com/ALN/B638>) at the end of the experiment. In addition, tissue myeloperoxidase was increased in both ventilated groups (fig. 1E) but not worsened by R428 (fig. 1F).

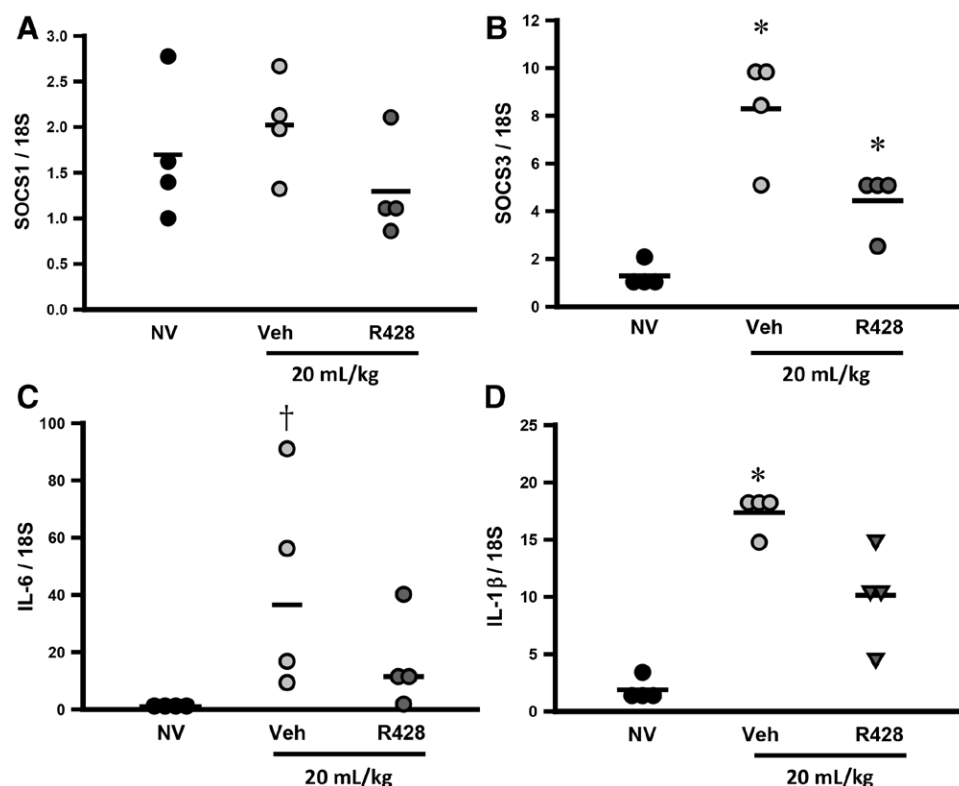


Fig. 2. Ventilation, Axl inhibition and expression of suppressor of cytokine signaling (SOCS) and cytokines. In ventilated murine lungs, Axl blockade with R428 suppressed lung tissue SOCS3 upregulation; SOCS1 was not affected (A and B, one-way ANOVA). Tissue cytokine messenger RNAs for interleukin (IL)-6 (C, ANOVA on ranks) and IL-1B (D, one-way ANOVA) were increased by ventilation. This increase was reduced in the presence of R428. **P* < 0.05 versus all other groups; †*P* < 0.05 versus nonventilated (NV); $n = 4$ per group. Veh = vehicle.

Axl Inhibition Reduced SOCS3 In Vivo Expression

Because SOCS expression has been reported downstream of Axl activation,^{12,13} we measured the expression of SOCS1,3 messenger RNA in lung tissue. Ventilation with high V_T induced SOCS3 (but not SOCS1) relative to nonventilated (control) mice (fig. 2, A and B). While R428 reduced SOCS3 expression relative to vehicle as hypothesized (fig. 2B), the expression of SOCS3 after pretreatment with R428 remained significantly higher than in nonventilated controls. R428 treatment did not result in increased expression of lung inflammatory markers such as interleukin-6 (fig. 2C) or interleukin-1 β compared to vehicle-treated, ventilated animals (fig. 2D). Additional markers (tumor necrosis factor- α , macrophage inflammatory protein-1 α) were not affected by mechanical ventilation in this model (Supplemental Digital Content 2, fig. S2, <http://links.lww.com/ALN/B638>) or by R428 treatment (Supplemental Digital Content 2, fig. S3, <http://links.lww.com/ALN/B638>).

Shedding of Soluble Axl in Bronchoalveolar Lavage Is Increased in Ventilated Mice

The ectodomain of Axl is shed as a soluble form (soluble Axl ectodomain) *via* the activity of A Disintegrin and Metalloprotease(ADAM)–10 and 17,¹² and can act as a decoy receptor that sequesters Gas6 and blocks activation of membrane-bound Axl.²³ We examined soluble Axl ectodomain in bronchoalveolar lavage fluid from ventilated mice and found that soluble Axl ectodomain concentrations increased 77% after 4 h of ventilation (V_T 20 ml/kg) compared to nonventilated mice (fig. 3A). In addition, Axl inhibition with R428 did not affect soluble Axl ectodomain concentrations in ventilated

mice ($P > 0.05$ *vs.* vehicle, fig. 3B). We hypothesized that increased soluble Axl ectodomain release due to lung cell stretch in ventilated mice might explain the lack of impact of R428 in worsening injury—*i.e.*, that soluble Axl ectodomain–mediated sequestration of Gas6 might limit Axl activity, thereby diminishing the capacity for “further antagonism” by R428.

Stretch-induced Inactivation of Axl in Cultured Rat Pulmonary Microvascular Endothelial Cells

Because Axl has been shown to exert immunoregulatory and antiapoptotic effects in endothelium,^{12,15} we employed cultured rat pulmonary microvascular endothelial cells to explore *in vitro* the mechanism of stretch-mediated desensitization of Axl. We measured Axl activation by immunoblotting with antiphosphotyrosine antibody after Axl proteins were immunoprecipitated from cell homogenates. Addition of Gas6 to culture medium activated Axl in cells that had not been stretched, but failed to activate Axl in cells that had been subjected to cyclic stretch (30 min; fig. 4).

Stretch-induced Inactivation Is Not Dependent on Axl Shedding

Soluble Axl ectodomain was constitutively shed from rat pulmonary microvascular endothelial cells and accumulated in the culture medium of both static and stretched cells, and shedding was inhibited by the ADAM17 inhibitor TAPI-2 (fig. 5A). Cyclic stretch did not increase soluble Axl ectodomain shedding (fig. 5B), and inhibition of shedding with TAPI-2 did not protect cells from the stretch-induced desensitization of Axl to its ligand Gas6 (fig. 5C, 5D).

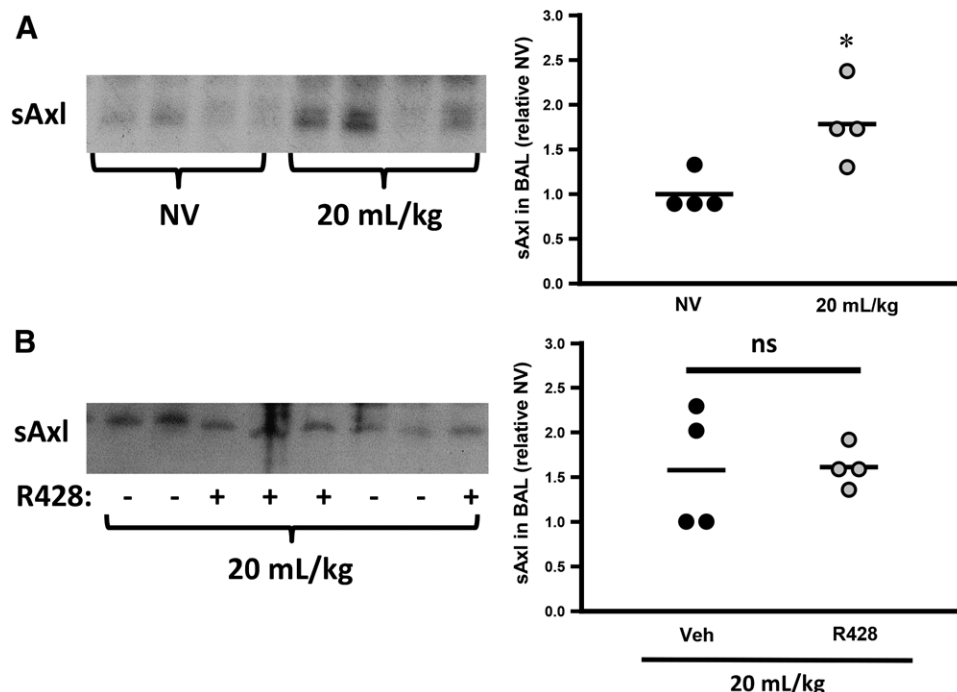


Fig. 3. Ventilation, Axl inhibition and Axl shedding. (A) Ventilation with high tidal volume (20 ml/kg) in mice increases shedding of soluble Axl ectodomain (sAxl) compared to nonventilated (NV) animals (* $P < 0.02$, t test, $n = 4$). (B) R428 administration does not affect sAxl concentration in ventilated animals (Mann-Whitney, $n = 4$). BAL = bronchoalveolar lavage; ns = nonsignificant; Veh = vehicle.

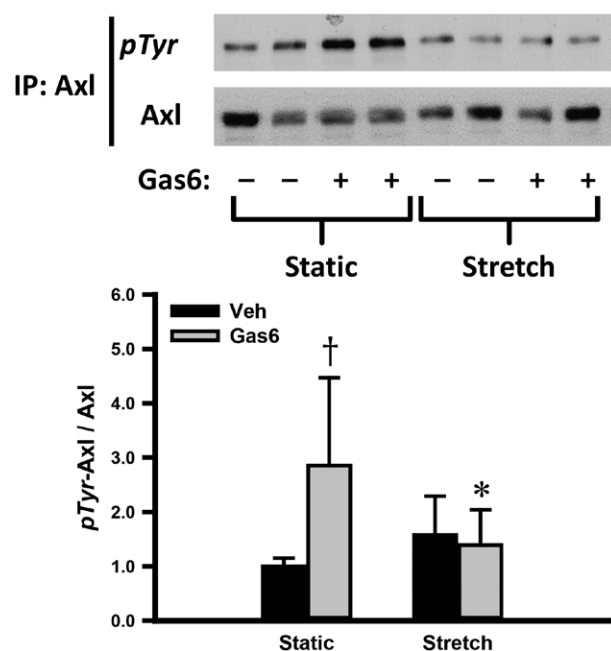


Fig. 4. Axl desensitization by cell stretch. Cyclic stretch abrogates Axl activation in response to growth arrest-specific 6 (Gas6) in rat pulmonary microvascular endothelial cells *in vitro*. Cells were stimulated (10 min) with 5 nM Gas6 after 30 min incubation under static or cyclic stretch conditions. Axl phosphorylation was detected in total cell lysates immunoprecipitated with anti-Axl antibody and immunoblotted sequentially for phosphotyrosine and for Axl. ^{*}*P* < 0.01 stretch versus static within Gas6; [†]*P* < 0.001 Gas6 versus vehicle within static (two-way ANOVA, for effects of stretch and Gas6). *n* = 9 to 10 wells from five independent experiments. IP = intraperitoneal; Veh = vehicle.

Stretch-induced Inactivation Is Not Dependent on rho Signaling

Because desensitization was not dependent on increased shedding of soluble Axl ectodomain, we investigated other well-characterized stretch-activated pathways. Activation of rho-guanosine triphosphatase signaling in endothelial cells by mechanical stretch is known to increase lung endothelial barrier injury,^{24,25} a key component of acute respiratory distress syndrome. Inhibition of this pathway with the rho-associated coiled-coil containing protein kinase (ROCK) inhibitor Y27632 did not prevent stretch-mediated desensitization of Axl (fig. 6).

Stretch-induced Inactivation of Axl Results from Influx through Stretch-activated Calcium Channels

We tested whether stretch-activated channels, important transducers of mechanical stretch implicated in acute respiratory distress syndrome,³ were involved in this mechanism. The presence of GdCl₃ in rat pulmonary microvascular endothelial cell media during cyclic mechanical stretch protected the sensitivity of cells to Gas6 (fig. 7A). Guanidinium ion-sensitive stretch-activated channels form a group capable of transporting a variety of cations into the cell. Therefore, we tested the effect of a selective Ca²⁺ ionophore, ionomycin,

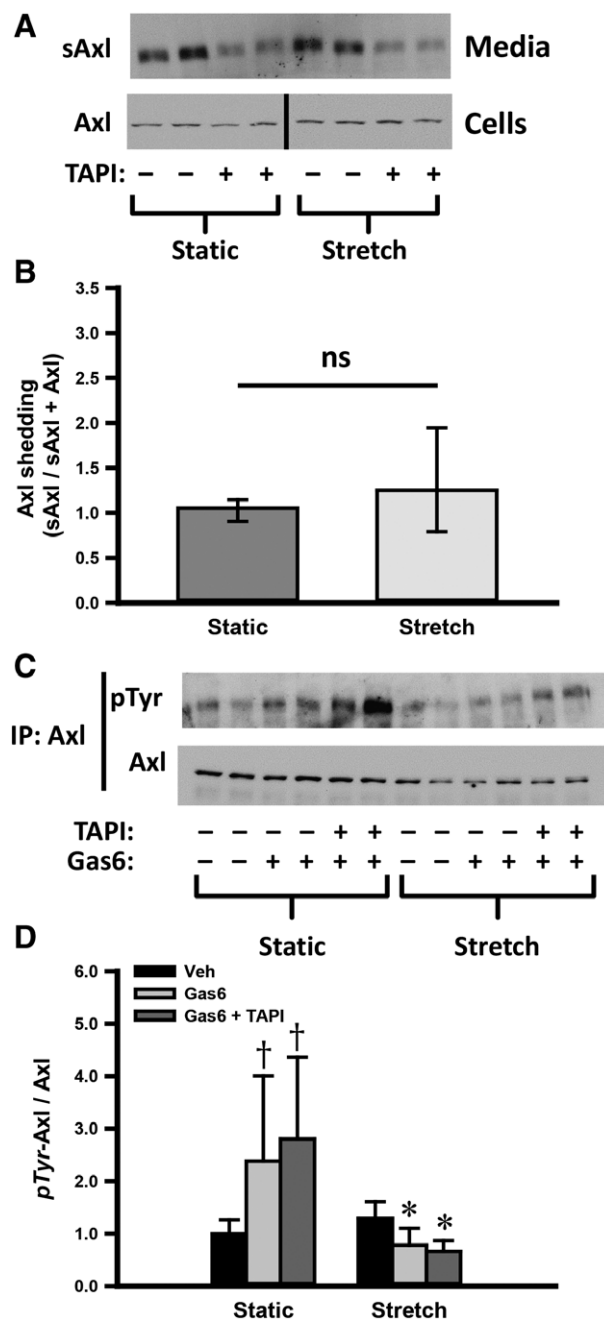


Fig. 5. Axl shedding and desensitization. Axl desensitization in stretched rat pulmonary microvascular endothelial cells is not dependent on shedding. Rat pulmonary microvascular endothelial cells constitutively shed soluble Axl ectodomain (sAxl), and shedding is inhibited by the A disintegrin and metalloprotease-17 (ADAM17) antagonist TAPI-2 (A); however, cyclic stretch does not increase shedding (B, Mann-Whitney, *n* = 13 wells from five independent experiments). Inhibition of ADAM17 does not prevent loss of Gas6-dependent Axl activation in stretched cells (C and D). ^{*}*P* < 0.002 versus static within stimulus (two-way ANOVA for effects of stretch and stimulus); [†]*P* < 0.001 versus vehicle within static. *n* = 6 wells from three independent experiments. Samples run on separate gels/blots (indicated by vertical line) were prepared and analyzed in parallel. IP = intraperitoneal; ns = not significant; Veh = vehicle.

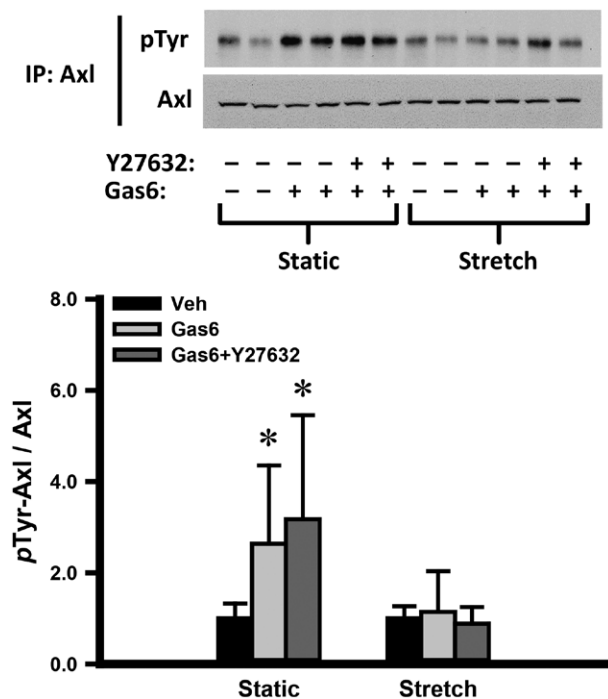


Fig. 6. Axl desensitization by cell stretch is not dependent on rho kinase activation. Inhibition of rho kinase with Y27632 in rat pulmonary microvascular endothelial cells does not prevent loss of growth arrest-specific 6–dependent Axl activation in stretched cells; * $P < 0.05$ versus vehicle within static (two-way ANOVA for effects of stretch and stimulus). $n = 6$ wells from three independent experiments per group. IP = intraperitoneal; Veh = vehicle.

on rat pulmonary microvascular endothelial cells before treatment with Gas6. Axl activation in response to Gas6 was significantly lower in ionomycin-treated rat pulmonary microvascular endothelial cells (fig. 7B). In addition, downstream signaling from Gas6-Axl to Akt was also significantly inhibited in ionomycin-treated cells (fig. 7B).

Discussion

Lung injury in acute respiratory distress syndrome is inflammatory in nature, and mechanical ventilation contributes to this inflammation, and thereby to adverse outcome. While a survival advantage from lower tidal volume has been established, further refining mechanical ventilation has not been demonstrated to improve survival. In addition, several classes of antiinflammatory agents have been tested in patients with acute respiratory distress syndrome, but none has proved successful in clinical trials.²⁶ Therefore, additional approaches to targeting inflammatory lung injury in acute respiratory distress syndrome are needed.

Axl and Acute Injury

Axl is a novel receptor in the TAM receptor family, stimulation of which elicits antiinflammatory responses^{12,27} via an independent postreceptor signaling pathway. Because of these properties, Axl has been proposed as a target through which inflammatory injury could be limited in several acute

organ injury states.^{12,13,28,29} In addition, Axl may be important in critically ill patients because its endogenous agonist (a protein called Gas6) is increased in patients with sepsis¹⁹; the concentrations in sepsis parallel the mortality rates,³⁰ and sepsis is the leading single cause of acute respiratory distress syndrome.³¹

The current data demonstrate that lung endothelial cell stretch reduces the sensitivity of Axl to activation by its ligand, Gas6. However, although soluble Axl can be shed from the membrane-bound receptor (and thus inactivated), the effects of mechanical stretch on Axl are not mediated by Axl shedding, but appear instead to be dependent on stretch activation of calcium channels. This explains why the blockade of an antiinflammatory receptor (Axl) by an antagonist (R428) did not result in increased inflammation (and therefore, did not increase *in vivo* lung injury) in the setting of mechanical ventilation.

Axl signaling may be important in the injured lung due to its effects in endothelial or in epithelial cells. In endothelial cells, Axl is activated by shear stress,¹⁵ and it blocks apoptosis *via* its downstream mediators.³² Axl also has well-characterized antiinflammatory effects in myeloid¹² and endothelial cells. Gas6 can inhibit neutrophil adhesion; conversely, inhibition by circulating soluble Axl ectodomain potentiates neutrophil adhesion.³³ Inhibition by Axl receptor blockade upregulates endothelial secretion of angiopoietin-2,³⁴ which in turn disrupts barrier function. Finally, Axl and Gas6 promote clearance of platelet microparticles by endothelial cells at sites of injury.³⁵ Together these findings suggest an antiinflammatory role for Axl in endothelial cells.

We have previously shown that cyclic stretch activates ADAM17 in alveolar epithelial cells *in vitro*, shedding amphiregulin, epiregulin, and tumor necrosis factor receptor, and inhibition with TAPI-2 protects murine ventilator-induced lung injury *in vivo*.³⁶ In the current study, soluble Axl ectodomain (also shed by ADAM17) was increased *in vivo* in lungs subjected to ventilator stretch, but not in endothelial cells subjected to cyclic stretch. Epithelial and immune cells could be alternative sources of soluble Axl ectodomain in the *in vivo* model. Additionally, *in vivo* phenomena such as cytokine release may induce soluble Axl ectodomain shedding *via* ADAM17.

Stretch and Mechanotransduction

Mechanical stretch activates multiple cellular pathways (fig. 8), including rho kinase, stretch-activated channels, mitogen-activated protein kinases, and phospholipase A2.²⁴ While rho signaling was not involved in Axl desensitization in the current study, inhibition of stretch-activated ion channels protected rat pulmonary microvascular endothelial cells from the desensitizing effects of stretch on Axl; furthermore, Ca^{2+} influx *via* ionomycin mimicked the effect of stretch, suggesting that the Axl desensitization is due to increased intracellular Ca^{2+} . Stretch-activated Ca^{2+} channels are important regulators of barrier and immune functions, including

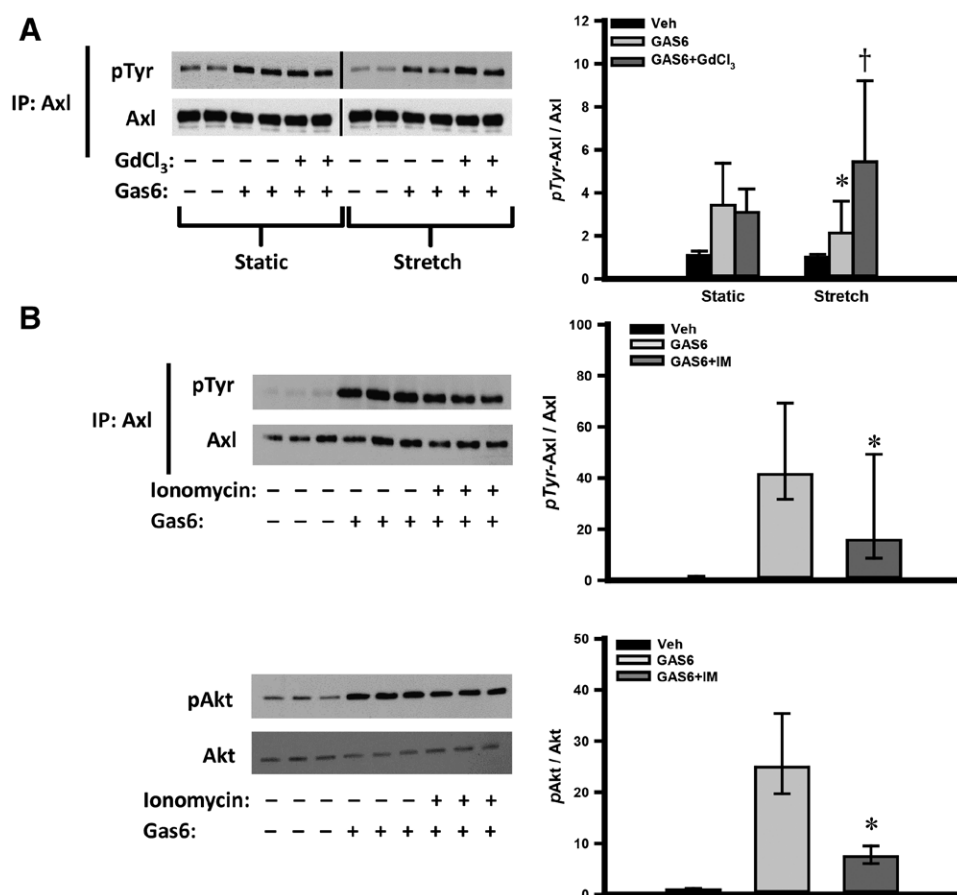


Fig. 7. Calcium influx and Axl desensitization. Axl desensitization to growth arrest–specific 6 (Gas6) in stretched rat pulmonary microvascular endothelial cells is caused by Ca^{2+} influx via stretch-activated Ca^{2+} channels. (A) Gas6-dependent Axl activation is rescued by GdCl_3 inhibition of stretch-activated channels. * $P < 0.05$ versus static within Gas6; † $P < 0.01$ versus Gas6 within stretch (two-way ANOVA for effects of stretch and stimulus). $n = 6$ wells from three independent experiments. (B) Treatment with the calcium ionophore (ionomycin) inhibits Gas6-induced Axl and Akt activation. * $P < 0.05$ versus Gas6 (ANOVA on ranks), $n = 6$ wells from two independent experiments. Samples run on separate gels/blots (indicated by vertical line) were prepared and analyzed in parallel. GdCl_3 = guanidinium chloride; IP = intraperitoneal; Veh = vehicle.

adhesion, cytokine secretion, and maintenance of tight junctions.^{37–40} Within this group, the transient receptor potential superfamily of nonselective cation channels mediates most of their effects *via* Ca^{2+} flux, and are key integrators of mechanotransduction, oxygen sensing, inflammatory activation, and barrier function.

While Axl receptor regulation by cyclic stretch appears to involve intracellular Ca^{2+} signaling, the pathway by which stretch and Ca^{2+} impact Axl sensitivity to activation by Gas6 is not defined. There are several possibilities, including receptor internalization,⁴¹ that could occur after stretch; while total Axl protein was not decreased after stretch (fig. 4), the receptors may undergo endosomal-to-plasma membrane cycling. Alternatively, lipid microdomains called caveolae are important mechanosensors, and caveolae-associated proteins (caveolins) can regulate signaling of several receptor tyrosine kinases.⁴¹ Indeed, in endothelial cells, Gas6 induced Axl localization to caveolae, and knockdown of caveolin-1 prevented phosphorylation of Axl by Gas6.⁴²

Potential Clinical Implications

Extrapolating the data to patients receiving mechanical ventilation requires caution. Our data suggest that targeting Axl activity with the aim of augmenting antiinflammatory activity might not confer benefit due to potential desensitization of endothelial cells in mechanically ventilated lungs. However, myeloid cells,¹⁴ because they are unlikely to be mechanosensitive, might remain a viable target. In addition, Axl antagonists are being proposed to target other conditions (*e.g.*, as an anticancer treatment); should this occur in ventilated patients, then the lung stretch from mechanical ventilation may ameliorate potentially harmful off-target effects of Axl antagonist on lung endothelium.

This would be in contrast to several other settings in which targeting Axl has been successfully exploited. For example, application of Gas6 to activate Axl is associated with less inflammation and organ dysfunction in experimental settings of cerebral hemorrhage,¹³ arthritis,²⁸ and autoimmune encephalomyelitis.⁴³ Consistent with this pattern, “knock-out” of the Axl receptor in mice is associated with increased

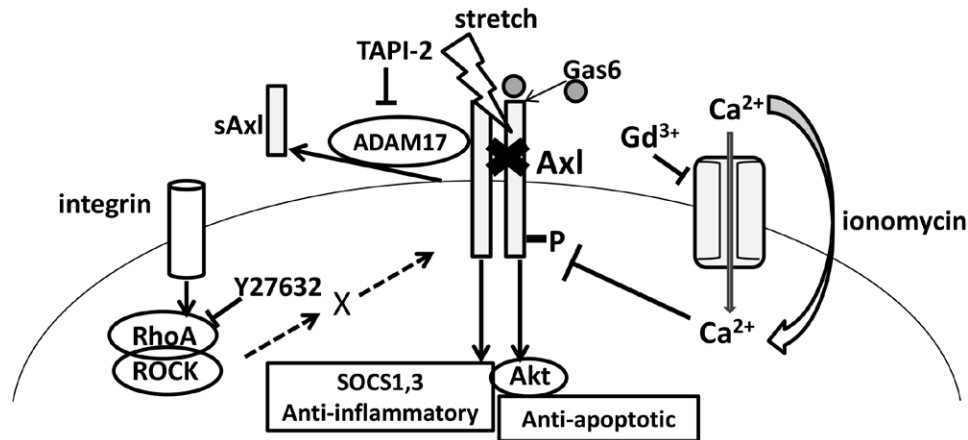


Fig. 8. Desensitization of Axl by cyclic cell stretch. Receptor tyrosine kinase, Axl, becomes phosphorylated in response to its ligand, Gas6, and can trigger antiinflammatory and antiapoptotic pathways. Shedding of the Axl ectodomain by ADAM17 creates a soluble form, soluble Axl ectodomain (sAxl), which can act as a decoy receptor. Sensitivity to Gas6 is lost after cyclic stretch. Stretch-activated signaling pathways in endothelial cells include activation of rho-kinase (mechanosensing of integrin interactions with extracellular matrix) and increased intracellular Ca^{2+} (opening of stretch-activated channels). Inhibition of the latter (but not the former) protected Axl sensitivity in stretched cells. Treatment with Ca^{2+} ionophore, ionomycin, mimicked the effects of stretch. Gd^{3+} = gadolinium ion; P = phosphate group.

mortality from influenza, notwithstanding preserved viral clearance from the lung, within an overall picture of immune hyperresponse to the viral infection.¹⁴

Study Limitations

This study of Axl function in lung injury has limitations. Our attempts to directly detect phosphorylated Axl by immunoprecipitation from lung tissue were unsuccessful, and therefore we used SOCS expression as a marker for Axl activity *in vivo*. The experiments were short term and limited to a single “hit,” where excess mechanical stretch was the underlying stimulus. While this approach facilitates identification and isolation of the contributions of mechanical ventilation in an experimental setting, in patients the milieu is invariably multifaceted, less clear, and of longer-term duration. Although both *in vivo* and *in vitro* systems were used, we did not examine epithelial or immune cells *in vitro*. Finally, we did note an increase in soluble Axl ectodomain *in vivo* that was not found in the simple *in vitro* endothelial cell system; this might have arisen from Axl expressed on other cell types, or due to secondary effects such as cytokine activation in the more complex *in vivo* system.

Conclusions

The current data demonstrating Axl dysfunction in stretched endothelial cells provides evidence that the antiapoptotic and immune regulatory effects of Axl in endothelial cells may be compromised by mechanical ventilation, despite increases in the circulating Axl agonist, Gas6, in such patients. Understanding the role of Axl (and other members of the TAM family) in multiple cell types will be important as novel receptor antagonists are translated into immune regulators or chemotherapeutics.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Kavanagh: Department of Critical Care Medicine, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. brian.kavanagh@utoronto.ca. Information on purchasing reprints may be found at www.anesthesiology.org or on the mast-head page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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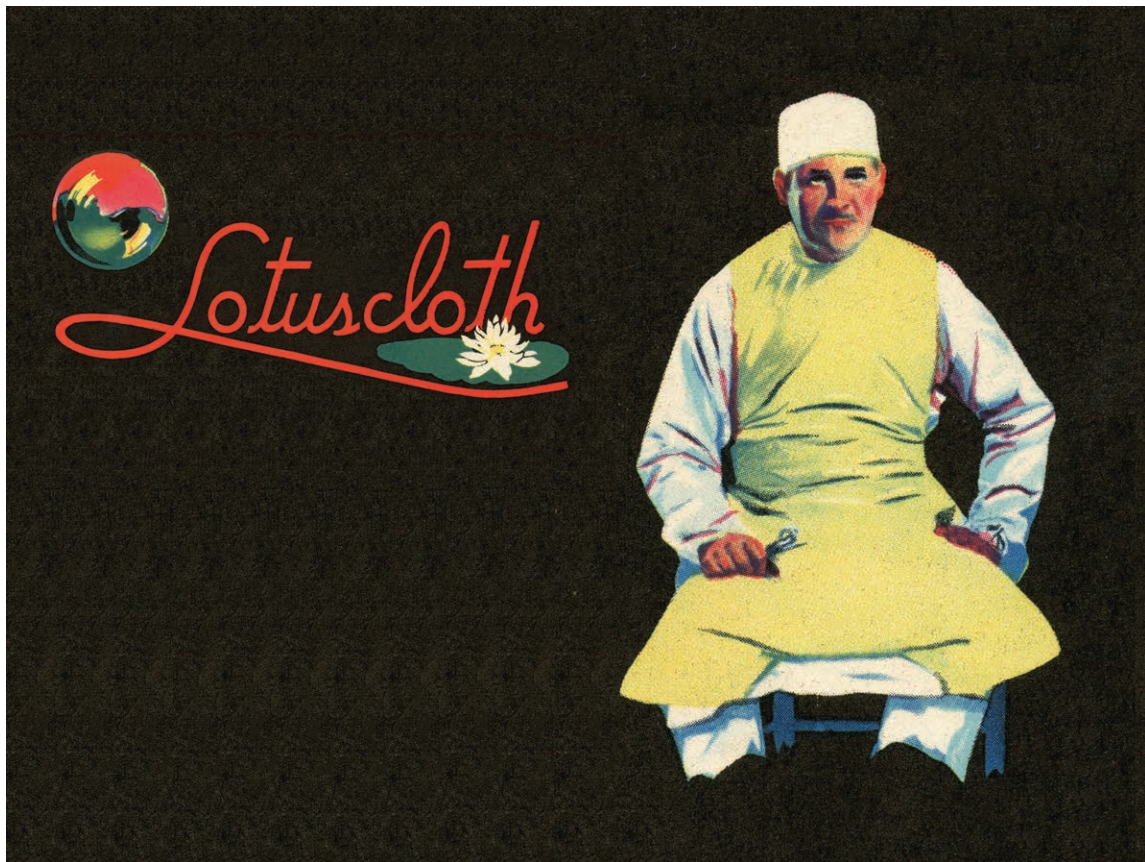
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Garbing Anesthetists in Lotuscloth: Impregnable Aprons of Latex-impregnated Silk



Advertised in the early 1930s with a flourish (*left*) and as “mercifully light in weight,” Lotuscloth was merely “silk, impregnated with pure latex-rubber.” Offered first to hospitals in the form of mattress covers, bed sheets, patient throws, and pillow covers, Lotuscloth was advertised to surgeons and anesthetists for garbing them in lightweight operating gowns and surgical aprons (*right*). Readily “washed, boiled, and sterilized,” Lotuscloth resisted chemical damage from disinfectants, such as “Lysol, Bi-Chloride solutions, Alcohol,” and, of interest to anesthetists, ether. Because the proprietary textile was both “non-porous, and waterproof,” a surgical gown or apron made of Lotuscloth could also spare anesthetists from self-inflicted skin burns resulting from chloroform spills. (Copyright © the American Society of Anesthesiologists’ Wood Library-Museum of Anesthesiology.)

George S. Bause, M.D., M.P.H., *Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio.* UJYC@aol.com.