Prevention of ischemia-reperfusion lung injury by sulfated Lewisα pentasaccharide

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Prevention of ischemia-reperfusion lung injury by sulfated Lewisα pentasaccharide. J. Appl. Physiol. 82(4): 1058–1063, 1997.—Inhibition of polymorphonuclear neutrophil (PMN) adhesion to the pulmonary endothelium attenuates ischemia-reperfusion (I/R) lung injury. We hypothesized that 3'-sulfated Lewisα (SuLa), a potent ligand for the selectin adhesion molecules, may have a beneficial effect on I/R lung injury, as measured by the filtration coefficient ($K_{fc}$), and reduce pulmonary sequestration of PMN as assessed by the lung myeloperoxidase (MPO) activity. Blood-perfused rat lungs were subjected to 30 min of perfusion, 60 min of warm ischemia, and 90 min of reperfusion after treatment with either SuLa (200 µg) or saline. Effects of SuLa on PMN adhesion to cultured human umbilical vein endothelial cells (HUVEC) stimulated with tumor necrosis factor-α and calcium ionophore were also investigated. Compared with preischemic conditions, I/R induced a significant increase in $K_{fc}$, which was attenuated with SuLa (80 ± 8 vs. 30 ± 5% P < 0.001). SuLa reduced lung MPO and PMN adhesion to stimulated HUVEC. These results indicate that SuLa reduces I/R-induced lung injury and PMN accumulation in lung. This protective effect might be related to inhibition of PMN adhesion to endothelial cells.

ISCHEMIA-REPERFUSION (I/R) lung injury occurs after lung transplantation, pulmonary thromboendarterectomy, or cardiopulmonary bypass. Increased microvascular permeability and polymorphonuclear neutrophils (PMN) lung sequestration are well-described consequences of lung I/R. The reperfusion of ischemic lungs initiates an inflammatory cascade characterized by the elaboration of cytokines and proinflammatory mediators, such as platelet-activating factor and leukotriene B₄ (10, 12, 27), the expression of cell adhesion molecules (6, 7, 13), the adhesion of PMN to the pulmonary endothelium (2, 19), and finally PMN-mediated lung injury (1, 3, 13). Studies aimed at the removal or inhibition of PMN have demonstrated a dramatic reduction in I/R lung injury and provide evidence that PMN play a major role in lung reperfusion injury (6, 21, 22).

The initial interaction between PMN and endothelial cells is mediated by the selectin adhesion molecules (9, 10, 29). The selectins initiate rolling and tethering of PMN to the endothelial surface and facilitate exposure to various PMN activators. This rolling is the first step in a sequence of events leading to firm adhesion of activated PMN to the endothelium, which is induced by PMN β₂-integrin (CD11/CD18) and its endothelial ligands, intercellular adhesion molecule-1 or -2 (ICAM-1 or ICAM-2) (6, 7). After adhesion to the pulmonary endothelium, PMN can undergo activation and release numerous toxic substances including oxygen-derived free radicals, inflammatory cytokines, platelet-activating factor, leukotriene B₄, elastase, myeloperoxidase (MPO), and other proteolytic enzymes (2).

Recent studies indicate that administration of monoclonal antibodies directed against P-selectin (8, 31) or both L- and E-selectins improves lung reperfusion injury (26). Soluble carbohydrate ligands to selectins may also have the potential to attenuate I/R-induced PMN-endothelial interactions (11). Indeed, each selectin shares a common molecular structure, most notably an NH₂-terminal lectin-like domain, which suggests that the selectins might bind to oligosaccharides on other cells. To date, three structures have been identified that appear to have binding affinity for selectins: 1) oligosaccharides related to sialyl Lewisα and sialyl Lewisβ, 2) phosphorylated mono- and polysaccharides, and 3) sulfated polysaccharides and lipids (17, 18, 29). The discovery that selectin-mediated adhesion is dependent on carbohydrate binding has generated a great deal of interest in creating bioactive and biostable analogs of Lewisα and Lewisβ. Sialyl Lewisα tri- and tetrasaccharides were recently shown to attenuate PMN accumulation and lung injury in rats subjected to acute lung inflammation mediated by either P- (18) or E-selectin (17). A sialyl Lewisβ analog was also shown to attenuate PMN accumulation and myocardial necrosis in a canine model of myocardial I/R (11). Recent in vitro studies demonstrate that the 3'-sulfated Lewisα (SuLa) pentasaccharide is a more potent ligand to E- and L-selectins compared with sialyl Lewisα analogs (5, 30).

Accordingly, we reasoned that SuLa might be able to attenuate the lung reperfusion insult and reduce PMN lung sequestration by decreasing PMN adhesion to endothelial cells. To test this hypothesis, we subjected isolated blood-perfused rat lungs to ischemia followed by reperfusion in the presence or in the absence of SuLa. More specifically, the objectives of the present study were 1) to investigate the ability of SuLa to reduce the I/R-induced increase in microvascular permeability, 2) to assess the effect of SuLa on the pulmonary sequestration of PMN during reperfusion, and 3) to investigate whether SuLa might inhibit PMN adhesion to endothelial cells in vitro.
METHODS

Isolated Perfused Rat Lung

Male Sprague-Dawley rats (250-350 g body wt; n = 18; Ifa Credo, France) were used. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 80-23, revised 1978). They were anesthetized with thiopental sodium (50 mg/kg ip), tracheostomized, and ventilated. After sternotomy and a median pericardotomy, a polyethylene cannula was inserted into the pulmonary artery through the right ventricle. A second cannula was placed in the left atrium through a midleft ventricular incision. The heart-lung preparation was then dissected free and suspended to a Statham force-displacement transducer into a thermostated and humidified chamber to monitor weight changes. The lungs were ventilated with a Harvard rodent ventilator (model 680) at 60 breaths/min, a tidal volume of 2.5 ml, and a positive end-expiratory pressure of 2 cmH2O. Ventilation was performed with a humidified, warmed gas mixture (20% O2-5% CO2-75% N2). The lungs were perfused via the pulmonary artery cannula with 30 ml heparinized blood obtained from two donor rats. The blood was recirculated by using a peristaltic pump (Lsamtec, Bioblock) at a flow rate of 0.04 ml·g body wt⁻¹·min⁻¹. Pulmonary effluent blood was collected into a plastic reservoir through the cannula ing material. The synthesis of the pentasaccharide was formed with the use of endothelial cells at second passage (106/ml) were added to washed HUVEC. After 30 min of contact, unbound PMN were removed by three washes with buffer. Adherent PMN were collected, lysed (1% Triton X-100), and sonicated three times at 10 s each. MPO in the adherent PMN fraction was measured as previously described (25). Adherence was expressed as the percentage ratio of the MPO activity, defined as the activity degrading 1 µmol of H2O2/min at 25°C.

Synthesis of the SuLa Pentasaccharide

The Lewisα pentasaccharide was sulfated at position 3 of the outer galactose (14). It was prepared by using the 4-methoxybenzyl glycoside of N-acetylgalactosamine as starting material. The synthesis of the pentasaccharide was achieved through a ψ-stereoselective coupling of an α-trichloroacetimidate-activated form of the N-acetamido-protected trisaccharide onto a 3-, 4-protected lactose derivative (14).

In Vitro Adhesion of PMN to Human Umbilical Vein Endothelial Cells

Human umbilical vein cords were treated with 0.05% collagenase, and human umbilical vein endothelial cells (HUVEC) were harvested as described elsewhere (25). Cells were suspended in the culture medium (M199 containing 20% heat-inactivated bovine calf serum, 1-glutamine, fungizone, penicillin-streptomycin) and initially seeded in gelatin-coated 35-mm dishes. Nonadherent cells were removed after 2 h. Confluent endothelial cells were passed after treatment with trypsin-EDTA (0.05–0.02%, respectively). Inverted microscopy was used to assess endothelial cell purity shown by a typical “cobblestone” appearance. All experiments were performed with the use of endothelial cells at second passage seeded in 24-well gelatin-coated culture plates.

PMN were recovered from heparinized blood of healthy donors and then purified as described elsewhere (25), by a process including sedimentation on 2% Dextran T-500 and centrifugation on a Ficoll-Paque density gradient, followed by hypotonic lysis of residual erythrocytes. The preparation contained 95% PMN, and viability as assessed by trypan blue exclusion was 97%.

HUVEC were stimulated with tumor necrosis factor-α (TNF-α) or calcium ionophore (A23187) for 4 h and 15 min, respectively. SuLa (100 µmol/l; n = 4) or saline (n = 4) were then added to stimulated HUVEC during 15 min at 4°C. PMN (10⁵/ml) were added to washed HUVEC. After 30 min of contact, unbound PMN were removed by three washes with buffer. Adherent PMN were collected, lysed (1% Triton X-100), and sonicated three times at 10 s each. MPO in the adherent PMN fraction was measured as previously described (25). Adherence was expressed as the percentage ratio of the MPO measured in adherent PMN to the MPO measured in the PMN (10⁵/ml) added initially.
Specific Protocols

After being placed in the temperature-controlled, humidified chamber, the lungs were allowed to equilibrate for 30 min and were made isogravimetric by adjusting Ppv. Then, after determination of the baseline values (Ppa, Ppv, Ppc, and $K_{fc}$), one of the following protocols was followed.

Time-control lungs (n = 6). After the baseline determinations, the lungs were subjected to 3 h of perfusion. Ppa, Ppv, Ppc, and $K_{fc}$ were again determined at the end of this period.

I/R lungs (n = 12). After the baseline determinations, ventilation and perfusion were interrupted, and the lungs were maintained in the humidified chamber at a temperature of 37°C for 60 min. After the arterial and venous cannulas were clamped, the recirculating blood was discarded, and the external circuit was flushed with saline. After this period of ischemia, the lungs were randomly allocated in two groups: 1) I/R control (n = 6; treated with saline), and 2) I/R SuLa (n = 6; treated with 200 µg of SuLa). New fresh blood was obtained from two donor rats, and saline or SuLa was then added. Hematocrit was adjusted to 28% at the beginning of each period (i.e., baseline and reperfusion) by addition of saline. After a 90-min isogravimetric period of reperfusion, measurements of Ppa, Ppv, Ppc, and $K_{fc}$ were performed.

Before and after the reperfusion period, platelets and white blood cell (WBC) counts (n = 6 in each group) were performed in the venous effluent to allow determination of the percentage decrease in circulating PMN count during reperfusion. At the end of experiment, the lungs were flushed with saline at a low flow of 5 ml/min during 5 min, and lungs were frozen for determination of MPO activity (n = 6 in each group).

Reagents

M199 culture medium, fetal bovine serum, l-glutamine (200 mM), penicillin-streptomycin (5,000 UI/ml-5,000 µg/ml), fungizone (250 µg/ml), trypsin-EDTA (0.5–0.2 g/l), and Hank’s (200 mM), penicillin-streptomycin (5,000 UI/ml-5,000 µg/ml), fungizone (250 µg/ml), trypsin-EDTA (0.5–0.2 g/l), and Hank’s balanced salt solution were from Gibco (Cergy-Pontoise, France). TNF-α was from Promo Cell (Heidelberg, Germany). Triton X-100, Cal., and ortho-dianisidine were from Sigma (St. Louis, MO). The 24-well gelatin-coated culture plates were obtained from Corning Glass (Corning, NY). Dextran T-500 and Ficoll-Paque were from Pharmacia Biotech (Uppsala, Sweden). Collagenase (Collostrum histidicum) was from Boehringer (Mannheim, Germany).

Statistics

All results are expressed as means ± SE. Baseline and final measurements of $K_{fc}$ and hemodynamic variables of different groups were compared using a two-way analysis of variance for repeated measurements. Newman-Keuls test was used as a post hoc test. Lung MPO activity and adherence were compared by using an independent Student’s t-test. Significance was determined when $P < 0.05$ was obtained.

RESULTS

Baseline values of Ppa, Ppv, Ppc, Rpa, Rpv, and $K_{fc}$ were similar in the three groups.

$K_{fc}$ values are shown in Fig. 1. After 3-h perfusion, $K_{fc}$ was not different from baseline in the time-control group. Compared with the respective baseline $K_{fc}$ values, I/R-induced increases of $K_{fc}$ were 80 ± 8% in the I/R-control group and 30 ± 5% in the I/R-SuLa group ($P < 0.001$).

Hemodynamic measurements are shown in Table 1. Compared with baseline values, Ppa, Ppv, Ppc, Rpa, and Rpv did not vary after 3-h perfusion in the time-control group (Table 1). After I/R, Ppa was lower in the I/R-SuLa group compared with the I/R-control group. After I/R, Ppv, Ppc, Rpa, and Rpv were not different between the two groups (Table 1).

After I/R, lung MPO activity in the I/R-control group was higher than in the time-control group (0.59 ± 0.04 vs. 0.32 ± 0.04 U/100 mg, respectively; $P < 0.01$). In the I/R-SuLa group, lung MPO activity after I/R was lower than in the I/R-control group and not different from lung MPO activity measured in the time-control group (Fig. 2).

During reperfusion, total WBC counts decreased similarly in both I/R groups (2,880 ± 163 and 2,700 ± 481 cells/µl before reperfusion vs. 1,900 ± 135 and 1,680 ± 246 cells/µl after reperfusion in the I/R-control and I/R-SuLa groups, respectively). Before reperfusion, blood PMN counts were 431 ± 41 and 404 ± 132 cells/µl in I/R-control and I/R-SuLa groups, respectively (NS). After I/R, the percentage decrease in blood PMN were similar in the I/R-control and I/R-SuLa groups (78 ± 5 and 87 ± 3% in the I/R-control and I/R-SuLa groups, respectively). During reperfusion, the variations of platelets, lymphocytes, monocytes, eosinophils, and basophil counts were not different between the two I/R groups.

An adhesion model was performed in vitro to confirm the efficiency of SuLa in inhibiting interaction of PMN and endothelial cells. Results reported in Fig. 3 show that SuLa inhibited PMN adhesion to TNF-α- or Cal-stimulated endothelial cells by ~40%.

DISCUSSION

This study shows that administration of the selectin ligand SuLa pentasaccharide attenuates the I/R-induced increase in lung microvascular permeability as well as the PMN accumulation in lung and reduces the adhesion of PMN to stimulated endothelial cells in vitro.

I/R lung injury is characterized by an increase in pulmonary microvascular permeability and a massive pulmonary PMN sequestration (1, 3, 6, 7). Because
infiltrating PMN have been implicated as key mediators of the reperfusion-induced lung damages, isolated rat lungs were perfused with blood rather than with buffer. Moreover, to prevent lung injury due to ischemia-derived blood-borne products, the lungs were reperfused with fresh blood obtained from rat donors. $K_{fc}$ was used to evaluate changes in lung microvascular permeability (22–24), and pulmonary sequestration of PMN during reperfusion was assessed by measuring lung MPO activity, which is a marker of tissue PMN infiltration (2, 16). In the time-control group, $K_{fc}$ remained unchanged after 3 h of normal perfusion, thus demonstrating the stability of our preparation. As expected, in the I/R-control group, I/R-induced increase of $K_{fc}$ and lung MPO activity after I/R were similar to those reported in our previous study (22).

A main step in the pathogenesis of I/R-induced lung injury is the adhesion of PMN to the vascular endothelium (19). Numerous studies conducted in various models of lung I/R consistently demonstrated that prevention of PMN adhesion by inhibition of leukocyte integrin component CD18 or endothelial cell adhesion molecule ICAM-1 improved reperfusion-induced lung injury (6, 7). One important property of the selectins is that they appear to be the initial adhesion molecules to influence the properties of PMN at the start of the inflammatory response (9, 10). Thus, selectins may serve as a target for therapeutic intervention in lung reperfusion injury. A recent study (26) indicates that survival after 6 h of lung reperfusion injury was improved by an antibody that binds and inhibits L- and E-selectins in intact sheep. However, the initial expression of lung reperfusion injury was not altered by blocking L- and E-selectins, suggesting that early lung injury induced by reperfusion was not dependent on L- and E-selectins (26). This was confirmed by a study in isolated rat lung, where monoclonal antibodies directed against P-selectin, but not those against L-selectin, protected the lungs against the I/R-induced permeability increase (15). Thus, contrary to P-selectin, L- and E-selectins would be projected to play only a minor role in the early phase of I/R lung injury.

One interesting aspect of selectins is that selectin-mediated adhesion is dependent on carbohydrate binding. A plethora of simple and complex carbohydrates have been reported to be recognized by the selectins.

### Table 1. Hemodynamic results

<table>
<thead>
<tr>
<th>Group</th>
<th>Ppa (cmH₂O)</th>
<th>Ppv (cmH₂O)</th>
<th>Ppc (cmH₂O)</th>
<th>Ra (cmH₂O·min·ml⁻¹)</th>
<th>Rv (cmH₂O·min·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
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<tr>
<td>Time control</td>
<td>13.6 ± 0.5</td>
<td>3.9 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>0.65 ± 0.05</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>I/R control</td>
<td>14.5 ± 0.9</td>
<td>4.1 ± 0.2</td>
<td>7.6 ± 0.5</td>
<td>0.69 ± 0.09</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>I/R SuLa</td>
<td>13.7 ± 0.8</td>
<td>3.6 ± 0.3</td>
<td>7.3 ± 0.4</td>
<td>0.64 ± 0.07</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td><strong>Time control</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Perfusion, 3 h</td>
<td>14.4 ± 0.4†</td>
<td>4.0 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td>0.74 ± 0.04</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>I/R control</td>
<td>16.8 ± 0.8*</td>
<td>4.4 ± 0.2</td>
<td>7.9 ± 0.5</td>
<td>0.90 ± 0.10</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>I/R SuLa</td>
<td>14.1 ± 0.4†</td>
<td>3.8 ± 0.2</td>
<td>6.7 ± 0.3</td>
<td>0.74 ± 0.05</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. Pressures are calculated in cmH₂O and resistances in cmH₂O·min·ml⁻¹. I/R, ischemia-reperfusion; SuLa, sulfated Lewisα; Ppa, pulmonary artery pressure; Ppv, pulmonary venous pressure; Ppc, pulmonary capillary pressure; Ra, pulmonary arterial resistance; Rv, pulmonary venous resistance. *P < 0.05 vs. I/R control at baseline; †P < 0.05 vs. I/R control after reperfusion.

**Fig. 2.** Pulmonary sequestration of neutrophils after I/R as assessed by lung myeloperoxidase activity in time-control, I/R-control, and SuLa groups. Results are expressed as means ± SE. †P < 0.01 vs. I/R-control group.

**Fig. 3.** Effect of SuLa on polymorphonuclear neutrophil adhesion to endothelial cells. Confluent human umbilical vein endothelial cells (HUVEC) in 24-well plates were stimulated with tumor necrosis factor-α (TNF; 20 ng/ml) or calcium ionophore (CaI; 2.10⁻³ M) for 4 h and 15 min, respectively. Cells were then washed and incubated with saline (SuLa⁻) or SuLa (SuLa+) (100 µM) for 15 min at 4°C. Adherence was assessed as described in METHODS; n = 4 experiments; C, control nonstimulated HUVEC. *P < 0.001 vs. saline-treated group.
tion of these oligosaccharides (18). More recently, administration of a pentasaccharide analog of sialyl Lewis $^a$ was demonstrated to attenuate myocardial injury and PMN myocardial accumulation after 90 min of regional ischemia and 4.5 h of reperfusion (11). In the present study, we have used the SuLa pentasaccharide, which was recently synthesized by Lubineau et al. (14). In vitro binding studies indicate that this pentasaccharide was a more potent ligand for E- and L-selectins than the sialyl Lewis $^a$ analogs (5, 30). Other studies suggested that such sulfated Lewis $^a$ analogs could also be ligands for P-selectin (29). Accordingly, in our in vitro study, SuLa induced a decrease in PMN adhesion to endothelial cells stimulated by either Cal or TNF-$\alpha$, which have been reported to induce expression of P-selectin and E-selectin respectively (10, 29). Moreover, the present study demonstrates that this SuLa pentasaccharide inhibits PMN-mediated lung reperfusion injury. Indeed, compared with baseline values, SuLa induced a 68% inhibition of lung injury and a virtually 100% inhibition in lung MPO content.

Interestingly, the circulating number of leukocytes decreased similarly after reperfusion in I/R-control and I/R-SuLa groups, whereas lung MPO activity was significantly lower in the treated group. Flushing the lungs with saline before determination of the lung MPO activity might account for this discrepancy. Flushing the lungs could have resulted in elimination of a significant part of the marginated PMN, leaving only firmly adherent PMN and/or PMN that have transmigrated through the endothelium. Taken together, all these observations suggest that SuLa inhibited not only PMN rolling but also PMN adhesion and/or transmigration. Although this hypothesis is presently purely speculative, various other sulfated polysaccharides have been shown to interfere strongly with leukocyte adhesion (8).

The development of selectin oligosaccharide ligands is attractive because of their lack of immune reactivity as well as their ease of handling for therapeutic use. Moreover, treatment with the SuLa pentasaccharide might effectively block the actions of all the selectins simultaneously, thus offering the advantage that it might protect the lung for longer periods of reperfusion. Additional studies in intact animals are necessary to confirm the applicability of this treatment.

In conclusion, we provide for the first time striking evidence that the SuLa pentasaccharide reduces lung PMN accumulation and adhesion to endothelial cells and exerts a significant degree of pulmonary protection in isolated rat lungs submitted to warm I/R.

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