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Failure of heparin to inhibit the expression of the thrombin receptor following endothelial injury of the rabbit carotid artery

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Abstract

The effect of heparin on thrombin receptor expression was evaluated in an experimental model of myointimal smooth muscle cell proliferation in rabbits. Myointimal hyperplasia was induced by an air-drying injury of the carotid artery and thrombin receptor expression following endothelial injury was measured by in situ hybridisation and immunohistochemistry. In healthy arteries, thrombin receptor mRNA and protein were detected in the endothelial cells only. In contrast, 14 days after endothelial injury, thrombin receptor mRNA expression increased in the smooth muscle cells present in the neointima, predominantly in areas of active cell proliferation. A 2-week subcutaneous treatment with heparin (10 mg/kg per day, s.c.) inhibited smooth muscle cell hyperplasia occurring in the intima following deendothelialization ($80 \pm 7.8\%$ inhibition, $P < 0.001$). The 14-day heparin treatment strongly reduced thrombin receptor gene and protein expression observed in the endothelial cells in healthy arteries but did not affect thrombin receptor expression which occurred in smooth muscle cells which have proliferated in the neointima as a consequence of endothelial injury. These results therefore establish that thrombin receptor expression during intimal hyperplasia is an heparin-insensitive event.

Keywords: Thrombin receptor; In situ hybridization; Heparin; Arterial injury

1. Introduction

Thrombin is generated in large amounts at the time of arterial injury (Eidt et al., 1989) and the local release of thrombin may play a significant role not only in thrombosis but also in the formation of proliferative vascular lesions (Chen and Buchanan, 1975). Indeed, thrombin has been shown to elicit a wide range of cellular responses and multiple effects on vascular cells have been described (Chen et al., 1976; Herbert et al., 1992; Vu et al., 1991; Rasmussen et al., 1991; Brass et al., 1992; McNamara et al., 1993). In particular, thrombin exhibits direct mitogenic effect on vascular smooth muscle cells, fibroblasts or vascular endothelial cells (Herbert et al., 1992; McNamara et al., 1993). Molecular cloning of a functional thrombin receptor on platelets and vascular endothelial cells revealed a novel proteolytic mechanism of receptor activation (Vu et al., 1991; Rasmussen et al., 1991). These works described a new signalling mechanism in which thrombin cleaved its receptor's amino-terminal extension to create a new receptor amino terminus that functioned as a 'tethered ligand' which activates the receptor.

Heparin and heparan sulphate are structurally complex glycosaminoglycans composed of repeating disaccharide units of alternating glucosamine and glucuronic acid sugars. Previous work from several laboratories demonstrated that heparin could inhibit the proliferation of smooth muscle cells and other selected cell types in vitro (Hoover et al., 1980) and in vivo (Guyton et al., 1980; Clowes and Clowes, 1986) but attempts to define a mechanism of action that accounts for all these effects of heparin have not as yet been successful. Although the antiproliferative action of heparin has been demonstrated not to be related to its anticoagulant activity (its anti-factor Xa and its anti-thrombin activity), but to other unrelated effects, we have shown that it could inhibit smooth muscle cell proliferation induced by selective activation of the thrombin receptor (Herbert et al., 1992). Moreover, it has been shown that heparin can affect the expression of several proteases involved in the coagulation or fibrinolysis (Au et al., 1992; Clowes et al., 1992; Kenagy et al., 1994; Kenagy and Clowes, 1995) but none of these studies evaluated the effect of heparin on the expression of the thrombin recep-

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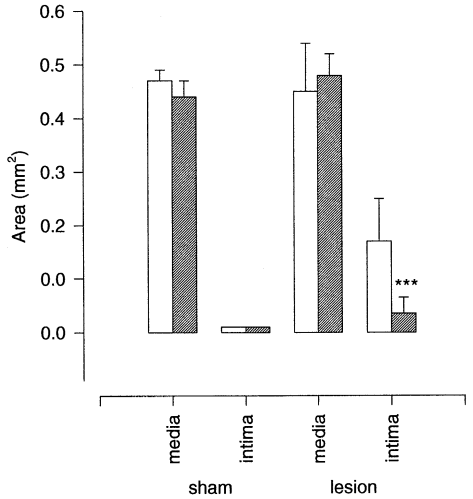


Fig. 1. Effect of heparin on the myointimal hyperplasia of carotid arteries. Heparin (10 mg/kg, s.c.) (shaded bars) or the vehicle (empty bars) was administered 2 h before and 5 min after air injury and daily for 14 days to rabbits. Intimal and medial surfaces were measured in sham controls or in animals whose arteries had been subjected to air-drying injury as described in Section 2. Data are mean ± S.D. ($n = 10$). Statistical significance: *** $P < 0.001$ (Mann-Whitney test).

tor following injury of the vascular wall. In an attempt to better define the mechanism of action of heparin with regard to smooth muscle cell proliferation, we therefore determined whether heparin could affect thrombin receptor expression, a cellular event which has been shown to occur as a consequence of mechanical manipulation of the artery (Harker et al., 1991) or to be present in lesions of atherosclerosis in humans (Wilcox et al., 1994; Nelken et al., 1992).

2. Materials and methods

2.1. Air-drying injury

Male New Zealand rabbits (Lago, Vonnas, France) weighing 2.5–3 kg were used. Air-drying injury was induced by applying an air flow through the carotid artery using a modification of the Fishman's method (Fishman and Ryan, 1975; Herbert et al., 1993). Rabbits were anaesthetised by i.v. injection of a mixture of acepromazine (0.3 mg/kg; Vetranquil, Sanofi-Winthrop, Gentilly, France) and ketamine (15 mg/kg; Imalgene 1000, Rhone-Merieux, Lyon, France). The left carotid artery was exposed and ligatured at 2 points 1.5 cm apart. A 27-gauge hypodermic needle was inserted into the proximal end of the segment by puncturing with an additional needle. After the lumen had been rinsed with saline, a stream of dry air was allowed to flow through the segment at 240 ml/min for 5 min. Following air-drying injury, ligatures were removed, allowing circulation to be re-established and hemostasis was ensured. The right carotid artery was manipulated but not submitted to air drying injury to serve as a control. In heparin-treated animals, the drug was administered by subcutaneous route (10 mg/kg per day) 2 h before injury, 5 min after and daily for 14 days.

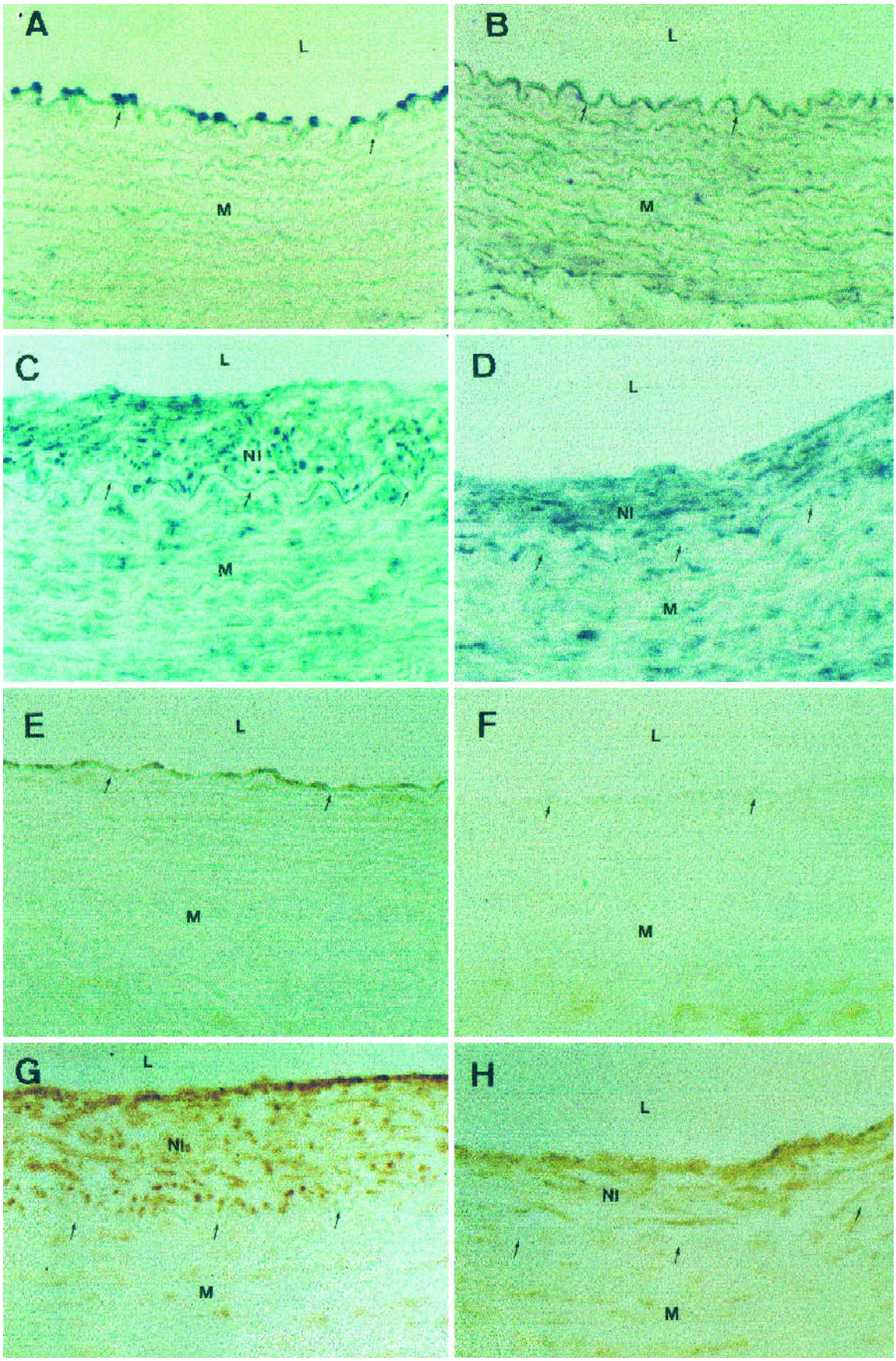
2.2. Tissue preparation and morphologic examination

Fourteen days after surgery, animals were anaesthetised with sodium pentobarbitone (30 mg/kg, i.v.). The carotid artery was isolated, rinsed with saline and fixed overnight with a 10% formaldehyde solution. The arterial segments were then dehydrated through graded solutions of alcohol, embedded in paraffin for serial cross-sectioning and stained with hematoxylin-eosin. Morphometric analysis of each arterial segment was performed with a computer-based Imagenia 2000 morphometric system (Biocom, Lyon, France). At least ten sections of each vessel were examined, and the measurements were averaged for statistical analysis. All morphometric analyses were carried out by a single examiner, who was blinded with respect to the experimental group to which each sample belonged. The cross-sectional surface areas of the vessel with the external elastic lamella (the total area), within the internal elastic lamina (the intimal area), and within the lumen (the lumen area) were measured. The degree of myointimal proliferation of the injured carotid artery was expressed as the absolute area of neointima.

2.3. In situ hybridisation and immunohistochemistry

In situ hybridisation of the thrombin receptor mRNA was performed on rabbit carotid artery, 14 days after injury as previously described (Bloch et al., 1986; Guitteny et al., 1988) by using a biotinylated synthetic 45-mer oligodeoxynucleotide probe, complementary to bases 960–1005 of the rat thrombin receptor cDNA (Zhong et al., 1992) (Appligene, Strasbourg, France). Since the rabbit thrombin receptor gene sequence is unknown, our probe was chosen in a region showing high interspecies homology between human, rat and hamster (Vu et al., 1991; Rasmussen et al., 1991; Zhong et al., 1992) cDNA. The oligodeoxynucleotide was biotinylated at its 3′ end by tailing with dUTP-16-biotine with terminal deoxynucleotidyl transferase (Amersham, Buckinghamshire, UK).

Fig. 2. Effect of heparin on the expression of the thrombin receptor mRNA and protein in the carotid artery after vascular injury. Thrombin receptor mRNA and protein were detected by in situ hybridisation (A–D) or immunohistochemistry (E–H) in sham (A, B, E, F) or air-injured (C, D, G, H) carotid arteries, 14 days after endothelial injury. The rabbits were treated with either saline (A, C, E, G) or heparin (10 mg/kg per day, s.c.) (B, D, F, H) for 14 days. In situ hybridisation and immunohistochemistry were performed as described in Section 2. Arrows indicate border of residual media and the beginning of the neointima. M, media; Ad, adventitia; L, lumen; NI, neointima; IEL, internal elastic lamina. Original magnification: ×350.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Carotid arteries were fixed in 1% formaldehyde and processed for sectioning as previously described (Bloch et al., 1986; Guitteny et al., 1988). Before hybridisation, cryosections were prehybridized in 100 μl pre-hybridisation buffer (4 × standard saline citrate; 1% Denhardt's solution) at 20°C for 90 min and dehydrated in absolute ethanol. For hybridisation, the sections were incubated 16 h at 40°C in 20 μl of hybridisation buffer (50% formamide, 5% 20 × sarcosyl solution, 10% phosphate buffer 1.2 M pH 7.2, 1% Denhardt's solution in 8 × standard saline citrate), containing 2–4 ng of biotinylated oligodeoxynucleotides. After hybridisation, the sections were washed several times with 4 × standard saline citrate buffer and treated with RNase A (Sigma). Sections were then washed in 4 × standard saline citrate followed by several washes in 1 × standard saline citrate for 1 h at 20°C, 1 × standard saline citrate for 1 h at 40°C, 0.5 × standard saline citrate for 1 h at 40°C and in 0.5 × standard saline citrate for 30 min at 50°C. Biotinilated hybrids were detected with a streptavidin alkaline phosphatase system with nitroblue tetrazolium and bromo-chloro-indolyl phosphate as substrates.

Thrombin receptor immunohistochemistry was performed on serial frozen sections which were post-fixed with 4% formaldehyde. Frozen tissue sections were reacted with an anti-thrombin receptor polyclonal antibody (IgY 948, prepared in the chicken), a kind gift of Dr F. Lanza (CRTS Strasbourg, France). Primary antibody was detected by indirect immunohistochemistry with a peroxidase-conjugated anti-IgG monoclonal antibody. Staining was visualised by using diaminobenzidine tetrahydrochloride and H_2O_2 as substrates.

2.4. Statistical analysis of data

All data are expressed as mean ± S.D. The *n* values indicate the number of animals studied. Grouped data were analysed for significance by comparison with the vehicle-treated group using the Mann-Whitney *U*-test. The level of significance was chosen as $P < 0.05$.

3. Results

3.1. Effect of heparin on the myointimal hyperplasia following carotid artery injury

In the right carotid artery (that had not been air-injured) of animals, we did not evidence any intimal proliferation or detect the presence of foam cells or thrombotic materials (platelets, fibrin, etc.) 14 days after the surgical procedure indicating that physical manipulation at the time of surgery but without air-drying injury was not sufficient to generate a lesion (Fig. 1). In the air-injured arteries, however, there was a considerable smooth muscle cell accumulation in the neointima and 2 weeks after injury, the intima area represented 38 ± 4% ($n = 10$) of the tunica media area. The newly formed intima was populated primarily by smooth muscle cells and macrophages were seen in the adventitia (not shown) but only rarely in the media or intima of injured arteries. After a 14-day subcutaneous administration of heparin (10 mg/kg per day, s.c.), the average cross-sectional area of the intima was strongly reduced showing that heparin affected smooth muscle hyperplasia occurring in the intima following deendothelialization (80 ± 7.8% inhibition, $n = 10$, $P < 0.001$) (Fig. 1). This effect of heparin on neointima formation occurred without noticeable modification of the media area (Fig. 1).

3.2. Effect of heparin on the expression of the thrombin receptor

In uninjured rabbit carotid arteries, in situ hybridisation with a biotinylated oligonucleotide probe as well as immunohistochemistry revealed the presence of thrombin receptor mRNA and protein in endothelial cells only (Fig. 2A and E) and no detectable expression of the thrombin receptor could be demonstrated in the media and adventitia of carotid arteries that had not been subjected to air injury. This detection was specific for thrombin receptor mRNA since no labelling could be observed with the biotinylated oligonucleotide control probe (corresponding sense oligodeoxynucleotide) or after competition with the unlabelled probe (not shown). Moreover, no immunostaining was observed with the isotype-matched monoclonal antibody used as a negative control (not shown). Serial sections were reacted with anti-von Willebrand factor antibodies to ascertain the identity of the endothelial cells (not shown). In contrast to the localized expression seen in the normal artery, 14 days after endothelial injury of the rabbit carotid artery, a sustained expression of the thrombin receptor mRNA and protein was detected in the neointima (Fig. 2C and G). Some smooth muscle cells and macrophages in the media and the adventitia were also labelled.

Following a 14-day treatment with heparin a strong reduction of thrombin receptor gene and protein expression was observed in endothelial cells of sham arteries (Fig. 2B and F). In air-injured carotid arteries, however, the heparin treatment did not affect thrombin receptor mRNA and protein expression which occurred in the neointima (Fig. 2D and H). Northern blots performed in parallel confirmed this observation (not shown).

4. Discussion

The highly sulphated glycosaminoglycan heparin acts as a potent modulator of smooth muscle cell growth. In vivo, heparin inhibits the migration and the proliferation of the cells in response to vascular damage (Guyton et al., 1980; Clowes and Clowes, 1986) and reduces the expression or transcription of several genes associated with cell cycle progression (Reilly et al., 1989; Wright et al., 1989) suggesting that heparin may have multiple targets for its

antiproliferative mechanism of action. In particular, it has been shown to affect the expression of several proteases involved in coagulation and fibrinolysis but none of these studies evaluated the effect of heparin on the expression of the thrombin receptor. In an attempt to better understand the mechanism by which heparin inhibits smooth muscle cell proliferation, we asked whether heparin modulates the expression of this protein in vivo following balloon injury of the rabbit carotid artery.

In normal carotid arteries, the thrombin receptor gene was expressed in endothelial cells only as shown by in situ hybridisation of normal carotid arteries with a thrombin receptor biotinylated oligonucleotide probe. Following air injury, 14 days after deendothelialization, thrombin receptor expression increased in some of the smooth muscle cells present in the media and in the neointima of the injured carotid arteries. In that respect, our results confirm those obtained by Wilcox et al. (1994) in baboons and suggest that thrombin may still exert an effect even at later times after vascular injury. However, in a recent work, we showed that topical application of an antisense oligodeoxynucleotide which selectively inhibited the synthesis of the thrombin receptor gene in vitro and in vivo did not generate a significant protective effect with regard to their response to injury (Herbert et al., 1997). Although these data confirmed previous in vivo observations indicating that there was an increased expression of the thrombin receptor mRNA and protein at sites within experimental vascular lesions, they showed that thrombin, acting as a single mitogen through its receptor on vascular cells is unlikely responsible for the initiation and maintenance of vascular lesion formation. This is consistent with observations of a cooperative effect between thrombin and other growth factors such as platelet-derived growth factor and basic fibroblast growth factor (Herbert et al., 1994; Weiss and Maduri, 1993) and is of particular importance in light of considering thrombin receptor antagonism as a single target to inhibit vascular lesion formation. These observations, however, raised a discrepancy with the results obtained with *r*-hirudin, demonstrating that specific inhibition of thrombin significantly reduced myointimal thickening in rabbits (Sarembock et al., 1991). However, this effect was likely to be the result of an inhibition of the combination of the multiple activities of thrombin, i.e. platelet activation, monocyte and macrophage chemotaxis, production of prothrombotic factors, etc., which seemed, however, of major importance in the process leading to vascular lesion formation.

Heparin inhibits the proliferation and migration of arterial smooth muscle cells in vivo and in vitro (Hoover et al., 1980; Guyton et al., 1980; Clowes and Clowes, 1986) and alters the amount and type of extracellular matrix (Snow et al., 1990). These effects may in part be accounted for by interference with the expression or action of extracellular proteinases. In particular, it has been previously reported that heparin prevents the increased expression of tissue-type plasminogen activator in vitro and in vivo (Au et al., 1992; Kenagy and Clowes, 1995) urokinase, interstitial collagenase, stromelysin, gelatinase or collagenase (Clowes and Clowes, 1986; Kenagy et al., 1994). In this respect, we evaluated the effect of heparin on the expression of the thrombin receptor in vivo. Our results in the present study suggest that, although heparin exhibited a strong antiproliferative effect in vivo, thrombin receptor mRNA and protein expression which occurred as a consequence of endothelial injury was insensitive to heparin inhibition. The concept of heparin-sensitive and insensitive pathways for smooth muscle cell activation is supported by several works including ours (Ottlinger et al., 1993; Wright et al., 1989; Barzu et al., 1994) and it has been recently suggested that the relative importance of each pathway induced by injury may account for the different responses to heparin and for the lack of effect of heparin and low molecular weight heparin in the prevention of coronary restenosis in humans (Geary et al., 1995). Heparin therefore showed a strong inhibition of smooth muscle cell proliferation without exhibiting an effect on the thrombin receptor expression measured simultaneously. This observation is by itself rather unexpected since an increased expression of thrombin receptor mRNA at sites within experimental vascular lesions has been shown to be typically associated with smooth muscle cell proliferation (Wilcox et al., 1994) but this lack of effect of heparin cannot be attributed to an effect of heparin which might not be optimal since doses higher than 10 mg/kg per day did not result in an enhanced effect on myointimal hyperplasia (not shown). Unexpected also was the observation of an effect of heparin on the basal thrombin receptor expression at the endothelial level of uninjured vessels. To our knowledge, this effect represents the first observation of such an effect on the expression of the thrombin receptor. The mechanism of action of this down-regulation is unclear but can be the consequence of the prolonged inhibition of the 'circulating' thrombin by the antithrombin-III/heparin complex. It has been recently described that heparin can durably modulate the procoagulant properties of stimulated endothelial cells (Cadroy et al., 1995). The etiology of such an effect has not been clearly determined but one can postulate that the effect of heparin on thrombin receptor expression of endothelial cells may contribute to the delayed long-acting increase of the anticoagulant potential of the luminal face of the endothelium reported by Cadroy et al. (1995).

To what extent the results described in the present work add to the current knowledge of the mechanism of action of heparin is largely unknown but this work represents the first report showing that (i) myointimal smooth muscle cell proliferation and thrombin receptor expression can be dissociated events and (ii) heparin can decrease the basal expression of the thrombin receptor on the endothelium of uninjured arteries hence contributing to its global antithrombotic activity.

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