Dermatan Sulphate Induces Plasminogen Activator Release in the Perfused Rat Hindquarters

By Marzia Abbadiini, Guang Jin Zhu, Antonio Maggi, Jerta Pangrazzi, Maria Benedetta Donati, and Luciana Mussoni

Heparin or heparin-like substances have been described to induce the release of plasminogen activator (PA) activity in different animal perfusion models. In this paper we report that Dermatan Sulphate (DS) is able to induce PA activity release in the perfused rat hindquarters. Perfusion of different doses of DS (0.1 to 0.8 mg/mL) stimulates a release of PA activity that is maximum after the initial two minutes of perfusion. The amount of PA activity released rises progressively within a certain concentration range of DS (0.1 to 0.4 mg/mL) and declines thereafter (0.6 to 0.8 mg/mL). The type of PA activity increased during DS perfusion was characterized by SDS-PAGE and fibrin autography as tissue-type PA (t-PA) on the basis of its mol wt (87,000 d) and inhibition by a specific anti t-PA antisera. This effect might be considered as potentially contributing to the antithrombotic activity of DS, at least at the local level.

Previously investigations have shown that heparin and heparin-like substances can interact with proteins of the fibrinolytic system, both in vivo and in vitro studies. In particular, Markwardt et al demonstrated that perfusion of heparin and sulphated polyanions enhance PA release, in the isolated pig ear model, in a dose-dependent manner. Moreover, high and low mol wt heparin preparations (HMW, LMW) induced fibrinolytic activity, both in vitro and in vivo in human volunteers. The same investigators have shown that this effect partially depends on the degree of sulphation of the molecule, since a synthetic polysulphate polysaccharide (SP54) was more active than other heparin preparations.

The antithrombotic effect of HMW and LMW heparins was lost when antifibrinolytic drugs were administered along with the heparins in the rabbit Wessler model. Further studies with SP54 indicated that this substance induced the release of t-PA if perfused in the rabbit ear and that this activity was involved in the drug’s antithrombotic action. Moreover in human volunteers SP54 activates the fibrinolytic system after both oral and subcutaneous administration. Recently it has been hypothesized that a direct interaction of heparin or heparin-like substances with plasminogen and t-PA or urokinase (UK) induces an increase in plasin formation.

Dermatan Sulphate (DS), like heparin, has been shown to be a potent antithrombotic agent in different experimental models of venous thrombosis, without affecting the coagulation tests. To clarify the possible mechanisms that may contribute to the antithrombotic efficacy of DS, we studied its interaction of this drug with the fibrinolytic system. In particular, we investigated if DS was able to modify the release of plasminogen activator (PA) from the vascular bed using the perfused rat hindquarters model. This model was proposed as a useful tool to study the physiological and pharmacological modulation of PA release.

MATERIALS AND METHODS

Dermatan Sulphate (DS) MF 701, isolated from pig intestinal mucosa, was kindly supplied by Mediolum Farmaceutici SRL, Milano, Italy. Heparin contamination was <1% as demonstrated by electrophoretic analysis and in the intravenanticoagulant activity was calculated to be around 5 U/mg USP.

Perfusion Model

The experimental model used was the perfused rat hindquarters described by Emeis. Briefly, 200 to 250 g male CD-COBS rats (Charles River, Italy) were anaesthetized with sodium pentobarbital (50 mg/kg), administered intraaperitoneally. After isolating both the aorta and vena cava, up to the iliofemoral bifurcation, a ligature was applied around each vessel. A 22 G needle was inserted, without stasis, into the aorta and perfusion was immediately started at a constant flow rate of 10 mL/min. A cannula was inserted into the vena cava to allow perfusate collection. Perfusion of the hindlimb vascular bed was performed using a Tyrode’s solution pH 7.4, containing 0.1% BSA (Sigma Chemical Co, St Louis) and oxygenated with 95% O2 and 5% CO2, at 37°C. The perfusion pressure on entry was continuously monitored using a transducer and polygraph (Grass Instrument Co, Quincy, MA).

Experimental procedure. Each experiment started with a 30 minute, wash-out period of the rat hindquarters using only Tyrode’s solution. After this initial period, the drug under investigation (dissolved in Tyrode’s) was perfused for three minutes, followed by a second wash-out with Tyrode’s solution. Samples (1 mL) were collected at regular intervals (five to ten minutes) from the cannulated vena cava during wash-out and every minute during drug perfusion. The samples, collected on ice, were centrifuged (12,000 g for 60 seconds) and analyzed for fibrinolytic activity. Each animal was perfused once only.

Fibrinolytic Activity

Fibrin plate assay. PA activity of the samples, collected during the perfusion period, was measured by a fibrin plate method using plasminogen-rich human fibrinogen (Grade L; Kabi, Stockcolm).

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Thirty microliters of each sample were placed (in duplicate) on the fibrin film and the diameter of the lysis areas were measured after 18 hours incubation at 37°C. The PA concentration was expressed as PA mU/mL of perfusate in comparison to a reference curve obtained, for each set of experiments, using known concentrations of t-PA standard (WHO, London, coded 831517). The results were analyzed statistically by Duncan’s test.12

Fibrin autography. The perfusion samples, collected as described in the experimental procedure, were kept at −20°C in 0.01% Tween 80 until tested. The molecular type of PA was evaluated by a fibrin autography technique13 after SDS-PAGE. The apparent mol wt of PA was calculated by comparing the migration distance of the lysis area with those of marker proteins of known mol wt (Pharmacia, Uppsala, Sweden), run simultaneously. The immunological characterization was performed with home-made rabbit antibodies against human t-PA or UK incorporated in the fibrin agar plate.

RESULTS

Using the rat hindquarters perfusion model, we observed that DS was able to stimulate the release of PA activity in a dose dependent manner (in the range of 0.1 to 0.4 mg/mL). At higher DS concentrations (0.6 to 0.8 mg/mL) there followed a gradual decrease in the lysis of fibrin plates (Fig 1). Throughout the whole of these experiments no increase in basal perfusion pressure (50 to 60 mmHg) was recorded.

If we examine the profile of PA activity measured in the perfusate with the DS dose giving the highest PA activity release (0.4 mg/mL), we can observe (Table 1) that the maximum PA level was achieved during the first initial minutes of perfusion progressively declining thereafter to basal values 50 to 60 minutes from the beginning of the experiment.

Table 1 reports PA activity released from vascular cells during the perfusion of DS at doses of 0.1 to 0.4 mg/mL. The release of PA activity induced by DS (0.2 to 0.4 mg/mL) was maximal and significantly different from baseline levels during the first two minutes of DS perfusion and decreased thereafter. Above 0.4 mg/mL a reduction in PA was also seen, 0.6 mg/mL inducing a similar release of PA as the 0.1 mg/mL dose.

Different heparin preparations, tested at concentrations comparable with DS, were found ineffective (Fig 1); moreover, also lower concentrations of heparin (0.0001 to 0.01 mg/mL) were devoid of activity (data not shown).

The samples collected from the vena cava both during

![Fig 1](https://example.com/fig1.png)  
**Fig 1.** PA activity released as a function of DS (●) and heparin (△) concentrations perfused. The data reported here refer to the first minute of perfusion. Each value is the mean of five experiments performed on different animals (SD never exceeded 10% of the mean).

![Fig 2](https://example.com/fig2.png)  
**Fig 2.** Fibrin autography of samples from perfused rat hindquarters. Line (C) corresponds to the perfusate collected after 25 minute wash-out with Tyrode’s solution alone and line (DS) to the sample collected after the first minute of perfusion with 0.4 mg/mL of the drug. The arrows indicate the apparent mol wt corresponding to the lysis area.

### Table 1. PA Activity Released in Rat Hindquarters Perfused With Different DS Concentrations

<table>
<thead>
<tr>
<th>Perfusion Time (min)</th>
<th>PA Released (mU/mL) After DS Perfusion*</th>
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<tbody>
<tr>
<td></td>
<td>0.1 mg/mL</td>
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<tr>
<td>25</td>
<td>Tyrode’s</td>
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<tr>
<td>30</td>
<td>DS</td>
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<tr>
<td>31</td>
<td>DS</td>
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<tr>
<td>32</td>
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<td>50</td>
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<tr>
<td>60</td>
<td>Tyrode’s</td>
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</tbody>
</table>

Note. n, numbers of animals; mg/mL, dose of DS.

*Mean ± SEM.

†P < .01 Duncan’s test (v Tyrode’s sample collected after 25 minutes).

Tyrode’s and DS perfusion were examined by fibrin autography after SDS-PAGE to determine the type of PA released. The perfusate contained two types of PA, which appeared on a fibrin-agar plate as lysis bands of 67,000 and 43,000 mol wt (Fig 2). It can clearly be seen, that both in control samples (collected during the first wash-out period) and in the stimulated samples (collected in the first minute of DS perfusion), both bands appeared and that perfusion of DS caused an increase only in the band corresponding to a 67,000 mol wt while the lower mol wt band was unaltered. The 67,000 mol wt band is blocked by an anti–PA antiserum and the 43,000 mol wt band partially inhibited by an anti-human UK antibody, as previously described.14

To verify whether DS had a direct fibrinolytic activity on fibrin films or an in vitro interaction with PA molecules, DS
(0.1 to 0.8 mg/mL) was dissolved in Tyrode's solution and placed directly on a fibrin plate, alone or together with different concentrations (10 to 100 mU/mL) of standard t-PA. DS alone did not lyse and did not potentiate the activity of standard t-PA. Moreover, no increased activity was observed when Tyrode's perfusion fluid was used as a source of PA instead of t-PA standard (data not shown).

**DISCUSSION**

DS is one of the components of the extracellular matrix; it is found alone or in association with other glycosaminoglycans in human coronaries, in bovine and Rhesus monkey aorta. Unlike heparin, DS inhibits thrombin formation by interacting with only heparin cofactor II and not with antithrombin III. Recently, it was shown that DS displays antithrombotic activity in models of experimental venous thrombosis. In contrast to heparin, the antithrombotic activity was achieved at doses devoid of hemorrhagic effects. These characteristics make DS a potentially interesting molecule for thrombosis prevention and call attention to its potential use as an alternative to heparin. The antithrombotic activity of DS was shown to be dose-dependent, and it was observed at doses of 10 to 100 mU/mL. The rise in fibrinolytic activity was concomitant with the presence of DS in the rat hindquarters model. The increase was dose-dependent, and it was observed at doses of 0.1 to 0.4 mg/mL, but decreased following a further increase in the doses used. The rise in fibrinolytic activity was concomitant with the presence of DS in the microvasculature; maximum release was achieved during the initial minutes of perfusion followed by a slight decrease during the subsequent minutes (Table 1). The fibrinolytic activity induced by DS has been identified as t-PA-like molecules on the basis of the relative mobility in SDS-PAGE.

**REFERENCES**


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