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SPECIAL REPORT

Endothelium-dependent and -independent responses to protease-activated receptor-2 (PAR-2) activation in mouse isolated renal arteries

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Protease-activated receptors (PARs) are receptors which require proteolytic cleavage to be self-activated by newly exposed N-terminal 'tethered ligands', and hence serve as sensors for protelytic enzymes. While both the thrombin receptor (PAR-1) and PAR-2 (activated by tryptic enzymes) have been shown to mediate endothelium-dependent vasorelaxation, only PAR-1 has been shown to cause direct vascular smooth muscle contraction. In this study, we report that trypsin and the PAR-2 selective peptide ligand SLIGRL-NH_2 not only caused endothelium-dependent relaxation of mouse renal arteries but also direct smooth muscle contraction if endothelial nitric oxide synthase was inhibited or if the endothelium was removed.

Keywords: Protease-activated receptor-2 (PAR-2); thrombin receptor; mouse renal artery; endothelium-dependent relaxation

Introduction Protease-activated receptors (PARs) are unique members of the seven transmembrane-spanning, G-protein-coupled receptor superfamily. Unlike other receptors, PARs can be self-activated by a peptide sequence upstream from the extracellular N-terminus of the molecule which is otherwise prevented from intramolecular binding by a downstream blocking peptide sequence (Coughlin *et al*., 1992). After specific proteolytic cleavage of the terminal peptide sequence, however, the new 'tethered-ligand' sequence is exposed, activating the receptor. The first PAR receptor was discovered after a lengthy search for a molecule which could account for the apparently receptor-mediated effects of the haemostatic serine protease thrombin (thrombin receptor, or PAR-1; Coughlin *et al*., 1992). 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 similar enzymes (e.g. mast cell tryptase (Mirza *et al*., 1997) and coagulation factor Xa (Fox *et al*., 1997)). Both PAR-1 and PAR-2 can be activated by synthetic peptide sequences which correspond to the reported tethered ligand sequence of each molecule, despite the presence of their unexposed native ligand sequence (Molino *et al*., 1997).

Thrombin is recognized as both an endothelium-dependent vasodilator as well as a potent mediator of vascular smooth muscle proliferation (Coughlin *et al*., 1992). Therefore, thrombin exerts both acute (vasodilatation) and chronic (vascular remodelling) effects. Furthermore, PAR-1 have been shown to cause smooth muscle contraction if the inhibitory influence of the endothelium is mechanically or pharmacologically removed (Laniyonu & Hollenberg, 1995). Similarly, PAR-2 activation has been shown to cause aortic smooth muscle proliferation (Bono *et al*., 1997) in accordance with the immunohistochemical localization of PAR-2 in the media of a range of human arteries (D'Andrea *et al*., 1998). However, unlike PAR-1, PAR-2 which also mediate endothelium-dependent relaxation (Hamilton *et al*., 1998; Hwa *et al*., 1996; Saifeddine *et al*., 1996; Laniyonu & Hollenberg, 1995) have not been reported to mediate direct contraction of vascular smooth muscle, even though PAR-2 mRNA (Saifeddine *et al*., 1996) and PAR-2 immunoreactivity (D'Andrea *et al*., 1998) have been localized to both endothelial and smooth muscle cells. We reasoned that the choice of large conduit vessels as bioassay preparations (Hwa *et al*., 1996; Saifeddine *et al*., 1996; Laniyonu & Hollenberg, 1995) may influence the potential for PAR-2 to have acute, direct actions on vascular smooth muscle. In this report, we provide functional evidence that activation of smooth muscle PAR-2 mediates contraction of isolated mouse renal arteries.

Methods *Tissue preparation* Male BALB/c mice (6–8 weeks old) were killed by cervical dislocation and both the left and right renal arteries were quickly excised and placed in ice cold modified Krebs' solution (composition (mM): Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, $SO4^{2-}$ 1.2, $H_2PO_4^-$ 1.2 and glucose 11.0). In order to remove the endothelium in some experiments, the renal arteries were excised after flushing the arterial tree (*via* a left ventricular cannula) with Krebs' containing Triton X-100 (0.1%) for 2 min, followed by a 2 min washout with Krebs' solution. In each case, histological examination confirmed complete removal of the endothelium with this technique. Each artery (1-.5–2 mm in length) was mounted on two 40 μm stainless steel wires in a Mulvany-Halpern-style myograph as previously described (Angus *et al*., 1988). After a 30 min equilibration period, during which the preparations were under no passive tension, a passive length-tension curve was constructed by applying cumulative stretches to the preparation. From this curve, equivalent transmural pressures were estimated and the vessel set at a level of tension equivalent to 90% of the diameter of the vessel at a distending pressure of 100 -mmHg (see Angus *et al*., 1988). After a further 30 min equilibration period, the preparations were contracted with a maximum concentration of phenylephrine (30 μM) in order to estimate the maximum active force developed (F_{max}) by each preparation. In five preliminary experiments, the preparations developed high spontaneous tone and phasic activity before and after washout of phenylephrine. In these preparations, spontaneous tone and activity was abolished by nifedipine

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(1 μM), which had no effect on the responsiveness of the preparations to the thromboxane mimetic U46619 used to contract preparations. Hence, in all experiments, nifedipine (1 μM) was added to the bath after washout of phenylephrine in order to control both spontaneous tone and activity.

Experimental protocol Thirty min after washout of phenylephrine, preparations were contracted to 30–50% of F_{max} with the thromboxane A_2 mimetic U46619 (1–10 nM). Cumulative concentration-response curves to trypsin (0.01–3.0 U ml^{-1}) and the PAR-1 and PAR-2 peptide ligands (SFLLRN-NH_2 and SLIGRL-NH_2 respectively; 0.1–30 μM) were then constructed. At the end of the experiment sodium nitroprusside (SNP, 10 μM) was added to establish the maximum relaxation of the tissue. In some experiments, tissues were desensitized to SFLLRN-NH_2 or SLIGRL-NH_2 by adding 30 μM of the peptide sequentially (without washout) until the response was diminished to less than 5% of the initial response. Endothelial production of nitric oxide (NO) was inhibited with the NO synthase inhibitor N^G-nitro-L-arginine (L-NOARG, 100 μM) which was added to the bath 20 min before contraction of preparations with U46619.

Drugs The peptide ligand sequences SFLLRN-NH_2 and SLIGRL-NH_2 were custom synthesized by Auspep (Melbourne, Australia). The following other drugs were used: trypsin (Wothington Biochemical, NJ, U.S.A.), L-NOARG (Sigma, MO, U.S.A.), U46619 (9-11-dideoxy-9α-methanoepoxy-prostaglandin $F_{2\alpha}$, Sapphire Bioscience, Sydney, Australia), sodium nitroprusside (SNP; Bull Laboratories, Melbourne, Australia), phenylephrine (Sigma, MO, U.S.A.) and nifedipine (Biomol, PA, U.S.A.).

Analysis All responses are expressed as a percentage of the maximum relaxation to SNP (means ± s.e.mean). Where the effects of drugs on PAR-mediated responses were assessed, one artery was used as a time control. The assignment of left and right arteries to treatment or control groups was randomized. Calculation of pEC_{50}'s were performed by computer-assisted non-linear regression (allowing for different slopes) of individual concentration-response curves using GraphPad Prism v.2.0 (Kemp & Cocks, 1997). Differences between control and treatment groups were assessed using Students' *t*-test; $P<0.05$ was accepted as significant.

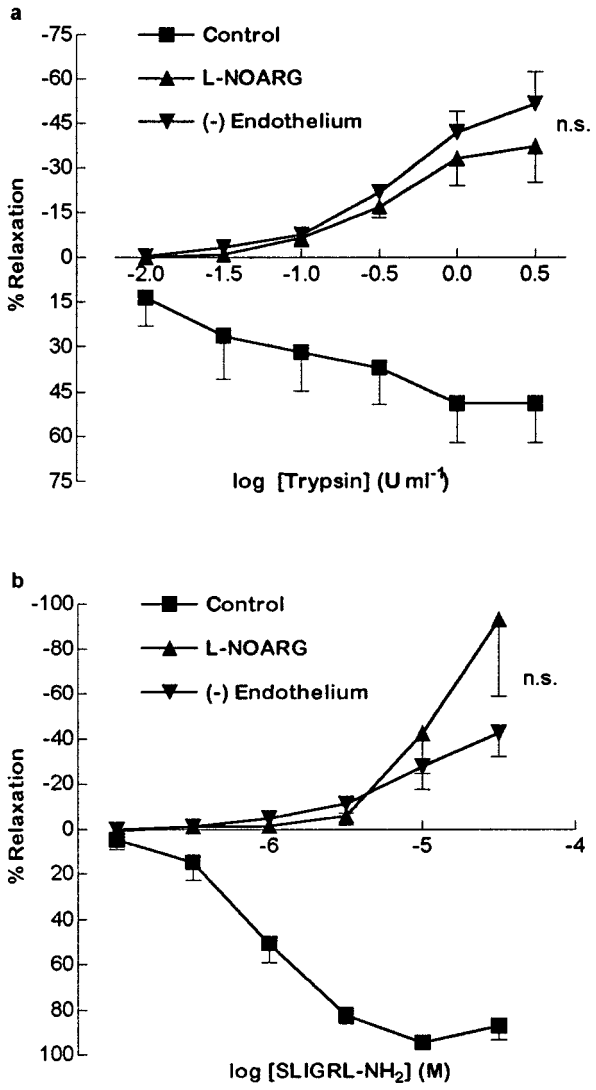

Figure 1 Cumulative concentration-response curves to (a) trypsin and (b) the PAR-2 peptide ligand SLIGRL-NH_2 in mouse isolated renal arteries contracted with U46619. The relaxations to trypsin and SLIGRL-NH_2 were reversed to contractions by L-NOARG (100 μM) or removal of the endothelium. n.s., no significant difference between maximum contractions in L-NOARG-treated or endothelium-denuded preparations.

Results *Effects of trypsin* Trypsin (0.01–3 U ml^{-1}) caused concentration-dependent relaxations with an EC_{50} of 0.15±0.11 U ml^{-1} and a maximum (R_{max}) of 86.93±6.47% ($n=6$; Figure 1a). In L-NOARG-pretreated preparations, however, trypsin caused only contraction ($EC_{50}=0.27\pm$ 0.11 U ml^{-1}; $n=6$; Figure 1a). Similarly, in preparations in which the endothelium had been removed, trypsin caused only contractions ($EC_{50}=0.40\pm0.11$ U ml^{-1}; $n=3$; Figure 1a).

Effects of PAR ligand peptides The PAR-2 tethered ligand sequence, SLIGRL-NH_2, elicited concentration-dependent relaxations (Figure 1b) with a pEC_{50} of 6.08±0.11 ($R_{max}=86.93\pm6.46\%$; $n=6$). In the presence of L-NOARG (100 μM), however, SLIGRL-NH_2 caused contractions ($n=6$; Figure 1b). In contrast, the PAR-1 tethered ligand sequence SLFFRN-NH_2 elicited contractions, regardless of whether NOARG was present or not (pEC_{50} 4.82±0.69; $n=6$; data not shown). In two preparations, however, SFLLRN-NH_2 elicited biphasic responses consisting of small transient relaxations followed by sustained contractions.

Effect of desensitization to SFLLRN-NH_2 or SLIGRL-NH_2 In three L-NOARG-treated preparations which had been desensitized to SFLLRN-NH_2, SLIGRL-NH_2 still elicited contractions (Figure 2a). Although it was not possible to complete full concentration-response curves for SLIGRL-NH_2 and hence determine pEC_{50} values, the maximum contraction to SLIGRL-NH_2 at 100 μM was significantly smaller following SFLLRN-NH_2 desensitization ($P=0.034$; unpaired *t*-test). By contrast, desensitization to SLIGRL-NH_2 did not abrogate the responses to SFLLRN-NH_2 ($n=4$, Figure 2b).

Discussion The endothelium-dependent vasodilator and direct vasoconstrictor effects of thrombin have been known for some time (Laniyonu & Hollenberg, 1995). Activation of vascular PAR-2, however, has only previously been reported

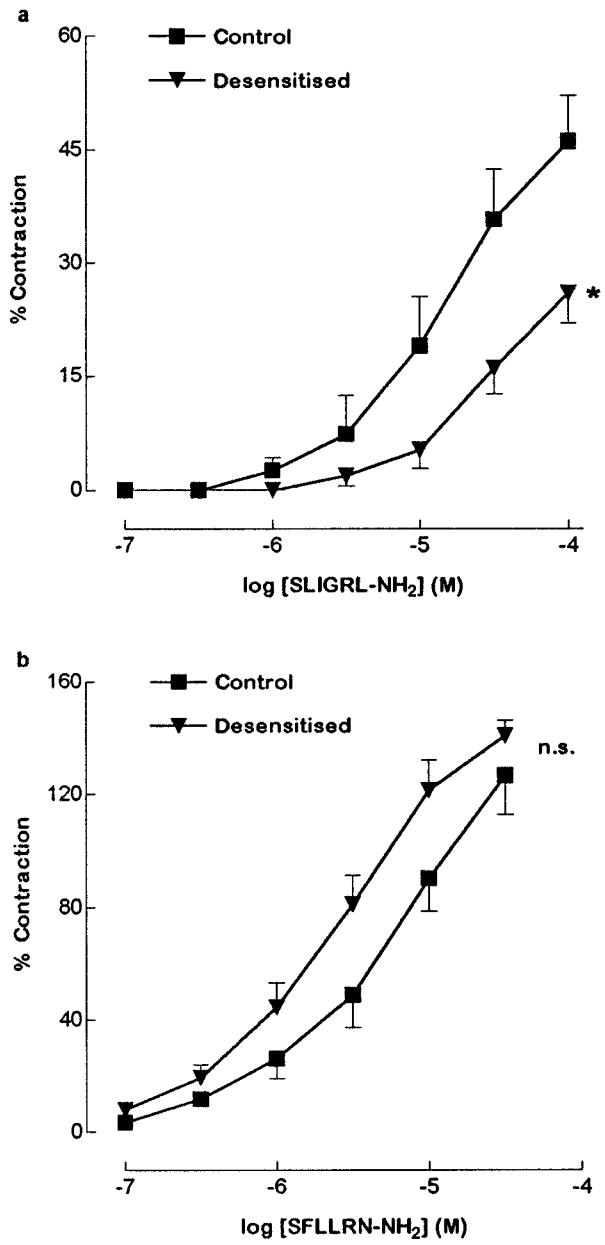

Figure 2 Cumulative concentration-response curves (in the presence of 100 μM L-NOARG) to (a) SLIGRL-NH_2 following desensitization to SFLLRN-NH_2 and (b) SFLLRN-NH_2 following desensitization with SLIGRL-NH_2. Asterisk indicates significant difference between maximum responses (*t*-test, $P<0.05$); n.s., no significant difference between maximum contractions.

to mediate endothelium-dependent vasorelaxation (Hwa *et al*., 1996; Saifeddine *et al*., 1996; Laniyonu & Hollenberg, 1995, Hamilton *et al*., 1998) and claimed to cause endothelin-dependent contraction (Roy *et al*., 1998). In this study, whilst we confirmed endothelium- and NO-dependent relaxation by PAR-2, our data indicate that PAR-2 are also located on the smooth muscle cells of mouse renal arteries and mediate c-ontraction.

Trypsin caused relaxation of precontracted arteries, a response reversed to contraction when endothelial NO synthesis was inhibited with L-NOARG as well as when the endothelium was removed. Both findings show that PAR-2 are located on both the endothelium (mediating NO synthesis) and smooth muscle (causing direct contraction) of mouse renal arteries. Further findings suggest that the effects of trypsin were mediated by PAR-2. Thus, the synthetic selective ligand sequence for PAR-2 (SLIGRL-NH_2) also elicited endothelium-dependent relaxation and endothelium-independent contraction. The possibility that SLIGRL-NH_2 may have activated PAR-1 at the higher concentrations required to elicit contraction was unlikely since desensitization of preparations to SFLLRN-NH_2 did not prevent SLIGRL-NH_2-mediated contractions. The rightward shift of the SLIGRL-NH_2 contraction curve following SFLLRN-NH_2 desensitization, however, agrees with previous studies which show that high concentrations of SFLLRN-NH_2 can activate PAR-2 (Blackhart *et al*., 1996). Furthermore, desensitization to SLIGRL-NH_2 had no effect on responses to SFLLRN-NH_2 in agreement with previous studies demonstrating that SLIGRL-NH_2 has no affinity for PAR-1 (Blackhart *et al*., 1996). Therefore, our results suggest that desensitization to SFLLRN-NH_2 also desensitized some of the PAR-2 present on smooth muscle and that contractile responses to SLIGRL-NH_2 most likely involved activation of PAR-2. It is also possible, however, that the receptor mediating contractions to SLIGRL-NH_2 differs slightly to PAR-2 (Roy *et al*., 1998). Furthermore, the affinity of the recently cloned PAR-4 (activated by both thrombin and trypsin; Xu *et al*., 1998) for SLIGRL-NH_2 is unknown. Therefore, further studies are required to determine if the receptor mediating contraction in mouse renal arteries is PAR-2, PAR-4 or perhaps an undescribed PAR. Our data to date, however, do not suggest that the receptor involved is not PAR-2.

Bono *et al*., (1997) demonstrated that activation of PAR-2 on isolated vascular smooth muscle cells initiated a mitogenic response which is in accordance with the accumulating evidence that G-protein-coupled receptors can be linked to mitogenic pathways (Malarkey *et al*., 1995). However, the mouse renal artery is the only preparation to date in which direct PAR-2-mediated vascular smooth muscle contraction has been reported. Since PAR-2 has been localized to smooth muscle in other blood vessels (Saifeddine *et al*., 1996; D'Andrea *et al*., 1998) but do not initiate contraction, these smooth muscle receptors most likely initiate mitogenesis without causing contraction. Our findings suggest that the simple localization of PAR-2 does not necessarily allow prediction of a physiological or pathophysiological response.

In conclusion, PAR-2 are present on both the endothelium and smooth muscle of mouse renal arteries, where they mediate indirect relaxation and direct contraction respectively. That smooth muscle PAR-2 activation can contract arteries is a novel and perhaps important finding, since the endogenous activator(s) of PAR-2 are uncertain. Mast cell tryptase is a large tetramer (Schwartz & Bradford, 1986) which is most likely to be released in the adventitia and hence contract arteries, whilst blood-borne Factor Xa is ordinarily confined to the circulation and is more likely to relax arteries *via* endothelium-derived factors like NO. We suggest that these two potential PAR-2 activating enzymes (Fox *et al*., 1997) may have opposite physiological effects during different pathophysiological situations. Therefore, PAR-2 may mediate appropriate vascular responses to diverse stimuli such as blood coagulation and mast cell activation.

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