British Journal of Pharmacology (1997) 122, 906–910

Inhibitory effect of locally administered heparin on leukocyte rolling and chemoattractant-induced firm adhesion in rat mesenteric venules *in vivo*

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1 Anti-inflammatory actions of heparin and related glycosaminoglycans have been described in the literature. Here, we used intravital microscopy of the rat mesentery microcirculation to examine effects of locally administered heparin on leukocyte rolling and chemoattractant-induced firm adhesion.

2 It was found that topical application of heparin reduced *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced leukocyte adhesion. Notably, the inhibitory action of heparin was not dose-dependent, but rather a biphasic dose-response was found, i.e. low (2 and 20 iu ml^{-1}) and high (1000 iu ml^{-1}) concentrations of heparin significantly reduced adhesion, whereas an intermediate dose (200 iu ml^{-1}) was less effective.

3 Heparin, 2 and 20 iu ml^{-1}, decreased rolling leukocyte flux, while having no effect on blood flow or total leukocyte flux. By contrast, heparin, 200 and 1000 iu ml^{-1}, increased total leukocyte flux in parallel with a rise in volume blood flow resulting in recovery of the rolling leukocyte flux at these doses. Thus, the biphasic inhibitory action of heparin on fMLP-induced firm adhesion could in part be attributed to changes in leukocyte delivery (i.e. blood flow) and rolling leukocyte flux induced by heparin.

4 When compensating for the influence of different rolling levels on fMLP-evoked adhesion, a dose-related inhibitory effect of heparin on the firm adhesive response *per se* was revealed. Similar results were obtained in a static adhesion assay *in vitro* where heparin 200 and 1000 iu ml^{-1} (but not 2 and 20 iu ml^{-1}) significantly inhibited fMLP-induced leukocyte adhesion in the absence of any modulatory influence on changes in rolling.

5 Our data show that locally administered heparin inhibits leukocyte rolling as well as chemoattractant-induced firm adhesion *in vivo* which thus may contribute to the postulated anti-inflammatory activity of this compound. However, because of interference with several microvascular functions, strict dose-dependent responses to heparin treatment were not found, which illustrates the complex interplay between local blood flow, leukocyte rolling and chemoattractant-induced adhesion as determinants of leukocyte recruitment to tissues in inflammation.

Keywords: Heparin; leukocyte adhesion; leukocyte rolling; inflammation; intravital microscopy; rat mesentery

Introduction

Leukocyte recruitment to tissues is a key component in inflammatory and immune reactions. However, misdirected accumulation of leukocytes may cause tissue damage in a wide variety of inflammatory diseases. The extravasation of leukocytes comprises a series of adhesion receptor-dependent events, among which initial rolling along the venular endothelium is known to be a precondition for subsequent firm adhesion and emigration of the leukocytes (Lawrence & Springer, 1991; Lindbom *et al*., 1992; von Andrian *et al*., 1992). The latter steps in this process depend mainly on adhesion molecules of the integrin family (Springer, 1994), whereas leukocyte rolling is predominantly mediated by the selectin molecules, i.e. L-, P- and E-selectin (Ley *et al*., 1991b; Mayadas *et al*., 1993; Kunkel *et al*., 1996). The function of the selectins in this process depends on recognition of specific glycoprotein ligands. However, a variety of carbohydrate structures, including heparin and related glycosaminoglycans may bind to these lectin-like molecules and interfere with natural ligand binding (Bevilacqua & Nelson, 1993; Nelson *et al*., 1993; Varki, 1994; Lasky, 1995).

Physiologically, glycosaminoglycans are known to function in cell-cell and cell-matrix interactions and to modulate growth factor and enzyme activity (Jackson *et al*., 1991). Commercial heparin has long been used for its anticoagulant activity. In addition, this compound has been shown to possess a variety of anti-inflammatory actions (Jaques, 1979), including the capacity to bind and neutralize products of the complement cascade, inhibition of chemoattractant-induced leukocyte adhesion and chemotaxis *in vitro* (Bazzoni *et al*., 1993; Matzer *et al*., 1984), and dermal accumulation of leukocytes *in vivo* (Teixeira & Hellewell, 1993). However, the effects of heparin on leukocyte-endothelium interactions in the extravasation process are unclear. For example, heparin and heparin-derived molecules have been shown to inhibit L- and P-selectin binding and to reduce leukocyte recruitment to tissues (Nelson *et al*., 1993), possibly via inhibition of leukocyte rolling (Ley *et al*., 1991a; Tangelder & Arfors, 1991). On the other hand, it was recently indicated that heparin interacts only minimally with L-selectin. Instead, heparin was found to be an adhesive ligand for CD11b/CD18 (Mac-1) and could also interfere with firm leukocyte adhesion under static conditions *in vitro* (Diamond *et al*., 1995).

In the present intravital microscopy study, effects of locally administered heparin on leukocyte rolling and chemoattractant (fMLP)-induced firm adhesion were examined in rat mesenteric microvessels *in vivo*.

Methods

Rat mesentery preparation

Adult female Wistar rats weighing 225–250 g were anaesthetized with equal parts of fluanison/fentanyl (Hypnorm, 10/0.2 mg ml^{-1}) and midazolam (Dormicum, 5 mg ml^{-1}) diluted

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1:1 with sterile water (2 ml kg^{-1}, i.m.). The trachea was cannulated to facilitate spontaneous breathing. A catheter was placed in the left femoral vein for i.v. administration of acridine orange and supplementary doses of anaesthetic. Body temperature was maintained at 37°C by a heating pad connected to a rectal thermistor. Laparotomy was performed by a midline incision and a segment of the ileum was exteriorized from the peritoneal cavity and placed on a heated transparent pedestal to allow microscopic observation of the mesenteric microcirculation. The exposed tissue was superfused with a thermostated (37°C) bicarbonate-buffered saline solution (composition in mM: NaCl 132, KCl 4,7, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 18) equilibrated with 5% CO_2 in nitrogen to maintain physiological pH.

The animal experiments performed in this study were approved by the regional ethical committee for animal experimentation.

Intravital microscopy

Observations of the mesenteric microcirculation were made with a Leitz Orthoplan microscope equipped with water immersion lenses ($\times 25$, NA 0.6 or $\times 55$, NA 0.8). The microscopic image was televised (Panasonic WV-1550, WV-1900 cameras) and recorded on video (Panasonic NV-F100) for subsequent off-line analysis. After positioning under the microscope, a 15 min equilibration period preceded quantitative measurements. Analyses of blood flow, leukocyte flux and leukocyte-endothelium interactions (rolling and adhesion) were made in small venules (inner diameter 15–25 μm) with stable blood flow.

The 'rolling leukocyte flux' was determined at various time intervals by counting the number of rolling leukocytes min^{-1} passing a reference point in the microvessel. Leukocyte adhesion was induced by the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) added topically via the superfusion buffer at a final concentration of 1 μM. At the end of the stimulation period, the number of stationary adherent leukocytes per 100 μm vessel length was counted. In order to evaluate the effects of heparin on leukocyte rolling and stimulus-evoked adhesion, heparin at indicated doses was administered topically to the tissue via the superfusion buffer. Before heparin treatment, challenge with fMLP was induced for 6 min followed by a washing period (buffer only for 10–15 min) during which the adherent leukocytes dislodged from the endothelial cell lining. Heparin was thereafter administered and, in the continuous presence of heparin, the tissue was again challenged with fMLP. There was no statistical difference in the adhesion response between the first and second challenge with fMLP when saline was given instead of heparin together with the second fMLP application. Acetylcholine, when used, was applied at a final concentration of 0.1 mM in parallel with heparin administration. In a separate series of experiments, the stimulation period with fMLP was prolonged to 20 min. In these experiments, heparin was added after 10 min of chemotactic stimulation (when leukocyte adhesion was already manifest) and co-administered with fMLP during the rest of the stimulation period.

The quantification of rolling and adherent leukocytes was made under ordinary light transillumination. In order to detect the free-flowing fraction of leukocytes for determination of the rolling/total leukocyte flux ratio (leukocyte rolling fraction), short periods of fluorescent light epi-illumination (Leitz Ploemopak, filter block I2) were used in combination with simultaneous i.v. infusion of the fluorochrome acridine orange (5 mg kg^{-1} body wt) to label the circulating leukocytes. This procedure also permitted, through frame-by-frame analysis of video-recordings, determination of the velocity of individual free-flowing leukocytes. Values for vessel radius (r) and highest detected white blood cell velocity (V_{max}) were used to calculate blood flow (Q) by use of the following relation: $Q = (V_{max}/2)\pi r^2$.

In vitro *adhesion assay*

Human umbilical vein endothelial cells (HUVEC) were cultured in 24 well tissue culture plates and grown to confluence. Human polymorphonuclear leukocytes were isolated by discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (74/55%) centrifugation and resuspended in Hanks balanced salt solution (HBSS) at a concentration of 10^6 cells ml^{-1} (purity > 99%). Five hundred microlitres of cell suspension was added to each well and incubated at room temperature for 10 min with fMLP (0.1 μM) and heparin at indicated concentrations. The wells were gently washed twice with HBSS to remove nonadherent leukocytes and then analysed for myeloperoxidase (MPO) activity in order to assess the number of adherent neutrophils. MPO is a reliable marker enzyme for polymorphonuclear leukocytes (Suzuki *et al.*, 1983).

Drugs

Heparin (porcine intestinal mucosa heparin; specific activity ~ 200 iu mg^{-1}) was from Kabi Pharmacia, Stockholm, Sweden. Acetylcholine, acridine orange, and fMLP were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dormicum was from Hoffman-La Roche (Basel, Switzerland). Hypnorm was from Janssen Pharmaceutica (Beerse, Belgium).

Statistical analysis

Statistical evaluations were performed by use of the Mann-Whitney test for unpaired samples and Wilcoxon signed rank test for paired observations. The results are presented as means ± s.e.mean, *n* represents number of animals.

Results

Effects of heparin on leukocyte-endothelium interactions in vivo

At the end of the equilibration period, the baseline rolling leukocyte flux along the venular endothelium was 20 ± 2 cells min^{-1} ($n = 31$), constituting 41 ± 3% of the total leukocyte flux (i.e. rolling plus free flowing leukocytes) in the observed vessels. When unstimulated preparations were followed for up to 1 h, the rolling leukocyte flux remained largely constant and only occasional cells adhered spontaneously during this time period (data not shown).

Local challenge with fMLP (1 μM) induced prompt firm adhesion of leukocytes to the venular endothelium, i.e. 6 and 20 min application of fMLP increased the number of adherent leukocytes from a baseline value of 0.9 ± 0.8 to 6.2 ± 0.4 and 10.4 ± 1.1 per 100 μm vessel length, respectively. Observations of the leukocyte-endothelium interactions were made in the proximal portion of the mesenteric venules. The number of rolling leukocytes entering this section did not change during the period of fMLP stimulation. In order to examine the effect of locally administered heparin on chemoattractant-induced adhesion, challenge with fMLP was induced in the presence of different concentrations of heparin. It was found that topically applied heparin could prevent leukocyte adhesion in response to fMLP-stimulation (Figure 1a). Moreover, heparin was able also to reduce already established stimulus-evoked firm adhesion, as indicated in separate experiments in which heparin was applied after induction of the chemotactic stimulus (Figure 1b). Interestingly, irrespective of whether heparin was given before or after induction of the fMLP-challenge, there was a clear biphasic dose-response to heparin administration. Notably, 2 and 20 iu ml^{-1} of heparin significantly reduced fMLP-induced leukocyte adhesion, while there was no significant inhibition at 200 iu ml^{-1} (Figure 1). At heparin 1000 iu ml^{-1}, leukocyte adhesion was almost abolished and not different from prechallenge values.

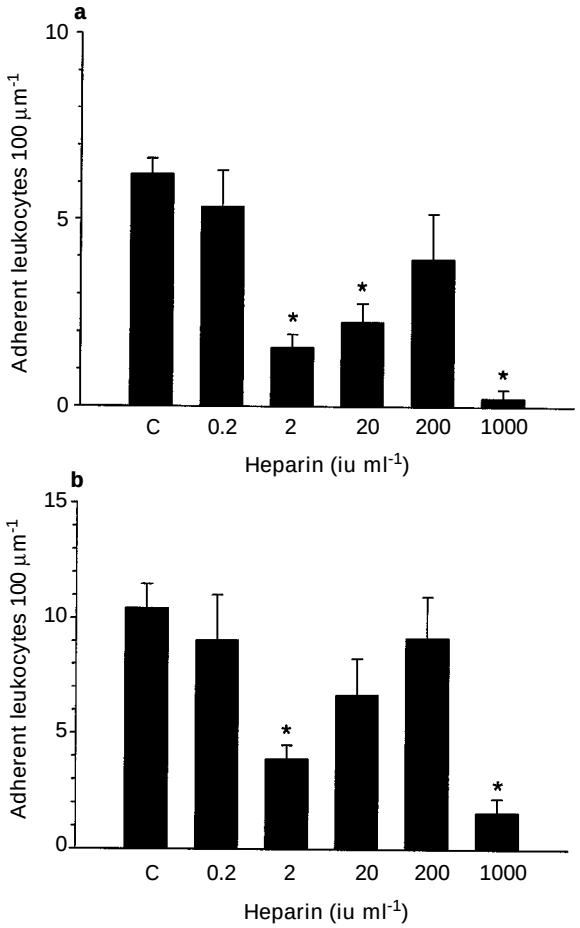

Figure 1 Effect of heparin on fMLP-induced (1 μM) firm leukocyte adhesion in rat mesenteric venules. Heparin at indicated concentrations (or buffer alone; C) was topically applied before and during a 6 min challenge with fMLP (a); or, in a separate set of experiments, administered 10 min after induction of a 20 min challenge with fMLP (b). The number of adherent leukocytes per 100 μm vessel length was counted at the end of the stimulation periods. Data are means $\pm$s.e.mean ($n=4-9$). *Significant difference from control ($P<0.05$).

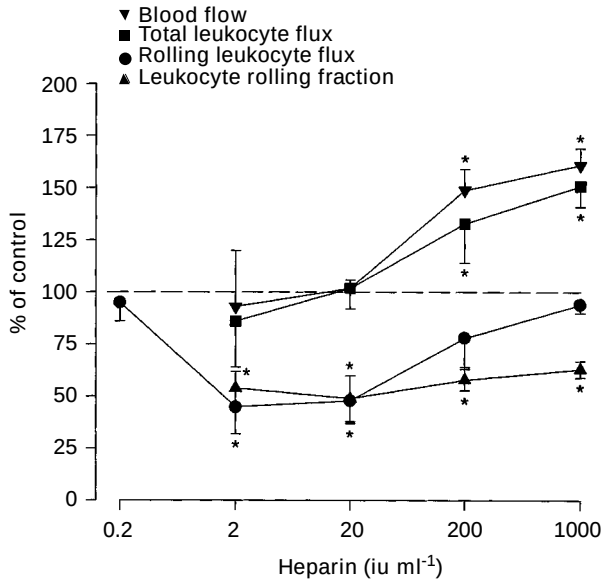

Figure 2 Effects of topically applied heparin on volume blood flow, total leukocyte flux, rolling leukocyte flux and leukocyte rolling fraction in rat mesenteric venules. Data are expressed as % of control values obtained before heparin application, and represent means with vertical lines showing s.e.mean ($n=4$). *Significant difference from control ($P<0.05$).

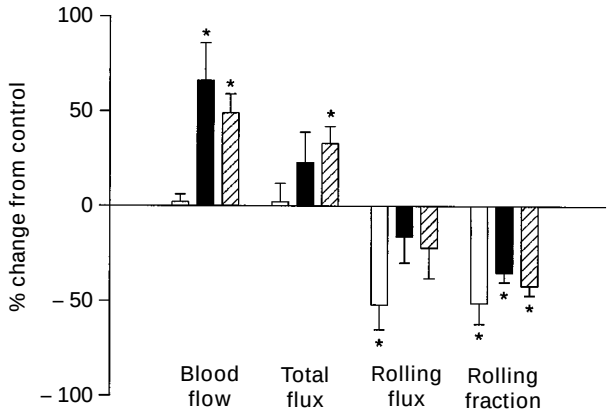

Figure 3 Changes in volume blood flow, total leukocyte flux, rolling leukocyte flux and leukocyte rolling fraction in rat mesenteric venules following topical administration of heparin 20 iu ml^{-1} together with acetylcholine 0.1 mM (solid columns) in comparison with effects of heparin 20 iu ml^{-1} alone (open columns) and heparin 200 iu ml^{-1} (hatched columns). (Data for heparin 20 iu ml^{-1} and 200 iu ml^{-1} are identical to those presented in Figure 2). Data are expressed as % change from control values obtained before heparin/acetylcholine application, and represent means$\pm$s.e.mean ($n=4$). *Significant difference from control ($P<0.05$).

We next examined whether this biphasic response curve for heparin on fMLP-induced leukocyte adhesion was related to changes in leukocyte delivery (i.e. blood flow) and/or leukocyte rolling which are critical determinants for the subsequent firm leukocyte adhesion *in vivo* (Lindbom *et al*., 1992). As shown in Figure 2, heparin at concentrations of 2 and 20 iu ml^{-1} significantly decreased rolling leukocyte flux while having no effect on blood flow or total leukocyte flux. This reduction in rolling leukocyte flux and the concomitant lack of effect on total leukocyte flux following 2 and 20 iu ml^{-1} of heparin is reflected also by a significant decrease in the rolling leukocyte flux fraction (Figure 2). On the other hand, heparin 200 and 1000 iu ml^{-1} increased total leukocyte flux in parallel to a rise in volume blood flow, indicating that the increased delivery of leukocytes (i.e. total leukocyte flux) was related to a vasodilator activity of heparin. Moreover, along with the increase in total leukocyte flux, the rolling leukocyte flux returned towards the control level at the higher doses of heparin, whereas leukocyte rolling fraction remained reduced (Figure 2). Thus, the leukocyte rolling fraction was reduced to a similar extent by heparin within the dose range of 2 to 1000 iu ml^{-1} (Figure 2). That the differences between the higher (200 and 1000 iu ml^{-1}) and lower (2 and 20 iu ml^{-1}) concentrations of heparin in the effect on rolling leukocyte flux were due to a vasodilator activity of heparin at the higher doses was also indicated in separate experiments performed with acetylcholine, a well-known vasodilator without direct effects on leukocyte-endothelium interactions *in vivo* (Thorlacius *et al*., 1995; unpublished observations). It was found that coapplication of 0.1 mM acetylcholine and 20 iu ml^{-1} heparin mimicked the actions of heparin at 200 iu ml^{-1} with regard to changes in blood flow and leukocyte rolling interactions (Figure 3).

Heparin inhibits chemoattractant-induced leukocyte adhesion

Under *in vivo* flow conditions, the level of leukocyte rolling will critically influence the extent of chemoattractant-induced firm adhesion (Lindbom *et al*., 1992). Therefore, it was of interest to analyse the effect of heparin on chemoattractant-induced leukocyte adhesion in a static adhesion assay *in vitro*. It was found

that heparin 200 and 1000 iu ml^{-1}, but not 2 and 20 iu ml^{-1}, significantly inhibited fMLP-induced leukocyte adhesion to HUVEC monolayers, suggesting a direct effect of heparin at high concentrations on the firm adhesive interaction (Figure 4). In order to discern if the response to heparin administration observed *in vivo* involved a direct effect on fMLP-induced adhesion, a ratio calculation of the data was made so as to compensate for the modulatory influence of the rolling leukocyte flux level on the firm adhesive response, i.e. the change in firm adhesion at each dose of heparin was related to the change in rolling leukocyte flux at the same dose level. In this way, a change in firm adhesion above that caused by the reduction in leukocyte rolling could be distinguished. In agreement with our *in vitro* findings described above, this ratio determination uncovered a dose-related inhibitory effect of heparin on firm leukocyte adhesion *in vivo* (Figure 5). While the change in firm adhesion and in rolling leukocyte flux were of equal magnitude in the lower dose range of heparin, there was a relatively larger inhibitory effect on firm adhesion than on rolling flux at the higher concentrations of heparin, as indicated by greater positive values for this ratio. Accordingly, the effect of 1000 iu ml^{-1} heparin on the fMLP-induced leukocyte response could be ascribed mainly to direct interference by heparin with the firm adhesive interaction.

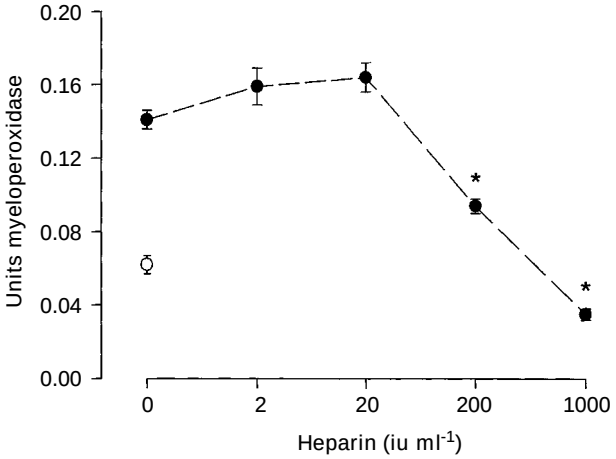

Figure 4 Effect of heparin on fMLP-induced (0.1 μM) neutrophil adhesion to HUVEC under static conditions *in vitro* (solid symbols). Adherent leukocytes were quantified through analysis of neutrophil myeloperoxidase activity. Open symbol represents spontaneous adhesion of non-stimulated neutrophils. Data are means and vertical lines show s.e.mean ($n=4$). *Significant difference from fMLP challenge without heparin ($P<0.05$).

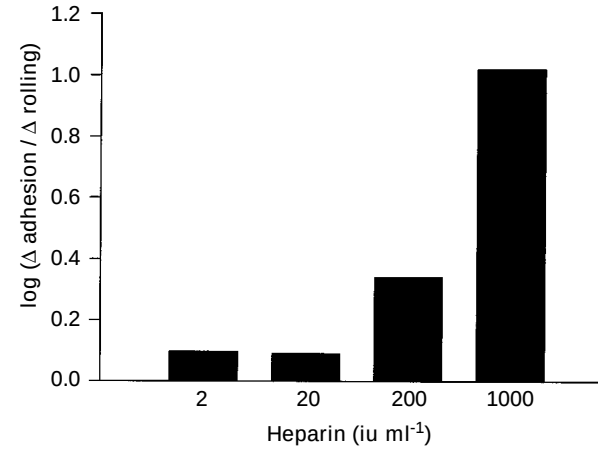

Figure 5 Change in fMLP-induced firm leukocyte adhesion in response to topical treatment with heparin normalized to the change in rolling leukocyte flux at the same concentrations of heparin. The logarithm of this ratio is presented in order to reach equality of deviations in either direction from unity. An effect of heparin on the firm adhesive response above that related to the change in leukocyte rolling is revealed by positive values for this ratio at the higher concentrations of heparin.

Discussion

In this study, we have documented that locally administered heparin inhibits leukocyte rolling and firm adhesion in the rat mesentery. These findings extend previous observations showing that local intradermal administration of heparin reduces chemoattractant-induced leukocyte accumulation in the skin (Teixeira & Hellewell, 1993), and may help explain the activity profile of heparin with regard to its anti-inflammatory properties.

The inhibitory action of locally applied heparin on leukocyte adhesion induced by fMLP in rat mesenteric venules was not dose-dependent. Rather, we found a biphasic response to increasing concentrations of heparin. Within the dose range used, low and high concentrations of heparin attenuated leukocyte adhesion, whereas an intermediate dose was less effective. In line with the close relationship between leukocyte rolling and chemoattractant-induced firm adhesion that exists in this tissue (Lindbom *et al*., 1992), the underlying mechanism for this biphasic response curve was attributed to the recovery of rolling leukocyte flux towards control levels at the higher concentrations of heparin (see Figure 2). The u-shaped response curve for rolling leukocyte flux to topical heparin was in turn probably due to the increased local blood flow and delivery of leukocytes (total leukocyte flux) with increasing doses of heparin. This notion is supported by our finding that administration of heparin 20 iu ml^{-1} together with acetylcholine, a vasodilator without effect on leukocyte rolling fraction (Thorlacius *et al*., 1995), mimicked the effects of heparin at 200 iu ml^{-1} on blood flow and rolling leukocyte flux. This is in agreement also with previous observations in the rat mesentery that increased blood flow and total leukocyte flux result in an increased number of rolling leukocytes, whereas the rolling flux fraction remains unchanged in spite of the changes in venular wall shear rate (Thorlacius *et al*., 1995; Xie *et al*., unpublished observations). Thus, the reversal of the inhibitory effect of heparin on fMLP-induced firm adhesion observed at the intermediate dose levels can be ascribed to a blood flow-dependent increase in rolling leukocyte flux induced by these concentrations of heparin. The mechanism for the heparin-induced vasodilatation is probably related to interference by heparin with calcium mobilization in the vascular smooth muscle, leading to relaxation of the arteriolar smooth muscle cells.

Part of the effect of heparin on fMLP-induced firm adhesion could thus be ascribed to inhibition of the rolling interaction. However, in the higher dose range of heparin, there was little contribution of this mechanism, because the rolling leukocyte flux was (due to the increase in blood flow) restored towards the control level, although the rolling fraction remained reduced. When compensating for the influence of different rolling levels on the fMLP-evoked adhesion, a dose-related inhibitory effect of heparin on firm adhesion was revealed. Notably, heparin at 1000 iu ml^{-1} almost completely inhibited leukocyte adhesion *in vivo*, in spite of the concomitant vasodilatation and increased rolling leukocyte flux observed at this concentration. A similar dose-dependent effect of heparin on fMLP-induced leukocyte adhesion was observed in the *in vitro* adhesion assay where no modulating influence of leukocyte rolling prevailed. Thus, our *in vivo* findings suggest that heparin may directly act to inhibit firm leukocyte adhesion, possibly through interference with leukocytic CD11/CD18-function (Diamond *et al*., 1995).

A substantial number of *in vitro* and *in vivo* studies have documented that leukocyte rolling is predominantly mediated

by the selectin family of adhesion molecules (McEver *et al*., 1995; Tedder *et al*., 1995). Even though the selectin function depends on recognition of specific glycoprotein ligands, these lectin-like molecules may interact with a variety of carbohydrate structures. Certain sulphated polysaccharides (*e.g*. heparin and fucoidin) bind to the selectins with high affinity (Nelson *et al*., 1993; Norgard-Sumnicht *et al*., 1993) and have also been shown to inhibit leukocyte rolling *in vivo* (Ley *et al*., 1991a; Tangelder & Arfors, 1991; Lindbom *et al*., 1992). However, studies on the effect of heparin on leukocyte rolling are incongruous. For example, microinfused or intravenously injected heparin has been shown to reduce significantly leukocyte rolling fraction in the rat and the rabbit (Ley *et al*., 1991a; Tangelder & Arfors, 1991), while others were unable to find such an effect of heparin in the hamster (Becker *et al*., 1994). In agreement with the latter study, we could observe no more than a weak transient effect on leukocyte rolling by intravenous injection of heparin in a dose range corresponding to the concentrations obtained with local administration (unpublished data). Consequently, local deposition of heparin may be important to achieve a more persistent inhibitory effect on leukocyte rolling and firm adhesion.

In summary, our data show that locally administered heparin inhibits leukocyte rolling as well as chemoattractant-induced firm adhesion *in vivo*, which thus may contribute to the postulated anti-inflammatory activity of this compound. However, because of interference with several microvascular functions, heparin treatment did not result in a strict dose-dependent inhibitory response, which further illustrates the complex interplay between local blood flow, leukocyte rolling and firm adhesion determining leukocyte recruitment to tissues in the inflammatory process *in vivo*.

This study was supported by the Swedish Medical Research Council (14X-4342, 04P-10738), the Swedish Foundation for Health Care Sciences and Allergy Research (A95093), and Karolinska Institutet.

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(Received May 29, 1997
Revised July 11, 1997
Accepted July 24, 1997)