Atypical Protease-Activated Receptor Mediates Endothelium-Dependent Relaxation of Human Coronary Arteries

Justin R. Hamilton, Paul B. Nguyen, Thomas M. Cocks

Abstract—Protease-activated receptors (PARs) are a family of G protein–coupled receptors activated by a tethered ligand sequence within the amino terminal that are revealed by site-specific proteolysis. The thrombin-sensitive PAR-1 and trypsin-activated PAR-2 mediate endothelium-dependent vascular relaxation in a number of species. Because both thrombin and trypsin-like enzymes have been implicated in coronary artery disease, the purpose of this study was to investigate whether similar receptors are present in human coronary arteries. Thrombin (0.001 to 0.1 U/mL) and trypsin (0.001 to 1 U/mL) caused concentration- and endothelium-dependent relaxations of human coronary artery ring segments suspended in organ chambers for isometric tension recording and contracted with the thromboxane A₂ mimic U46619. These relaxations were dependent on the catalytic activity of each enzyme and were inhibited by the NO synthase inhibitor N⁵-nitro-L-arginine (100 μmol/L) and the NO scavenger oxyhemoglobin (20 μmol/L). The synthetic PAR-1 tethered ligand sequence SFLLRN-NH₂ (0.01 to 10 μmol/L) also caused endothelium-dependent relaxation of U46619-contracted human coronary arteries; however, the equivalent PAR-2 ligand SLIGKV-NH₂ caused almost no relaxation. In addition, desensitization to either thrombin or trypsin resulted in cross-desensitization to the other enzyme but had only a minimal affect on the response to SFLLRN-NH₂. Therefore, we conclude that human coronary artery endothelial cells possess a PAR-1–like receptor that is potently activated by thrombin, trypsin, and SFLLRN-NH₂ to cause NO-mediated vascular relaxation. Once cleaved, this receptor is recycled in a truncated form, able to respond to exogenous application of only its tethered ligand sequence, suggesting the presence of another endogenous activator possibly acting independently of receptor cleavage. (Circ Res. 1998;82:1306-1311.)

Key Words: endothelium-dependent relaxation ■ human coronary artery ■ protease-activated receptor ■ thrombin ■ trypsin

Protease-activated receptors are a novel subclass of the superfamily of seven transmembrane domain, G protein–coupled receptors activated by site-specific, proteolytic cleavage of the amino-terminal exodomain to expose a neo-N-terminal (or tethered ligand), which self-activates the receptor.1–5 Interestingly, these receptors are also activated by synthetic peptides corresponding to their tethered ligand sequences.2,5,6 Three PARs have been cloned: 2 thrombin receptors (PAR-12 and PAR-37) and the trypsin-activated PAR-2.4,5 These receptors have been located in a variety of cell types, including platelets7,8 and gastric smooth muscle cells.9,10 In the vasculature, both thrombin and trypsin induce endothelium-dependent relaxation of pig coronary arteries11 and rat aortas10 via activation of PAR-1 and PAR-2, respectively, and it has been proposed that both receptors modulate vascular inflammatory responses.10,12 In the human vasculature, localization of PAR-1 mRNA has been reported in hepatic artery endothelial cells,13 and several studies have demonstrated thrombin-induced vasorelaxation in a variety of human vascular preparations.14–17 Furthermore, both PAR-1- and PAR-2–mediated calcium fluxes have been measured in transfected cell lines18 and umbilical vein endothelial cells.19,20 However, the present study indicates that endothelium-dependent relaxations of human coronary arteries induced by thrombin and trypsin are mimicked only by the synthetic PAR-1 tethered ligand sequence SFLLRN-NH₂ and are likely to occur via the same receptor. In addition, our findings suggest that once activated, this receptor is positioned in the membrane without its tethered ligand sequence, unable to respond to thrombin or trypsin, yet it maintains its responsiveness to SFLLRN-NH₂.

Materials and Methods

Preparation of Human Coronary Arteries

Left anterior descending, distal right, and middle right coronary arteries were dissected from the explanted hearts of 12 patients undergoing heart transplantation at the Alfred Hospital, Melbourne, Australia, after informed patient consent and approval from the Alfred Hospital and University of Melbourne Ethics Committees had been obtained. Eight patients were diagnosed with end-stage dilated...
cardiomyopathy, 3 with congenital septal defects, and 1 with ischemic heart disease.

Arteries, which were used only if they were macroscopically free of atheromatous plaques, were isolated immediately after explantation and transported to the laboratory in ice-cold Krebs solution (composition in mmol/L: Na\(^+\) 144, Cl\(^-\) 128.7, HCO\(_3\)\(^-\) 25.0, K\(^+\) 5.9, Ca\(^2+\) 2.5, Mg\(^2+\) 1.2, HPO\(_4\)\(^2-\) 1.2, SO\(_4\)\(^2-\) 1.2, and glucose 11, pH 7.4).

Three-millimeter-long ring segments, some with the endothelium removed by abrasion of the luminal surface with a Krebs solution—moistened filter paper taper, were mounted between two parallel stainless steel wire hooks in 30-mL organ baths containing Krebs solution maintained at 37°C and continuously bubbled with 95% O\(_2\) and 5% CO\(_2\). One hook was attached to a micrometer-adjustable support leg and the other to an isometric force transducer (model FT03C, Grass Instruments) to record changes in isometric circumferential force, which were amplified and displayed on flatbed chart recorders (W & W Scientific Instruments).

**Tissue Equilibration**

After a 60-minute equilibration period, artery ring preparations were stretched to 5 g passive force and allowed to recover for 30 minutes before again being stretched to 5 g. After another 30 minutes, tissues were exposed to an isotonic KPSS solution (composition in mmol/L: K\(^+\) 124.9, Cl\(^-\) 128.7, Na\(^+\) 25.0, HCO\(_3\)\(^-\) 25.0, Ca\(^2+\) 2.5, Mg\(^2+\) 1.2, SO\(_4\)\(^2-\) 1.2, HPO\(_4\)\(^2-\) 1.2, and glucose 6.1) to obtain a maximum contraction for each artery ring. The KPSS was then replaced with normal Krebs solution, and the tissues were allowed to return to their original passive-force level for 30 to 60 minutes. Nifedipine (0.3 mmol/L) and indomethacin (3 mmol/L) were added to all tissues to inhibit spontaneous contractile activity and prostanoiad release, respectively.

**Preparation of Pig Coronary Arteries**

Right coronary arteries were dissected from the hearts of Large White pigs (either sex, weighing 30 to 40 kg, freshly slaughtered at the local abattoir), and ring segments were prepared for isometric tension measurements and equilibrated as described for the human coronary arteries.

**Responses to PAR Activators**

Artery ring segments were contracted to ~50% KPSS\(_{max}\) with titrated concentrations of the thromboxane A\(_2\) mimetic U46619 (1 to 10 mmol/L). Once the U46619-induced contraction had reached a stable level, cumulative concentration-response curves to thrombin and trypsin (0.0001 to 1 U/mL) were generated in the absence or presence of a functional endothelium, soybean trypsin inhibitor (100 μmol/L), the endothelial NO synthase inhibitor L-NOARG (100 μmol/L), the NO scavenger HbO\(_2\) (20 μmol/L), or a combination of L-NOARG and HbO\(_2\). For comparison with non–PAR-mediated, endothelium-dependent relaxations, a similar protocol was carried out for bradykinin.

Cumulative concentration-response curves to the human PAR-1 tethered ligand sequence SFLLRN-NH\(_2\), the mouse PAR-2 tethered ligand sequence SLIGKV-NH\(_2\), and the mouse PAR-2 tethered ligand sequence SLIGRL-NH\(_2\) (0.01 to 100 μmol/L each) were generated in human coronary artery preparations in the absence or presence of a combination of the aminopeptidase A inhibitor amastatin (10 μmol/L), the aminopeptidase B inhibitor bestatin (10 μmol/L), and the endopeptidase inhibitor phosphoramidon (10 μmol/L). In addition, responses to SLIGKV-NH\(_2\) and SLIGRL-NH\(_2\) were recorded in U46619-contracted pig coronary artery preparations, which have been shown to contain both PAR-1 and PAR-2, to confirm the activity of the peptides.

All curves were generated in the presence of BSA (0.005%) to prevent protein adherence to the glass walls of the organ bath. At the completion of each curve, maximum endothelium-dependent and -independent relaxations for each ring segment were determined with the addition of substance P (3 mmol/L) and isoprenaline (1 μmol/L), respectively.

**Desensitization Experiments**

Tissues either were left untreated or were treated with cumulative additions of one of thrombin (0.1 U/mL) or trypsin (0.1 U/mL) every 30 minutes for 2 hours in the presence of BSA (0.005%). After this step, tissues were washed thoroughly with Krebs solution and contracted to ~50% KPSS\(_{max}\) with U46619. Tissues were then repeatedly exposed to the enzyme (0.1 U/mL) with which they had previously been treated until no further relaxation was observed. Importantly, tissues were washed with Krebs solution (containing an appropriate concentration of U46619 to maintain the precontraction) between treatments with each activating enzyme to ensure that receptor desensitization was not masked by occupation of the receptor by the tethered ligand sequence. Once desensitization was achieved, cross-desensitization was investigated by addition of the enzyme (0.1 U/mL) not used in the desensitization process. After this procedure, cumulative concentration-response curves to SFLLRN-NH\(_2\) were generated. Again, substance P (3 mmol/L) and isoprenaline (1 μmol/L) were then added to determine maximal endothelium-dependent and -independent relaxations, respectively.

**Chemicals**

Amastatin, bestatin, BSA, bradykinin triacetate, Hb (bovine plasma), indomethacin, (−)-isoprenaline, L-NOARG, phosphoramidon, soybean trypsin inhibitor, substance P (acetate salt), and α-thrombin (bovine serum) were obtained from Sigma Chemical Co. U46619 (9,11-dideoxy-9α,11β-epoxymethanoprostaglandin F\(_2\)α) and nifedipine were from Saffhire Bioscience. Trypsin (bovine pancreas) was from Worthington Biochemicals; the peptides SLIGRL-NH\(_2\), SLIGKV-NH\(_2\), and SFLLRN-NH\(_2\) were from Auspep; and PPCK was obtained from Calbiochem. PPCK-thrombin was prepared by adding excess PPCK (10-fold) to thrombin at room temperature for 15 minutes. Excess unbound PPCK was dialyzed overnight in distilled water at 4°C. Stock solutions of Hb (1 mmol/L) were dissolved in 0.9% NaCl and then reduced with Na\(_2\)S\(_2\)O\(_4\). Excess Na\(_2\)S\(_2\)O\(_4\) was removed by passing the solution through a Sephadex PD10 size-exclusion column. PPCK was dissolved in 1 mmol/L HCl. Stock solutions of nifedipine (10 mmol/L), U46619 (1 mmol/L), and phosphoramidon (10 mmol/L) were made in absolute ethanol, whereas those for indomethacin (100 mmol/L) and L-NOARG (100 mmol/L) were made in aqueous Na\(_2\)CO\(_3\) and NaHCO\(_3\), respectively. All subsequent dilutions of these drugs were made in distilled water, as were solutions of all other drugs.

**Statistical Analysis**

Concentration-response curves were normalized as the percent reversal of the U46619-induced contraction, and data were expressed as mean±SEM. Each normalized curve was fitted by computer to a sigmoidal regression curve (Graphpad Prism, Graphpad Software Inc) to generate values for median sensitivity (pEC\(_{50}\)). Differences in mean pEC\(_{50}\) and R\(_{max}\) were tested for significance by either unpaired Student’s t test or 1-way ANOVA with a Tukey-Kramer modified t statistic for multiple comparisons. In all cases, differences were considered significant at P<0.05.

**Results**

**Responses to Thrombin and Trypsin**

Thrombin (0.001 to 0.1 μmol/L) and trypsin (0.001 to 1 μmol/L) each caused rapid, enzyme activity–dependent relaxations of...
U46619-contracted human coronary artery ring segments, relaxations that were abolished on removal of the endothelium (Figure 1). Sensitivity (pEC_{50}; negative logarithm of the median concentration in units per milliliter) and R_{max} (percent contraction reversal) values for thrombin were 2.460.2 and 88.964.9%, respectively (n=6 from 6 patients). Relaxations to trypsin had an R_{max} similar to that of thrombin (88.162.9%) but a significantly decreased pEC_{50} (1.760.1) (P<0.05, n=6 from 6 patients).

Soybean trypsin inhibitor (10 μg/mL) abolished responses to trypsin but did not affect thrombin-induced relaxations (n=6 from 2 patients) (Figure 1), and PPACK-inactivated thrombin caused no response (n=6 from 3 patients) (Figure 1), indicating a requirement for proteolytic activity in the relaxations induced by both enzymes.

The endothelial NO synthase inhibitor L-NOARG (100 μmol/L) in combination with the NO scavenger HbO (20 μmol/L) significantly (P<0.05) decreased both the pEC_{50} and the R_{max} of thrombin (pEC_{50} 1.060.4, R_{max} 14.267.1%; n=5 from 5 patients) (Figure 2A) and of trypsin (pEC_{50} 1.360.2, R_{max} 17.2610.7%; n=5 from 5 patients) (Figure 2B). For both enzymes the effect of L-NOARG in combination with HbO on PAR-mediated relaxations was not significantly different from either HbO or L-NOARG alone (n=5 from 5 patients; data not shown). Also, the effect of these NO inhibitors on PAR-mediated responses was not different from their effect on relaxations to bradykinin (n=7 from 7 patients) (Figure 2C). Thus, as with other endothelium-dependent dilators of human coronary arteries, PAR-induced relaxations appear to be mediated predominantly by endothelial cell–derived NO.

Responses to Synthetic Tethered Ligand Sequences
The human PAR-1 tethered ligand sequence SFLLRN-NH_{2} also caused relaxation of U46619-contracted human coronary

![Figure 1. Top, Digitized traces of original chart recordings showing typical relaxations to thrombin (A) and trypsin (B) in human coronary artery ring preparations contracted to ~50% of their maximum contraction to 125 mmol/L KCl with U46619. Vertical calibration bar represents 1 g, and the horizontal time bar denotes 20 minutes before and 1 minute after the arrow. SP indicates substance P (3 nmol/L) and ISO, isoprenaline (1 μmol/L). Bottom, Group data from these experiments, showing mean concentration-response curves to thrombin (C) and trypsin (D) in endothelium-intact (open circle) and -denuded (filled circle) artery ring segments and in the presence of soybean trypsin inhibitor (open square). Responses to PPACK-thrombin are also shown (filled square). Relaxations are expressed as percent reversal of U46619 contraction and are mean±SEM from 5 to 7 separate experiments (patients).](http://circres.ahajournals.org/)

![Figure 2. Effect of inhibitors of NO on relaxations to thrombin (A), trypsin (B), and bradykinin (C) in human coronary artery ring preparations contracted to ~50% of their maximum contraction to 125 mmol/L KCl with U46619. Responses to each agonist were examined in control tissues (open circle) and tissues treated with a combination of L-NOARG (100 μmol/L) and HbO (20 μmol/L) (filled circle). Relaxations are expressed as percent reversal of U46619 contraction and are mean±SEM from 5 to 7 separate experiments (patients).](http://circres.ahajournals.org/)
Interestingly, the human PAR-2 tethered ligand sequence SLIGKV-NH$_2$ caused almost no relaxation ($R_{\text{max}}$ 14.5±7.1%; $n=10$ from 5 patients) (Figure 3A). In addition, the mouse PAR-2 tethered ligand sequence SLIGRL-NH$_2$, which has been shown to activate human PAR-2 with a potency equivalent to that of SLIGKV-NH$_2$, caused no relaxation of human artery preparations ($n=10$ from 5 patients; data not shown). Importantly, both SLIGKV-NH$_2$ and SLIGRL-NH$_2$ caused relaxation of porcine coronary artery rings, a preparation that has been shown to contain functional PAR-2, ($n=6$) (Figure 3B), indicating that the peptides were indeed active. The lack of peptide activity was unlikely to be due to tissue-stimulated enzymatic degradation, because responses to SLIGKV-NH$_2$ or SLIGRL-NH$_2$ were not observed in the presence of inhibitors of aminopeptidases A and B and endopeptidase-amastatin (10 μmol/L), bestatin (10 μmol/L), and phosphoramidon (10 μmol/L), respectively ($n=6$ from 2 patients; data not shown).

**Desensitization of PARs**

Desensitization to either thrombin or trypsin caused loss of responsiveness to maximum relaxation–inducing concentrations of both enzymes (Figure 4A and 4B), indicating that the receptor types involved are activated by either enzyme. Interestingly, under these desensitizing conditions, the $R_{\text{max}}$ to SFLLRN-NH$_2$ was unaffected (92.0±5.0), although there was a small but significant ($P<0.05$) decrease in pEC$_{50}$ (7.0±0.1 versus 6.4±0.2; $n=8$ from 4 patients) (Figure 4C).

**Discussion**

PAR-1 and PAR-2 mediate endothelium-dependent vascular relaxation in a number of species; however, there is limited evidence that this phenomenon occurs in the human vasculature. As has been shown in rat, pig, and dog vessels, responses to thrombin and trypsin were dependent on the catalytic activity of these serine proteases and were predominantly mediated by endothelium-derived NO, because relaxations were virtually abolished by a combination of L-NOARG–mediated inhibition of endothelial NO production and scavenging of residual NO by HbO. Bradykinin-induced relaxations were similarly inhibited with these agents, consistent with other reports that endothelium-dependent relaxation of human coronary vessels is mediated predominantly by NO.

In contrast to previous reports describing thrombin contraction of endothelium-denuded preparations of dog and pig coronary artery, neither enzyme induced contraction of endothelium-denuded human artery preparations in the present study. The lack of contraction to thrombin may be explained by the observation of Nelken et al that the mRNA for PAR-1 was...
present only in endothelial cells of normal, nonatherosclerotic human arteries. It will be of interest to see whether thrombin or the PAR-1 tethered ligand sequence causes contraction of endothelium-free atherosclerotic vessels, because in the same study Nelken et al also located PAR-1 mRNA in smooth muscle cells in the diseased state.

Interestingly, the present study demonstrates that the thrombin- and trypsin-induced endothelium-dependent relaxations of human coronary arteries can be mimicked by the synthetic PAR-1 tethered ligand sequence SFLLRN-NH$_2$, whereas the PAR-2 tethered ligand sequences SLIGKV-NH$_2$ (human) and SLIGRL-NH$_2$ (mouse) caused almost no response. Importantly, near-maximal responses to both of these peptides were obtained in pig coronary artery preparations (this study) and mouse fundus strips (T.M.C. et al, unpublished observations, 1998), both of which have been shown to contain functional PAR-1 and PAR-2. It is unlikely that the lack of activity of SLIGKV-NH$_2$ and SLIGRL-NH$_2$ in human coronary arteries was due to enzymatic degradation of the peptides, because inhibitors of aminopeptidases and endopeptidases were unable to promote responses. In addition, the two peptides are protected from degradation by carboxypeptidases by their carboxy-terminal amidation.

Therefore, the lack of activity of the PAR-2 tethered ligand sequences in human coronary arteries may indicate the sole presence of PAR-1, because receptor agonist specificity is often lost at high concentrations and trypsin can cleave and activate PAR-1. However, the concentrations of trypsin previously demonstrated to activate PAR-1 are substantially greater than those capable of inducing endothelium-dependent relaxation of human coronary arteries in this study. Also, the extended human PAR-2 tethered ligand sequence SLIGKVD-NH$_2$ was found to not activate PAR-1 in human platelets at concentrations up to 1 mmol/L, far in excess of the concentrations of SLIGKV-NH$_2$ and SLIGRL-NH$_2$ used in this study. In addition, structure-activity studies have shown that PAR-1–activating peptides lacking an aromatic residue at position 2 (as is the case with SLIGKV-NH$_2$) are incapable of activating PAR-1 in transfected cell lines and human platelets. Therefore, it is possible that both PAR-1 and PAR-2 are present in human coronary endothelial cells but that PAR-2 is unable to be potently activated by its tethered ligand sequence, as is the case with the recently cloned PAR-3. This possibility is unlikely, however, given that heterologous desensitization occurred with either thrombin or trypsin. In porcine coronary arteries, which are known to express both PAR-1 and PAR-2, heterologous desensitization was observed with trypsin, but only homologous desensitization was seen with thrombin (J.R.H. et al, unpublished observations, 1997). Thus, although cross-desensitization and the poor sensitivity of SLIGKV-NH$_2$ point to the involvement of a single receptor population, the ability of relatively low concentrations of trypsin to mediate relaxations similar to those of thrombin argues against the involvement of a “typical” thrombin receptor in the human coronary artery.

Low concentrations of thrombin cause rapid activation of PAR-1 and PAR-3 by means of a specific recognition site for this enzyme, termed the hirudin-like binding domain, which is located in the receptors’ extracellular amino terminal, immediately distal to the Arg$_{41}$-Ser$_{42}$ cleavage point required for receptor activation. Such specific binding results in close alignment of thrombin’s catalytic site with this peptide bond to ensure efficient receptor cleavage and rapid signal transduction, which are essential for efficient cellular responsiveness. PAR-2 lacks the hirudin-like binding domain and is consequently unresponsive to thrombin. PAR-2s, however, most likely have a similar amino-terminal recognition site for trypsin, because like thrombin, trypsin causes rapid, efficacious responses, most likely due to targeting of the enzyme to the PAR-2 cleavage site. If the human coronary artery endothelial cell PAR is an atypical thrombin receptor, then its activation by both thrombin and trypsin may occur via either a common or a dual enzyme-binding site.

Further support for the existence of an atypical PAR in human coronary artery endothelial cells is our observation that SLIGKV-NH$_2$ caused relaxation despite the lack of the critical aromatic residue pharmacophore at position 2. Therefore, we propose that the receptor responsible for endothelium-dependent relaxation of human coronary arteries is a PAR-1–like receptor, which has a modified amino-terminal exodomain containing binding domains for thrombin and trypsin and a modified tethered ligand binding region containing different pharmacophore specificities.

A further novel finding of this study was that desensitization of responses to both thrombin and trypsin caused only a small inhibition of responses to SFLLRN-NH$_2$. Using a similar protocol in the pig coronary artery, we obtained homologous desensitization with thrombin and heterologous desensitization with trypsin and again in each case, responses to SFLLRN-NH$_2$ and SLIGRL-NH$_2$ were maintained (J.R.H. et al, unpublished observations, 1997). In the same preparation, however, Hwa et al used a desensitization technique with a shorter contact time (10 to 20 minutes) and reported loss of responses to SFLLRN-NH$_2$ after homologous desensitization with thrombin and loss of responses to both SFLLRN-NH$_2$ and SLIGKV-NH$_2$ after heterologous desensitization with trypsin. The retention and loss of responses to the tethered ligand sequences after desensitization in our studies and those of Hwa et al, respectively, may indicate how PARs internalize and recycle after enzymatic activation. For example, the rapid internalization of PAR-1 and PAR-2 after enzymatic activation has been reported to stimulate the mobilization of a pool of intact, preformed receptors that are inserted into the cell membrane within 30 minutes. Thus, the loss of subsequent enzyme-induced responses observed by Hwa et al using a rapid desensitization technique could be explained by the inability of the cell to replenish cell surface receptors from its intracellular reserve. By contrast, the prolonged desensitization technique used in our studies may have depleted intracellular receptor reserves. Despite this, we found that the sensitivity of SFLLRN-NH$_2$ was only slightly decreased after enzyme desensitization. Therefore, we propose that once activated, human endothelial cell PARs are internalized into early endosomes, as previously reported for human erythroleukemia cells, and returned to the membrane without their amino-terminal exodomain, able only to respond to synthetic tethered ligand sequences. Such a proposal implies the presence of an endogenous activator other than thrombin or trypsin, which may act independently of receptor cleavage.
In conclusion, our studies provide evidence for endothelium-dependent, NO-mediated relaxation of human coronary arteries in vitro via a PAR-1–like receptor. This receptor either has a common, low-stringency, hirudin-like thrombin-binding domain or multiple, specific binding domains to enable serine proteases other than thrombin, such as trypsin, to potently activate it. Also, this receptor appears to be recycled via a novel mechanism, whereby cleaved (activated) receptors are returned to the membrane with the ability to respond to agonists acting independently of receptor cleavage. While pathophysiological roles of endothelial cell PARs in human coronary arteries are unknown, they may provide a level of protection against inflammation, because activated mast cells release several proteases, including the trypsin-like enzyme trypstatin, which activates PAR-1 and PAR-2.  

Finally, it remains to be determined whether the nonselectivity of this novel PAR extends to other proteases.

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References

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