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Evidence that Heparin but Not Hirudin Reduces PAI-1 Expression in Cultured Human Endothelial Cells

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Abstract

Heparin and other antithrombotic drugs besides their anticoagulant action could have a profibrinolytic effect. We have analyzed the effect of unfractionated heparin (UFH) and hirudin on PAI-1 gene expression in human umbilical vein endothelial cells (HUVEC). Cells were stimulated with UFH (1 and 10 IU/ml) and hirudin (20 and 100 TIU/ml). Samples were obtained before and 2, 6, and 24 hours after stimulation. mRNA analysis was conducted by reverse transcription followed by polymerase chain reaction, and PAI-1 antigen was determined by ELISA. Addition of UFH (10 IU/ml) to HUVEC resulted in a decrease of PAI-1 mRNA at 6 hours (40% reduction) and 24 hours (60% reduction) and PAI-1 antigen. Hirudin, however, did not modify significantly the PAI-1 mRNA nor the inhibitor secretion. The addition of UFH (10 or 100 IU/ml) to endotoxin-stimulated HUVEC also reduced the increased PAI-1 mRNA and antigen secretion (45%), whereas no effect could be observed with hirudin. Our results suggest that UFH, but not hirudin, by reducing the endothelial expression of PAI-1 might have a profibrinolytic effect.

Key Words: Heparin; mRNA; Hirudin; Endotoxin; PAI-1; HUVEC

Vascular endothelial cells actively participate in the regulation of fibrinolysis through the synthesis and secretion of plasminogen activators (tissue plasminogen activator [t-PA] and urokinase plasminogen activator [u-PA]) and plasminogen activator inhibitor-1 (PAI-1) [1]. Several studies suggest that PAI-1, the main plasminogen activator inhibitor found in plasma, plays an important pathophysiological role. Raised plasma levels have been associated with thrombosis [2–4], while defective PAI-1 is associated with a bleeding tendency [5].

PAI-1 is a 50-kD glycoprotein containing 379 aminoacids and 3 N-glycosylation sites, which exists in different conformations in vivo. Endothelial cells synthesize an active form that spontaneously converts to the latent or inactive form due to conformational changes of protein tertiary structure [6,7]. The PAI-1 gene is located in chromosome 7, and it has 12.2 kb organized in nine exons separated by eight introns [8,9]. The 3′ untranslated region contains several potential polyadenylation signals, which result in two mRNA species of PAI-1 with 2.3 and 3.2 kb approximately. The 5′ untranslated region contains the promotor with several regulatory sequences. Polymorphisms in the promoter of PAI-1 gene also have been associated with an increased risk of thrombosis [10]. Endothelial cell

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Abbreviations: HUVEC, human umbilical vein endothelial cells; UFH, unfractionated heparin; PAI-1, plasminogen activator inhibitor type I; RT-PCR, reverse transcription–polymerase chain reaction; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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synthesis and secretion of PAI-1 is regulated by a number of factors including hormones, growth factors, endotoxin, and cytokines [11–14].

Some reports have shown that heparin and other antithrombotic substances might have a profibrinolytic effect by modulating some endothelial fibrinolysis parameters [15,16], although direct proof of impairment at the molecular level has yet to be established. The aim of the present work was to analyze the PAI-1 gene expression by human endothelial cells in response to unfractionated heparin (UFH) and recombinant hirudin, on the basis that these substances could alter the vascular fibrinolytic potential [17,18].

1. Materials and Methods

1.1. Materials

Medium 199 with Earle's salts (MEM 199) and Hank's balanced salt solution were purchased from BioWhittaker (Verviers, Belgium); collagenase A from *Clostridium histolyticum* from Boehringer Mannheim (Mannheim, Germany); phosphate buffered saline (PBS) Dulbecco's, trypsin-EDTA, penicillin-streptomycin mixture, endothelial cell serum-free Medium and L-glutamine, from Gibco (Paisley, UK); Lipopolysaccharide from *Escherichia coli* 0127:B8 and bovine gelatine from Sigma (St. Louis, MO, USA); unfractionated heparin from Roger (Barcelona, Spain); recombinant desulfatohirudin (Revasc^{TM}) from Ciba Geigy (Basel, Switzerland).

1.2. Endothelial Cell Culture

Endothelial cells were isolated from human umbilical cords (HUVEC) obtained less than 8 hours after delivery, essentially as described by Jaffe et al. [19]. The umbilical vein was cannulated, perfused with PBS, and incubated at 37°C with collagenase A (0.5 mg/ml) for 15 min. Cells thus obtained were centrifuged at 250*g* for 5 minutes, resuspended in culture medium (MEM 199 containing 20% pooled human serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin) and seeded in 25-cm^2 culture flasks precoated with 0.1% gelatine in PBS (v/v). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed 24 hours after seeding and again every 48 hours. When cultures reached confluence, they were passaged by adding 1 ml trypsin-EDTA to flasks and incubated at 37°C for 2 minutes. Cells were then subcultured at ratio 1:3. Gram negative bacterial contamination was ruled out by using a *Limulus*-based assay. Cell viability was determined in a Neubauer chamber after Trypan blue staining.

1.3. Addition of UFH and Hirudin to HUVEC in the Absence and Presence of Endotoxin

All studies were performed with confluent cultures on the third passage. Twenty-four hours before stimulation, cultures were washed with Hank's balanced salt solution after which fresh endothelial cell serum-free medium with penicillin (50 U/ml) and streptomycin (50 μg/ml) was added. Cultures derived from the same umbilical cord were incubated with UFH (1 and 10 IU/ml) and hirudin (20 and 100 TIU/ml). Additional nonstimulated cultures were maintained as negative controls. Culture supernates and cells were harvested before stimulation (basal samples) and 2, 6, and 24 hours afterwards.

An additional experiment was performed by adding UFH (10 and 100 IU/ml) and hirudin (100 and 1000 TIU/ml) to cultures stimulated with endotoxin (50 ng/ml) to induce an increase of PAI-1 expression [11]. Samples were collected before and 2 and 6 hours after stimulation. Cultures only stimulated with endotoxin were used as positive controls.

1.4. Isolation of mRNAs

Cell mRNA was obtained by hybridizing the polyadenylated tails of mRNA molecules to oligo dT primers coupled to a solid phase matrix (Oligotex TM; Qiagen, Hilden, Germany) [20]. Briefly, confluent cultures were trypsinized and collected as a cell pellet. Lysis buffer containing guanidinium isothiocyanate and β-mercaptoethanol was added to generate an immediate RNase-free environment. Cell lysates were homogenized and centrifuged for 3 minutes at 14000*g* to remove the cell debris and protein. The supernates were incubated with 2 mg of the oligotex suspension for 10 minutes at room temperature to allow hybridization between the oligo dT30 and poly A tails of mRNAs. The hybrids were washed, and the mRNA was eluted by lowering the ionic strength followed by precipitation with 2.5 v ethanol. The resultant pellet was washed with ethanol 70% and vacuum-dried and resuspended in diethyl pyrocarbonate-treated water. After determining the concentration spectrophotometrically, the mRNA was stored at −80°C.

1.5. Isolation of PAI-1 cDNA by Reverse Transcription–PCR

The reverse transcription (RT) reaction was performed in a final volume of 20 μl by using 200 U Moloney murine leukemia virus RT (GIBCO BRL, Paisley, UK), 2 μl RT buffer, 100 ng/μl random hexamers (Boehringer Mannheim), 1 mM dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), 20 U RNAse inhibitor (Amersham Pharmacia Biotech), 5 mM DTT (Gibco BRL), and 35 ng of mRNA at 37°C for 1 hour.

PCR primer pairs used in this procedure were (5′-ACAGGAGGAGAAACCCAGCAG-3′) and (5′-CCGTCTGATTTGTGGAAGAGG-3′) upstream and downstream, respectively, giving a PCR product of 434 bp (nucleotides 217–651) from human PAI-1 cDNA [12]. Oligonucleotides (5′–3′) d(CCAAGGTCATCCATGACAAC) and d(TGT CATACCAGGAAATGAGC) were used to amplify a 464-bp fragment for human G3PDH cDNA located between nucleotides 476 and 940 [21]. cDNA was amplified in a final volume of 50 μl in the presence of 10 and 20 ng/ml each primer of PAI-1 and G3PDH, respectively, and PCR master mix (2 U Taq polymerase from Boehringer Mannheim, 1.5 mM $MgCl_2$, 40 mM KCl, 16 mM Tris-ClH, pH 8.3) [22]. PCR was performed using the GeneAmp 2400 PCR system (Perkin Elmer, Norwalk, CT, USA) with the following amplification profile: 40 seconds at 95°C, then 23 cycles (20 seconds denaturation at 95°C; 15 seconds annealing at 56°C for G3PDH and 58°C for PAI-1; 15 seconds extension time at 72°C) followed by a final extension at 72°C for 5 minutes. Fithteen microlitres of the reaction mixture was electrophoresed in 1.5% agarose gel and the amplified bands visualized by ethidium bromide. Intensity of PCR bands was determined by densitometric analysis with the Gel Doc 1000 UV fluorescent system and Molecular Analyst software for quantification of images (Bio-Rad, Hercules, CA, USA). Values corresponding to PAI-1 amplification were normalized with those for G3PDH. Averages of three experiments performed with samples obtained from independent cultures before stimulation and 2, 6, and 24 hours afterwards are reported for each condition.

1.6. Analysis of PAI-1 Antigen Levels in HUVEC Conditioned Medium

Cultured medium from cells treated with UFH and hirudin was collected before stimulation and at 2, 6, and 24 hours and stored at −40°C. Samples from cultures stimulated with UFH (10 and 100 IU/ml) and hirudin (100 and 1000 TIU/ml) added simultaneously to endotoxin were collected before and at 2 and 6 hours after stimulation. The PAI-1 antigen levels were determined using an ELISA assay (TintElize PAI-1 from Biopool, Umea, Sweden) with a monoclonal antibody that detects latent, active, and t-PA/PAI-1 complex with equal sensitivity [23]. Averages of three independent experiments are reported for each condition.

1.7. Statistical Analysis

Data are expressed as percentages with respect to baseline and presented as mean±SEM. The significance of differences between stimulated and control groups was assessed by Student's *t*-test or Mann-Whitney U test, as appropriate. A *p* value <0.05 was considered to be significant.

2. Results

2.1. PCR Amplification of Sequences Encoding for PAI-1 and G3PDH

Amplification profiles for PAI-1 and G3PDH were made to test the amount of mRNA input and cycle number needed to find the exponential range in the RT-PCR reaction. The amounts chosen in the linear range of the standard curves were 15 ng mRNA and 23 cycles for PAI-1 and 20 ng mRNA and 23 cycles for G3PDH (Figure 1). To assess the reproducibility of our assay, we calculated the index of intra-assay variation and the mean coefficient of inter-assay variation in eight samples (9 and 18%, respectively).

The identity of PCR product from PAI-1 cDNA amplification was demonstrated after digestion with BclI and SacI yielded the predicted fragments.

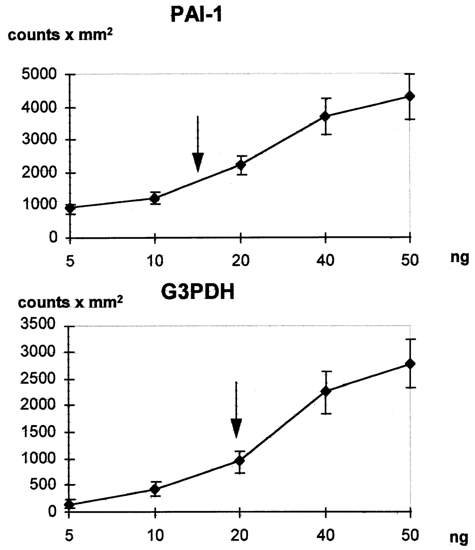

Fig. 1. Line graphs show PAI-1 (top) and G3PDH (bottom) amplification profile at 23 cycles. To find the exponential range in the PCR, we conducted a series of RT-PCR reactions in which different amounts of mRNA input were tested at different cycles and the obtained values (counts × mm^2) were plotted on a curve. Arrows indicate the final amount of mRNA chosen for each cDNA amplification assay.

2.2. Effect of UFH and Hirudin on PAI-1 Expression and Secretion from Cultured HUVEC

As shown in Figure 2 and Table 1 the PAI-1 mRNA remained unchanged over the 24-hour incubation period in unstimulated cultures, whereas PAI-1 antigen secretion increased linearly, suggesting a continuous release from endothelial cells.

When cultured HUVECs were incubated with UFH in the absence of serum, a progressive decrease in the level of PAI-1 gene expression was observed (30% reduction in relation to baseline at 2 hours of incubation with 1 IU/ml), to normalize to the levels observed in unstimulated cultures at 6 and 24 hours. At a dose of 10 IU/ml, a progressive time-course decrease was observed, starting at 2 hours (15% reduction with respect to basal levels), reaching 40% at 6 hours and 60% at 24 hours (Figure 2).

The effect of UFH on the PAI-1 antigen levels in the conditioned medium also was examined to determine whether the decrease in PAI-1 mRNA correlated with a reduced level of PAI-1 release from HUVEC. As shown in Table 1, we found that UFH attenuated the synthesis of PAI-1 antigen in HUVEC with respect to control cultures throughout the experiment (17% reduction).

When confluent cultures were incubated for various times in the presence of high (100 TIU/ml) and low (20 TIU/ml) r-hirudin doses, no significant changes were detected in the PAI-1 mRNA levels (Figure 2). Likewise, PAI-1 antigen in the conditioned medium did not change significantly after addition of different hirudin doses with respect to unstimulated cultures (Table 1).

2.3. Effect of UFH and Hirudin Treatment on the Endotoxin-Induced PAI-1 Expression and Secretion by HUVEC

Additional experiments were performed to assess whether the anticoagulant treatment was able to inhibit the endotoxin-induced PAI-1 mRNA expression as well as PAI-1 antigen release. To that purpose confluent cultures from HUVEC were incubated with endotoxin without or with UFH or r-hirudin. As shown in Figure 3 and Table 2, whereas both PAI-1 mRNA and protein showed a twofold increase at 6 hours in response to endotoxin stimulation with respect to control cultures, the simultaneous addition of UFH resulted in a significant decrease of PAI-1 mRNA (35% reduction at 2 hours, $p<0.01$ and 22.5% at 6 hours). Furthermore, the PAI-1 mRNA levels were greater reduced by addition of 100 IU/ml of UFH to endotoxin-stimulated HUVEC (55% at 6 hours, $p<0.01$). At these high UFH doses a significant reduction of PAI-1 antigen secreted into the medium was also observed (45% reduction at 6 hours, $p<0.05$) (Table 2).

No significant differences in the ability to reduce the increased PAI-1 mRNA and protein by endotoxin-stimulted HUVEC were seen after the simultaneous addition of any r-hirudin dose (Figure 3 and Table 2).

3. Discussion

Vascular endothelium plays a pivotal role in initiation and control of fibrinolysis through the synthesis of t-PA and PAI-1. In vivo studies have emphasized the pathophysiological role of PAI-1 (reviewed in references [2–4]), so that the possibility of modulat-

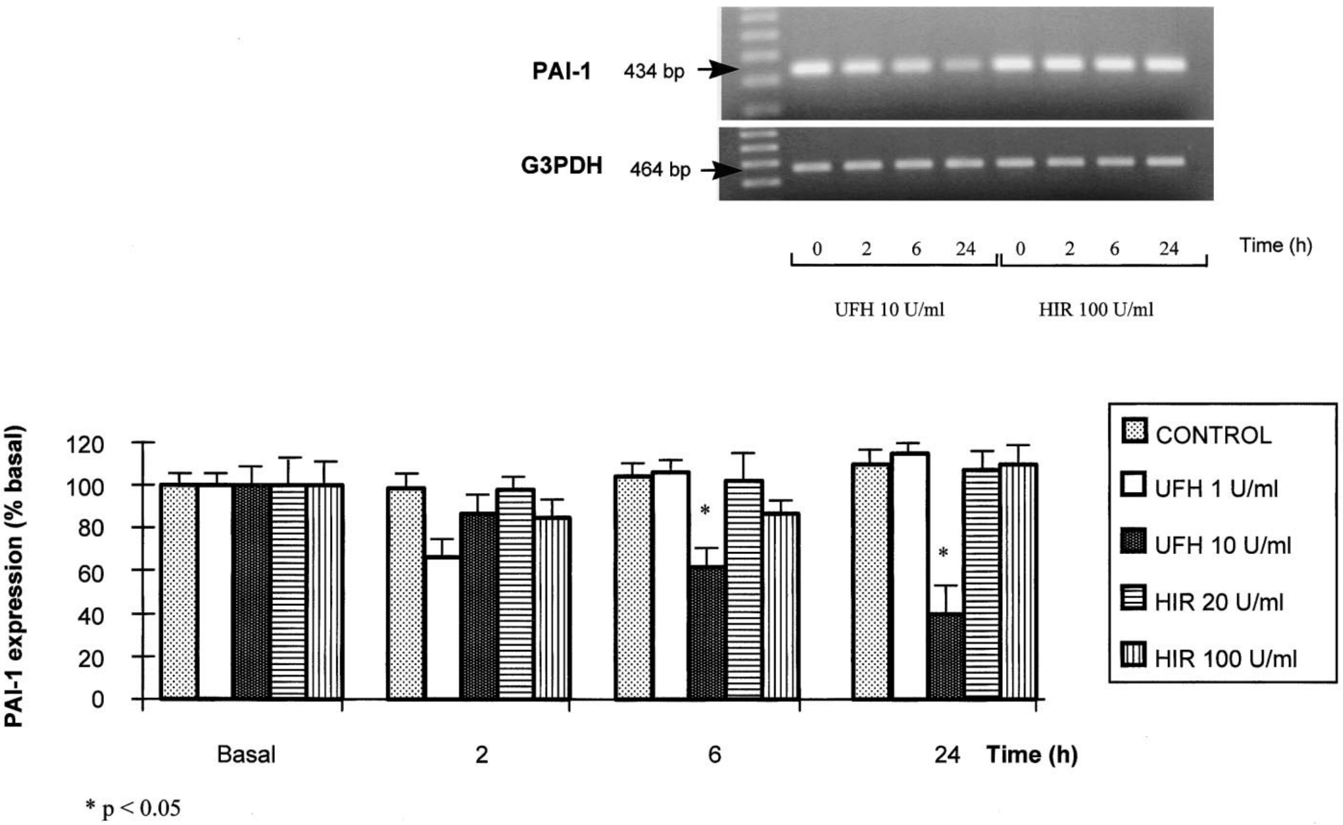

Fig. 2. Time course of PAI-1 mRNA induction by UFH and hirudin. Confluent cultures of HUVEC were preincubated for 24 hours with UFH (1 and 10 U/ml) and hirudin (20 and 100 U/ml) in serum-free medium. Unstimulated cultures were used as controls. PAI-1 mRNA (15 ng) and G3PDH (20 ng) were analyzed by RT-PCR. The PCR products (15 μl) were electrophoresed in agarose gels (top) and quantitated by densitometric analysis (see Materials and Methods). Each bar represents the data for PAI-1 mRNA normalized to the level of G3PDH. Asterisks indicate $p<0.05$ as compared with control cultures.

ing the endothelial inhibitor expression represents an attractive therapeutic approach.

In the present study, we have analyzed PAI-1 gene expression in HUVEC stimulated with UFH and hirudin on the basis that these agents, besides their anticoagulant action, also could modify the vascular fibrinolytic potential [15–18]. By using a RT-PCR method, which allows detection of changes in gene expression [24,25], we have shown that heparin reduces PAI-1 expression in cultured HUVEC, while hirudin has no effect on endothelial inhibitor expression.

The addition of UFH reduced PAI-1 mRNA levels even at a concentration as low as 1 IU/ml,

Table 1. Effect of the addition of UFH and hirudin to HUVEC on PAI-1 antigen (ng/10^5 cells)

		UFH		Hirudin	
	Control	**1 U/ml**	**10 U/ml**	**20 U/ml**	**100 U/ml**
Basal	416.6±15.2	420.1±19.1	400.1±11.7	420.4±14.2	424.9±5.4
2 hours	425.1±17.1	513.2±12.7	373.2±8.7	420.4±32.5	450.4±27.3
6 hours	533.3±19.4	583.2±27.3	453.2±12.6	746.6±27.1	600.1±16.9
24 hours	933.2±40.7	896.6±13.7	826.6±32.9	960.1±26.9	910.3±11.7

Mean±SEM of triplicate experiments is shown.

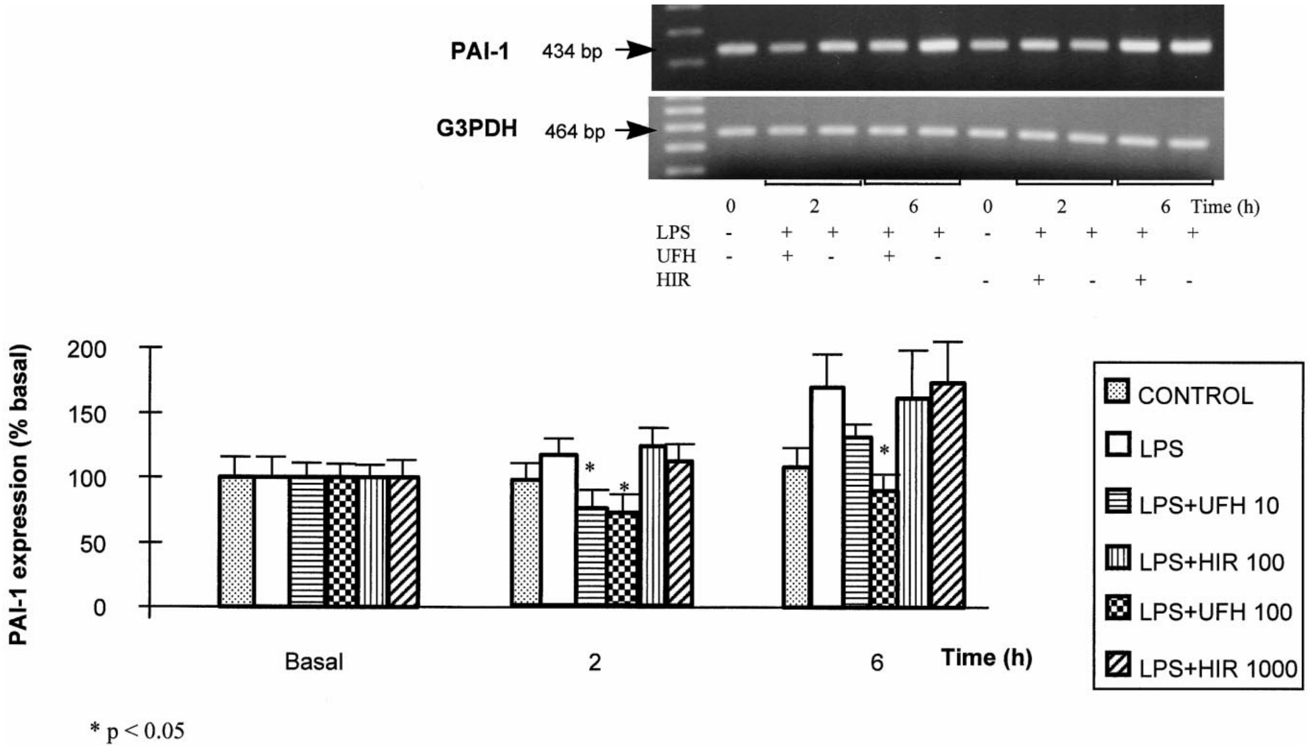

Fig. 3. Decrease of endotoxin-induced PAI-1 mRNA in HUVEC with addition of heparin. Confluent cultures of HUVEC were incubated with 50 ng/ml LPS alone or LPS plus UFH (10 and 100 IU/ml) or r-hirudin (100 and 1000 TIU/ml). Unstimulated cultures were used as controls. At indicated times the PAI-1 mRNA (15 ng) and G3PDH (20 ng) were analyzed by RT-PCR. The PCR products (15 μl) were electrophoresed in agarose gels (top) and quantitated by densitometric analysis. Each bar represents the data for PAI-1 mRNA normalized to the level of G3PDH. Asterisks indicate $p<0.05$ as compared with control cultures.

which is within the plasma level reached when used as antithrombotic agent in humans [26]. The rapid PAI-1 mRNA decrease initially observed with low-dose heparin as well as the significant delay and long-lasting reduction observed with higher UFH doses can be explained by the different modulation of some endothelial cell properties in the presence of heparin: a transient effect, probably related to the presence of heparin molecules bound at the membrane surface, and a delayed one mediated by an increase in the membrane heparan sulfate molecules [27]. The possibility that the observed PAI-1 mRNA decrease might be related to an inhibitory effect of heparin on enzymes present in the RT-PCR [28,29] can be ruled out since G3PDH amplification was not affected.

Studies evaluating the changes in vascular endothelium induced by glycosaminoglycans have focused primarily on the modulation of the procoagulant properties of stimulated endothelial cultures

Table 2. Effect of the addition of UFH and hirudin to HUVEC on PAI-1 antigen (ng/10^5 cells) in the presence of endotoxin

			Endotoxin+UFH		**Endotoxin+HIR**	
	Control (nonstimulated)	**Endotoxin (50 ng/ml)**	**10 IU/ml**	**100 IU/ml**	**100 TIU/ml**	**1000 TIU/ml**
Basal	416.6±15.2	375.1±15.3	369.9±18.6	356.6±17.2	426.4±22.8	416.6±19.8
2 hours	425.1±17.1	660.3±19.7	647.3±24.2	404.1±18.3	614.5±39.4	586.6±27.9
6 hours	533.3±19.4	1031±43.3	894.1±14.7	612.6±26.7[a]	979.2±29.6	1137.2±34.1

Mean±SEM of triplicate experiments is shown.

[a] $p<0.05$.

in the presence of heparin [30–32]. Our results also indicate a profibrinolytic effect of heparin via reduction of PAI-1 mRNA expression, thus differing from previous reports in which such an effect was not observed [33,34], because it was masked by the addition of either serum or thrombin to the medium, which are known to induce PAI-1 expression [35].

As regards the PAI-1 antigen, we observed an accumulative protein secretion to the medium in long-term HUVEC cultures, which agrees with previous findings [36]. The addition of 10 IU/ml UFH attenuated the PAI-1 antigen secreted to the medium with respect to control cultures, although higher heparin doses are required to show a greater effect on protein secretion, as previously shown [37]. A net balance of synthesis and accumulation rate, also observed in control cultures, could explain the observed differences between PAI-1 mRNA and protein secretion.

In contrast, recombinant hirudin at the doses used in this study did not alter the inhibitor expression or the protein secretion with respect to controls. While few reports have analyzed the effect of hirudin on the fibrinolytic potential of vascular endothelium [37,38], this is to our knowledge the first study demonstrating that r-hirudin has no direct effect on PAI-1 endothelial expression. However, an indirect effect on inhibitor expression cannot be ruled out, since hirudin is able to block the thrombin induced PAI-1 mRNA increase by smooth muscle cells [38], and in vivo studies also indicate that hirudin can improve some fibrinolytic parameters [39,40].

To further analyze the effects of UFH and hirudin on the inhibitor expression both agents were added simultaneously to HUVEC incubated with endotoxin, which is known to enhance the PAI-1 mRNA expression and induce PAI-1 antigen release [12,15]. High UFH doses significantly suppressed the endotoxin-induced PAI-1 mRNA and protein secretion. Although the mechanism responsible for this effect is far from being understood, the presence of heparitinase-sensitive sites on the HUVEC surface could play an important role [41]. In contrast, r-hirudin did not affect the endotoxin-induced inhibitor expression by HUVEC.

We conclude that heparin, besides regulating blood coagulation, also would modulate vascular fibrinolysis by reducing the endothelial expression of PAI-1, which could explain some of its profibrinolytic properties observed in vivo [18,19]. The ability of heparin to reduce the LPS-induced enhancement of endothelial PAI-1 expression might be an additional mechanism in the prevention of thrombosis, operating at the local level. Hirudin had no effect in this in vitro model.

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