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Proteinase-activated receptor-1 (PAR-1) activation contracts the isolated human renal artery *in vitro*

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1 The *in vitro* motor function of protease-activated recepter-1 (PAR-1), PAR-2 and PAR-4 and the presence by immunohistochemistry of PAR-1 in the human renal artery have been investigated.

2 Thrombin and the human PAR-1 (SFLLRN-NH_2) activating peptide, but not the PAR-1 reverse peptide (NRLLFS-NH_2), contracted both endothelial-intact and endothelial-denuded human renal artery strips, whereas no relaxation was observed either in strips non-precontracted or precontracted with phenylephrine. Maximum contraction by thrombin or SFLLRN-NH_2 was about 60% of phenylephrine. However, thrombin was approximately 1000-fold more potent than SFLLRN-NH_2.

3 PAR-1 desensitisation, using repeated applications of SFLLRN-NH_2, almost completely blocked the response to thrombin. The contractile effect produced by thrombin and SFLLRN-NH_2 was not affected by nitric oxide synthase inhibition, but was significantly reduced by cyclooxygenase blockade.

4 Trypsin, the PAR-2 (SLIGKV-NH_2 and SLIGRL-NH_2) and PAR-4 (GYPGQV-NH_2 and AYPGKF-NH_2) activating peptides did not produce any significant contraction or relaxation.

5 In agreement with the motor function data immunohistochemistry showed specific staining patterns for PAR-1 in the human renal artery.

6 Combined, these studies would suggest a possible role for PAR-1 in renal vascular homeostasis.
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Keywords: Protease-activated receptor-1 (PAR-1); thrombin; human renal artery

Abbreviations: PAR, protease-activated receptor

Introduction

Protease-activated receptors (PARs) are unique members of the heptaelical G protein-coupled receptor superfamily. The first PAR receptor was discovered after a lengthy search for a molecule that could account for the apparent receptor-mediated effect of the haemostatic serine-protease, thrombin (thrombin receptor, or PAR-1) (Vu *et al*., 1991). Subsequently, a second PAR (PAR-2) was identified and was found to be insensitive to thrombin, but readily activated by trypsin (Nystedt *et al*., 1994) and other serine-proteases (e.g. mast cell tryptase and coagulation factor Xa) (Fox *et al*., 1997; Mirza *et al*., 1997). Successively, additional PARs have been cloned: PAR-3, activated by thrombin (Ishihara *et al*., 1997), and PAR-4 (Kahn *et al*., 1998; Xu *et al*., 1998), activated by both trypsin and thrombin. Unlike other receptors, PAR activation involves the proteolytic unmasking of a cryptic N-terminal sequence, which remains tethered and acts as a ligand binding to the extracellular domain (Coughlin *et al*., 1992; Dery *et al*., 1998; Hollenberg & Compton, 2002).

PAR-1, PAR-2 and PAR-4 can be activated by synthetic peptide sequences (SFLLRN-NH_2, SLIGKV-NH_2, SLIGRL-NH_2 and GYPGQV-NH_2, respectively), which correspond to the reported tethered ligand sequence of each receptor (Molino *et al*., 1997; Dery *et al*., 1998). Furthermore, a novel PAR-4 agonist peptide, AYPGKF-NH_2, has been demonstrated to be more potent and specific than those peptides with the same sequence of the natural amino terminus (Faruqi *et al*., 2000). Thrombin, apart from its widely recognised role as a coagulant via the proteolytic cleavage of fibrinogen, through PAR activation stimulates platelet aggregation, and regulates vascular contractility (Coughlin *et al*., 1992). Thrombin and SFLLRN-NH_2 mediate a substantial endothelium-dependent relaxation of aortic and coronary blood vessels from different experimental animals, including rats (Hamilton *et al*., 2001a), guinea pigs (Muramatsu *et al*., 1992) and dogs (Ku & Zaleski, 1993; Tesfamariam, 1994) as well as human pulmonary arteries (Hamilton *et al*., 2001b). However, following removal of the endothelium, thrombin generates a robust contractile response in dog (Ku & Zaleski, 1993) and human coronary arteries (Ku & Dai, 1997). Trypsin and PAR-2 activating peptide cause an endothelium-dependent relaxation of isolated vessels (Saifeddine *et al*., 1996) from rats (Al-Ani *et al*., 1995), rabbits (Roy *et al*., 1998) and pigs (Hamilton *et al*., 1998; Hwa *et al*., 1996; Sobey & Cocks, 1998; Sobey *et al*., 1999).

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In human vessels, the role of thrombin and PAR-1 has been studied in the coronary arteries (Ku & Dai, 1997; Hamilton *et al*., 1998), whereas information on other vascular tissues is absent. In the present study, we have investigated the motor function of three of the four known PARs in human renal artery, by using PAR-1, PAR-2 and PAR-4 agonists under basal conditions or after precontraction with phenylephrine. Only stimulation of PAR-1 resulted in a robust contraction of the human renal artery, an effect that occurred even in the presence of a functional endothelium. With specific antisera directed against the human form of PAR-1, we have localised the receptor in the different layers of the human renal artery.

Methods

Tissue preparation

Histologically normal sections of the main branch of the renal artery were obtained from 29 individuals (35 – 74 years), undergoing nephrectomy for adenocarcinoma or transitional cell carcinoma, and were immediately transferred to cold oxygenated Krebs solution and experimental procedures began within 40 min. Excised kidneys were functional and of normal size. Biochemical functions were normal and renal ultrasound showed no evidence of hydronephrosis. All patients gave their informed consent prior to surgery and the Ethical Committee of the University of Ferrara approved the present study.

Organ bath studies

Human renal arterial rings (2 – 3 mm) were used either intact or after mechanical removal of the endothelium and mounted in an organ bath (5 ml) and bathed at 37°C in a gassed (95% O_2 5% CO_2) Krebs buffer (pH 7.4) solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10. Tissues were equilibrated for 90 min before the beginning of the experiments and the tension ($1.5 \times g$) was monitored by means of an isometric transducer (Basile, Comerio, Italy), and continuously adjusted during this time. The presence of a functional endothelium or efficacy of its removal was checked by testing the relaxant response to acetylcholine (1 mM) in preparations precontracted with phenylephrine (10 μM). Relaxation produced by acetylcholine in tissues with and without endothelium was 221 ± 35 mg ($n = 6$) and 32 ± 12 mg ($n = 6$, $P < 0.01$), respectively. Cumulative concentration – response curves were performed in basal or in phenylephrine (10 μM) precontracted tissues by adding a higher concentration as soon as the effect of the previous concentration had reached a plateau. To produce the noncumulative concentration – response curve, a single concentration was added at one time, and once the contractile response had reached a plateau, the tissue was washed and allowed to stabilise for 30 min before another concentration was added. In these experiments, only three different concentrations of the PAR agonists were tested in each tissue. In all samples, phenylephrine (10 μM) was administered prior to the challenge with PAR agonists to test viability and establish the maximum contraction of the tissues.

Cumulative concentration – response curves to human thrombin (0.1 – 1 nM), trypsin (0.1 – 1 nM), PAR-1 (SFLLRN-NH_2), PAR-2 (SLIGKV-NH_2) or PAR-4 (GYPGQV-NH_2) activating peptide (1 – 100 μM) and the reverse peptides for PAR-1 (NRLLFS-NH_2), PAR-2 (VKGILS-NH_2) and PAR-4 (VQGPYG-NH_2) were constructed. Additionally, the more selective PAR-1, PAR-2 and PAR-4 agonists (TFLLR-NH_2, SLIGRL-NH_2 and AYPGKF-NH_2, respectively) were also tested (1 – 100 μM) in a noncumulative manner. Contractions to PAR agonists were expressed as a percentage of the contraction produced by phenylephrine (10 μM). In another set of experiments, production of nitric oxide (NO) was inhibited with the NO synthase inhibitor (N^G-nitro-L-arginine methyl ester (L-NAME), 100 μM), or prostanoid production was inhibited with indomethacin (5 μM). These inhibitors were added to the bath 20 or 45 min prior to the administration of PAR agonists, respectively. Finally, to test homologous desensitisation of PAR-1, some tissues were challenged with the PAR-1 activating peptide (SFLLRN-NH_2) or its vehicle (0.9% saline) for three consecutive times (without washing) and then challenged with thrombin.

Immunohistochemistry

Human renal arteries were placed immediately into 10% neutral buffered formalin (48 h). Tissues were trimmed and processed for paraffin embedding according to conventional methods (D'Andrea *et al*., 1998, 2000). Briefly, tissue sections (5 μm) on microscopic slides were dewaxed, rehydrated and microwaved in target buffer, washed in phosphate-buffered saline (PBS, pH 7.4) and treated with 3.0% H_2O_2 for 10 min. Tissues were incubated with normal blocking serum for 10 min. After a brief rinse in PBS, sections were treated with polyclonal antibody (1 μg ml^{-1}) for PAR-1 (LLSSR VPMSQOESERC, Smith-Swintosky *et al*., 1997). The following antibodies were also used to characterise the vessels: smooth muscle actin, proliferating cell nuclear protein and the von Willebrand factor VIII. Slides were then washed in PBS and treated with biotinylated goat anti-rabbit antibodies, and avidin – biotin – horseradish peroxidase complex reagent. All incubations (30 min) and washes were performed at room temperature. Slides were washed and treated with 3,3′-diaminobenzidine twice for 5 min, rinsed in dH_2O and counterstained with haematoxylin. Preabsorption controls were prepared using 10-fold titre excess of PAR-1 antigen preincubated with antibody overnight at 4°C. This mixture was then placed on the slides as the primary antibodies.

Materials

The following were used: antibodies (proliferating cell nuclear protein, smooth muscle actin and the von Willebrand factor VIII), target buffer (DAKO, Carpenturia, CA, U.S.A.); avidin – biotin – horseradish peroxidase complex reagent, biotinylated goat anti-rabbit antibodies, blocking serum (Vector Labs, Burlingham, CA, U.S.A.); polyclonal antibody for PAR-1 (Spring House, PA, U.S.A.); 3,3′-diaminobenzidine (Biomeda, Foster City, CA, U.S.A.); trypsin ($\geqslant$10,350 BAEE units mg^{-1} protein, from bovine pancreas code TRL; Worthington Biochemical Co., Freehold, NJ, U.S.A.); thrombin (1000 NIH units mg^{-1} protein, from human plasma Cat. No. T7009), indomethacin, L-NAME (Sigma Chemical Co., St Louis, MO, U.S.A.). Human PAR-1 activating peptide (SFLLRN-NH_2 and TFLLR-NH_2), reverse PAR-1 peptide (NRLLFS-NH_2), PAR-2 activating peptide (SLIGKV-NH_2), reverse PAR-2 peptide

(VKGILS-NH_2), PAR-4 activating peptides (GYPGQV-NH_2 and AYPGKF-NH_2) and reverse PAR-4 peptide (VQGPYG-NH_2) and the mouse PAR-1 activating (SLIGRL-NH_2) peptide were synthesised at the Laboratory of Pharmaceutical Sciences of the University of Ferrara.

Statistical analysis

All values are expressed as mean ± s.e.m. Statistical analysis was performed by the Student's *t*-test for unpaired data or with the one-way analysis of variance (ANOVA) and the Dunnett's test for multiple comparisons when necessary. In all cases, a value of $P<0.05$ was considered significant.

Results

Organ bath studies

All the tissues studied responded to phenylephrine (10 μM) with a contractile response that was 423 ± 39 mg ($n=29$). In 95% of endothelium-intact and non precontracted rings of human renal artery, increasing concentrations of thrombin resulted in a concentration-dependent contraction. Maximum contraction obtained with thrombin (100 nM) was 63 ± 9% of phenylephrine ($n=9$) (Figures 1 and 2). The human PAR-1 activating peptide (SFLLRN-NH_2, 1 – 100 μM) also caused a concentration-dependent contraction. Maximum contraction by SFLLRN-NH_2 (100 μM) was 61 ± 8% of phenylephrine ($n=8$) (Figures 1 and 2). No significant difference was observed between the contractile response performed with SFLLRN-NH_2 administered cumulatively and noncumulatively (Figure 3a). In contrast, the human PAR-1 reverse peptide (NRLLFS-NH_2) even at the highest concentration (100 μM) caused only a negligible response (Figures 1, 2 and 3b). In isolated rings of human renal artery, trypsin and the human PAR-2 activating peptide caused a small (Figure 2) contractile response at the highest concentrations tested (0.1 and 10 μM, respectively). In comparison, the PAR-4 activating peptide given cumulatively (1 – 100 μM, Figure 2) was unable to cause any significant variation of baseline tension. In another set of experiments, response to a single concentration of agonists (100 μM) was studied. In addition to SFLLRN-NH_2, only the other PAR-1 agonist, TFLLR-NH_2, produced a significant contractile response that was not significantly different from that of SFLLRN-NH_2 (Figure 3b). Activating peptides for PAR-2 and PAR-4 produced minor responses that were not significantly different from that produced by the PAR-1 reverse peptide (Figure 3b), and for this reason we considered that these responses were not receptor mediated.

The contractile response to thrombin and PAR-1 activating peptide was not affected by removal of a functional endothelium (Figure 4a and b) or by the presence of the NO synthase inhibitor, L-NAME (100 μM). In contrast, the response to PAR-1 agonists was partially, although

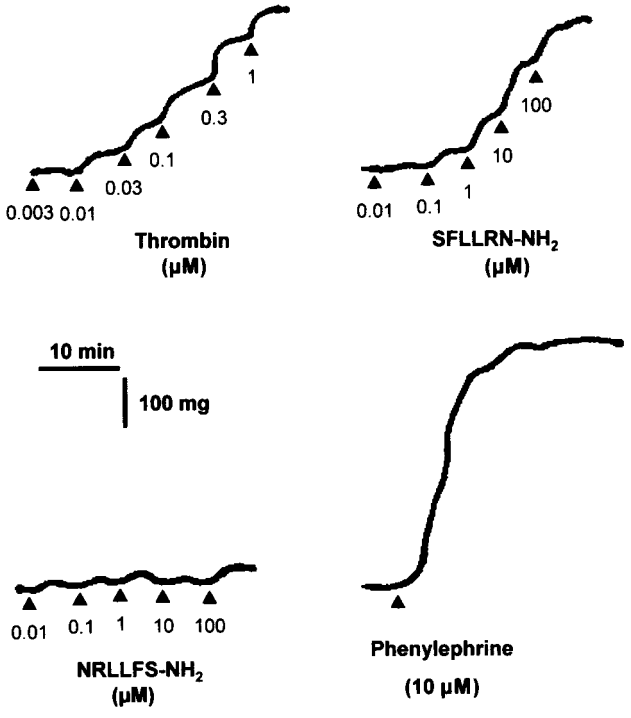

Figure 1 Typical tracings of the motor effect of thrombin, the PAR-1 (SFLLRN-NH_2) activating peptide, the PAR-1 (NRLLFS-NH_2) reverse peptide and phenylephrine in isolated rings of the human renal artery.

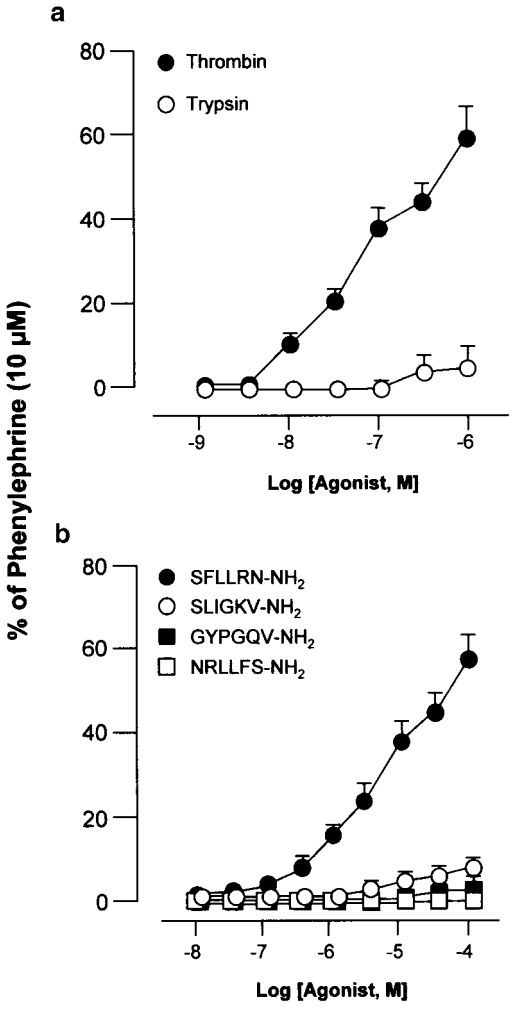

Figure 2 Concentration-dependent motor responses to (a) thrombin and trypsin and (b) PAR-1 (SFLLRN-NH_2), PAR-2 (SLIGKV-NH_2) and PAR-4 (GYPGQV-NH_2) activating peptides and PAR-1 (NRLLFS-NH_2) reverse peptide in isolated rings of human renal artery. Each entry is the mean ± s.e.m. of at least five experiments.

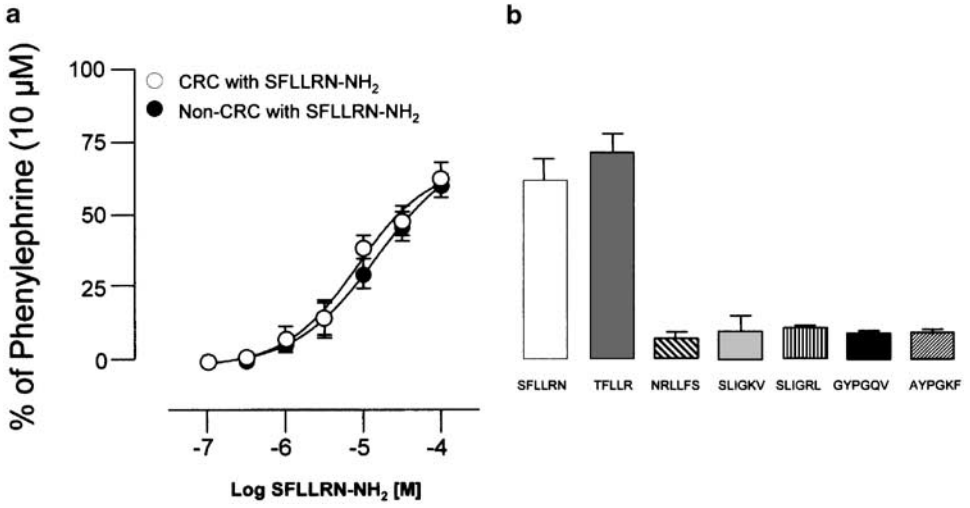

Figure 3 Concentration-dependent motor responses to (a) PAR-1 (SFLLRN-NH_2) activating peptide administered cumulatively (CRC) and noncumulatively (Non-CRC, washing between each administration of agonist) and (b) a single administration (100 μM) of PAR-1 (SFLLRN-NH_2 and TFLLR-NH_2), PAR-2 (SLIGKV-NH_2 and SLIGRL-NH_2) and PAR-4 (GYPGQV-NH_2 and AYPGKF-NH_2) activating and PAR-1 (NRLLFS-NH_2) reverse peptides in isolated rings of human renal artery. Each entry is the mean $\pm$ s.e.m. of at least five experiments.

significantly, decreased by the presence of indomethacin (Figure 4a and b). The contraction in response to thrombin was reduced by indomethacin also in endothelium-denuded preparations (data not shown). Phenylephrine (10 μM) produced a sustained contractile response for at least 20 min. In endothelial-intact tissues precontracted with phenylephrine, thrombin and SFLLRN-NH_2 caused a contractile response that was superimposed to the contraction caused by phenylephrine (data not shown). In these precontracted tissues, neither trypsin nor the activating peptides for PAR-2 and PAR-4 caused any appreciable effect on isolated rings of endothelial-intact human renal artery (data not shown).

In the study of homologous desensitisation of PAR-1, at the third consecutive administration (without washing), the contractile response to SFLLRN-NH_2 (100 μM) in isolated rings of human renal artery was significantly reduced from the response obtained at the first challenge (8 $\pm$ 2% of the response obtained at the first administration), indicating desensitisation (Table 1). Following this procedure of desensitisation, the response to thrombin was decreased to 9.5 $\pm$ 3% of the response to thrombin obtained in preparations challenged three times with the vehicle (0.9% saline) of SFLLRN-NH_2 (Table 1). In preparations challenged three times with SFLLRN-NH_2, the response to phenylephrine (10 μM) was similar to the response obtained in tissues challenged three times with the vehicle of SFLLRN-NH_2 (data not shown).

Immunohistochemistry

Immunohistochemical analysis was performed on the renal vessels. PAR-1-positive immunohistochemical labelling was

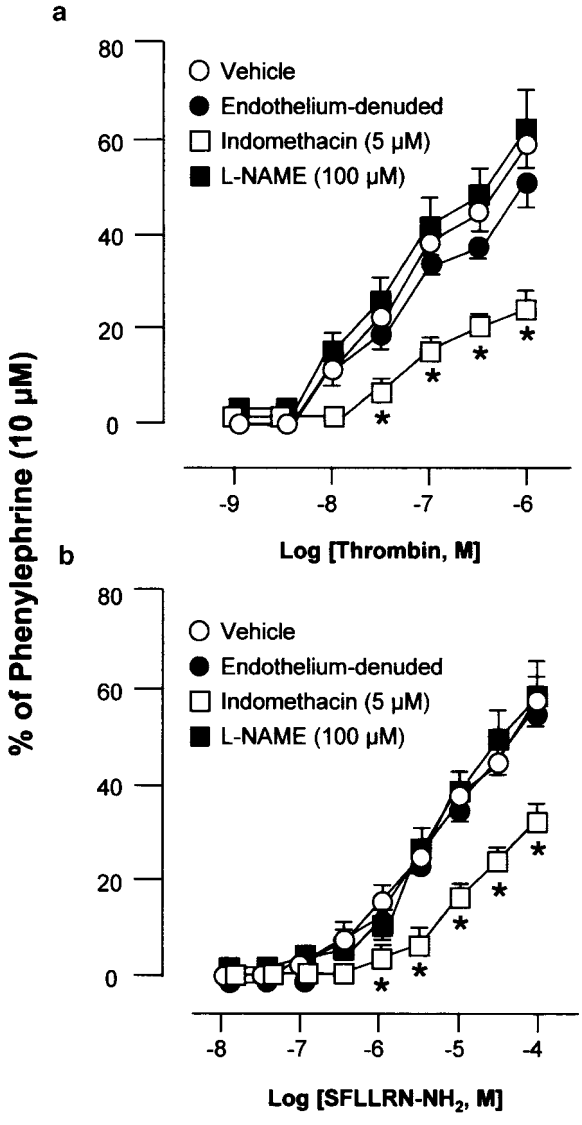

Figure 4 Concentration-dependent motor responses to (a) thrombin and (b) PAR-1 activating peptide (SFLLRN-NH_2) in isolated rings of human renal artery. Experiments were performed in preparations pretreated with indomethacin, L-NAME or their respective vehicles (Vehicle) or in endothelial-denuded preparations. Each entry is the mean $\pm$ s.e.m. of at least six experiments. *$P<0.05$ vs vehicle.

Table 1 Contractile response (% phenylephrine (PE) 10 μM) to SFLLRN-NH_2 or thrombin in isolated rings of human renal artery

Additions	*Agonist*	*% PE*	*Additions*	*%PE*
I	SFLLRN-NH_2 (100 μM)	60.0 $\pm$ 9.0	Vehicle (0.9% saline)	ND
II	SFLLRN-NH_2 (100 μM)	29.0 $\pm$ 5.0	Vehicle (0.9% saline)	ND
III	SFLLRN-NH_2 (100 μM)	4.6 $\pm$ 2.0*	Vehicle (0.9% saline)	ND
IV	Thrombin (100 nM)	5.8 $\pm$ 2.5*,**	Thrombin (100 nM)	63.8 $\pm$ 11.0

Administrations (I–III) of SFLLRN-NH_2 or its vehicle were performed at 20 min intervals without washing. At 20 min after the last administration of SFLLRN-NH_2, thrombin was added. *$P<0.05$ vs I-SFLLRN-NH_2. **$P<0.05$ vs thrombin after vehicle. ND, no response was detected.

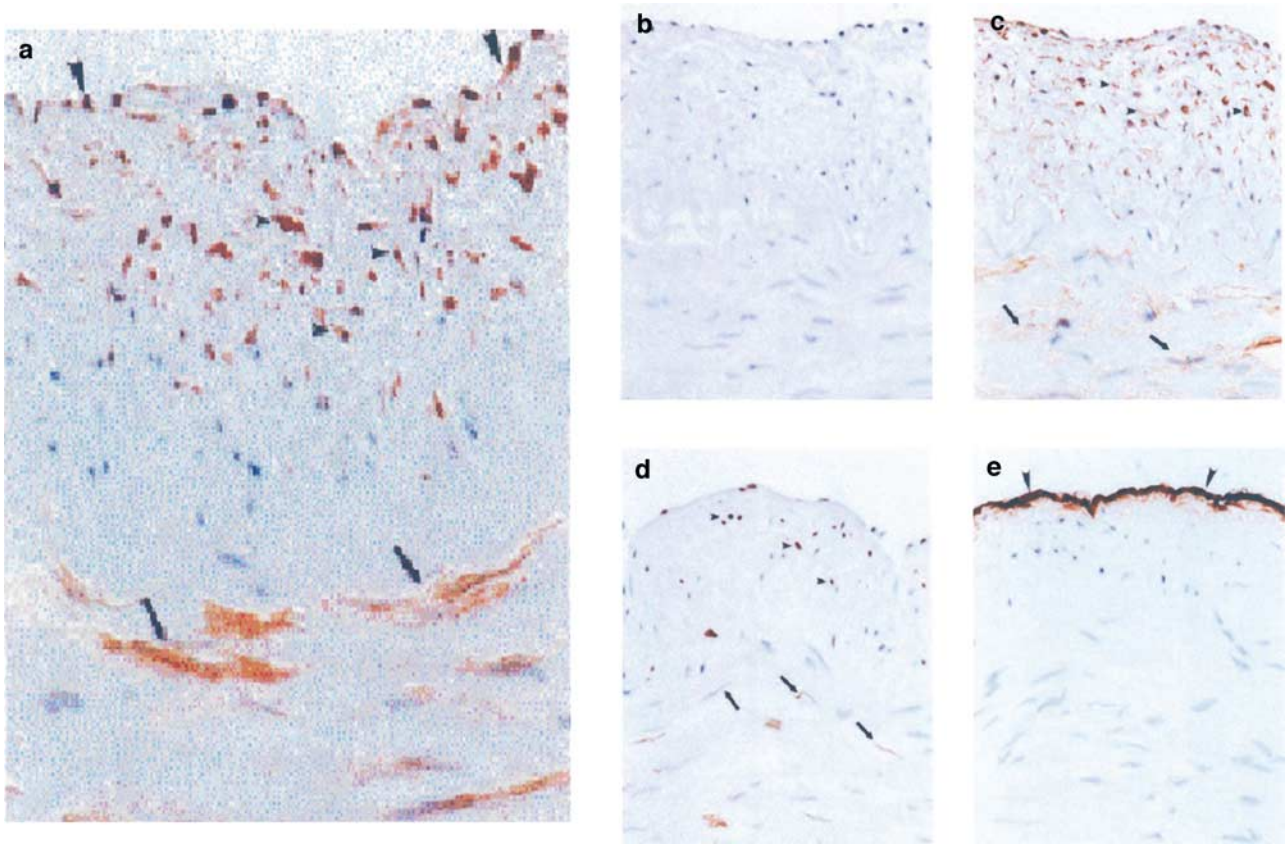

Figure 5 Application of immunohistochemistry to localise various proteins in human renal arteries using the following primary antibodies: PAR-1 (a), smooth muscle actin (c), proliferating cell nuclear antigen (d), von Willebrand factor VIII (e). Immunolabelling was absent after preabsorption of the PAR-1 antiserum with the PAR-1 antigen (b). Arrows indicate labelling in the media, small arrowheads indicate labelling in the neointima, and large arrowheads indicate labelling in the endothelial cells. Magnification $\times$ 600.

presented by brown staining. Preabsorption with an excess of PAR-1 antigen did not produce any detectable immunolabelling (Figure 5b). Prominent smooth muscle actin immunolabelling was observed in the media (arrows) and neointima (small arrowheads) of the vessels (Figure 5c). As presented in Figure 5d, serial tissue sections determined that the smooth muscle cells in the neointima (small arrowheads) and media (arrows) consist of PCNA-positive cells, as previously described (Damiano *et al*., 1999). Positive von Willebrand factor VIII immunolabelling (large arrowheads) was only observed along the leading lumenal edge of the neointima (Figure 5e) suggesting the presence of an endothelium.

Prominent PAR-1 immunolabelling (small arrowheads) was observed in the smooth muscle cells of the neointima (Figure 5a) and in the smooth muscle cells of the media (arrows). PAR-1 immunolabelling was also present along the lumenal edge cells (large arrowheads), which may be endothelial cells based on the von Willebrand factor VIII immunolabelling (Figure 5e). PAR-1 immunolabelling was less intense and diffuse than the von Willebrand factor VIII immunolabelling. A possible explanation for this discrepancy derives from the observation that at higher magnification not all the endothelial cells showed PAR-1 immunolabelling.

Discussion

Proteases via their role as signalling molecules have been recognised as important mediators for the control of vascular

resistance in experimental animals (Hollenberg, 1996; Hwa *et al*., 1996; Saifeddine *et al*., 1996; Hamilton & Cocks, 2000). However, minimal information is available regarding the role of PARs in the regulation of vascular tone in man (Hamilton *et al*., 1998; 2001a, b). The renal artery contributes significantly in the cardiovascular homeostasis since renal blood flow is a critical factor for maintenance of kidney function. For these reasons, we considered the importance of the study of the motor functions of the four known PARs in the human renal artery and the precise localisation of those PARs that produce a motor response in this tissue.

We found that thrombin produced a robust contraction of isolated rings of human renal artery. The possibility that this response was mediated by PAR-1 is indicated by the following findings. Firstly, administration of the PAR-1 activating peptides (SFLLRN-NH_2 and TFLLRN-NH_2) resulted in a marked and concentration-dependent contraction of this tissue. The observation that the concentration – response curve to SFLLRN-NH_2 was shifted to the right approximately by two logarithmic units as compared to that of thrombin suggests that the potency of SFLLRN-NH_2 is about 100 times lower than that of the protease, consistent with previous literature (Hollenberg, 1996; Dery *et al*., 1998).

Secondly, activating peptides with different levels of potencies for PAR-2 and PAR-4 did not show any significant activity in the isolated human renal artery. These findings, coupled to the observation that trypsin, an agonist for both PAR-2 and PAR-4, was only weakly active at high and probably nonselective concentrations (above 0.1 nM), excluded any possible role for these two receptors in the contraction induced by thrombin and PAR-1 activating peptides.

Following desensitisation to the PAR-1 activating peptide, the contractile response to thrombin was negligible, whereas the response to phenylephrine was unaffected. Since it is unlikely that the PAR-1 activating peptide causes activation/desensitisation of PAR-3 (Kinlough-Rathbone *et al*., 1993; Connolly *et al*., 1996), the most parsimonious explanation for the present results indicates that the abolition of the response to thrombin is the consequence of PAR-1 desensitisation. This implies that thrombin-induced contraction of the human renal artery results from PAR-1 activation.

There is abundant evidence that relaxant responses produced by PARs in different vessels are mediated by endothelium-derived relaxing factors (Laniyonu & Hollenberg, 1995; Hwa *et al*., 1996; Saifeddine *et al*., 1996; Hamilton *et al*., 1998). There is also evidence that contraction produced by PARs can be mediated by endothelial factors (Roy *et al*., 1998; Derkach *et al*., 2000). However, the endothelium does not seem to play a significant role in the present preparation, as the contractile responses to PAR-1 agonists were unchanged by the removal of a functional endothelium. In agreement with these findings, inhibition of the release of the major endothelium-derived relaxing agent, NO, did not affect the contraction of PAR-1 agonists. The present results are somewhat surprising because the expression of PAR-1 in the human renal artery was not exclusively confined to the smooth muscle cells, but was also found on endothelial cells. However, there are additional examples of human vessels, including the human umbilical and placental arteries, where a contractile component of the motor response to thrombin prevailed even in the presence of the endothelium (Tay-Uyboco *et al*., 1995). Similar findings have also been reported in mouse renal artery, in which the PAR-1 activating peptide elicited contraction both in the absence and presence of the NO synthase inhibitor L-NOARG (Moffatt & Cocks, 1998). Less recent observation showed that thrombin causes contraction of the canine basilar artery (White *et al*., 1980) and the rabbit thoracic aorta even in the presence of the endothelium (Walz *et al*., 1985). There is evidence suggesting that these effects of thrombin are mediated by PAR-1 activation (Laniyonu & Hollenberg, 1995).

Cyclooxygenase inhibition caused a significant attenuation of the contraction of the human renal artery produced by both thrombin and SFLLRN-NH_2. Since the effect of indomethacin was also found in endothelium-denuded preparations, the source of the contractile prostanoids released upon PAR-1 activation may therefore not be the endothelial cells, but rather other cells of the vessel wall, including the smooth muscle cells themselves. The possibility that contractile prostanoids are released by PAR activation has been already shown in the rat stomach (Saifeddine *et al*., 1996). The lack of potentiation of the contractile response to PAR-1 agonists by indomethacin and L-NAME is in agreement with the observation that PAR-1 activation did not result in any appreciable relaxant effect in human renal artery rings in resting conditions and even after precontraction with phenylephrine.

Present findings showing the expression and motor functions of PAR-1 in the human renal artery underline a possible role of this receptor in renal vascular homeostasis in man. However, it should be emphasised that the effect of PAR-1 activation in the conductance vessels tested in the present study may or may not reflect the function of this receptor in resistance vessels of the kidney. The current research for PAR-1 antagonists focuses on the inhibition of thrombin-induced platelet, their aggregation and the consequent thrombus formation. Our study adds a further possible potential beneficial effect to those predicted for PAR-1 antagonists. These antagonists could be of importance to maintain an adequate blood supply to the kidney during pathological conditions if elevated concentrations of thrombin are released in the bloodstream. The sole predictable role of PAR-1 antagonists as vasodilators of the human renal artery is underlined by the fact that, apparently, thrombin does not produce any endothelium-dependent relaxation in this tissue.

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