Increased Expression of Protease-Activated Receptor-2 (PAR2) and PAR4 in Human Coronary Artery by Inflammatory Stimuli Unveils Endothelium-Dependent Relaxations to PAR2 and PAR4 Agonists

Justin R. Hamilton, Albert G. Frauman, Thomas M. Cocks

Abstract—Protease-activated receptor (PAR)1 and PAR2 are expressed on vascular endothelial cells and mediate endothelium-dependent relaxation in several species, and PAR4 agonists cause similar responses in rat aortas. To date, only PAR1 has been reported to mediate relaxation of human arteries despite endothelial cell expression of both PAR1 and PAR2 in these tissues. Because inflammatory stimuli increase PAR2 expression in human endothelial cells in culture, the present study investigated the effect of similar stimuli on PARs in human isolated coronary arteries (HCAs). In HCA ring segments suspended for isometric tension measurements, the selective PAR1-activating peptide, TFLLR (0.01 to 10 μmol/L), caused endothelium-dependent relaxation of precontracted preparations. Little or no change in vascular tension was elicited by either the PAR2- or PAR4-activating peptides, SLIGKV and GYPGQV, respectively (up to 100 μmol/L). Exposure of HCAs to interleukin (IL)-1α (1 ng/mL, 12 hours) or tumor necrosis factor-α (3 nmol/L, 12 hours) did not affect PAR1 expression but increased PAR2 and PAR4 mRNA levels by ≈5- and 4-fold, respectively, as determined by quantitative polymerase chain reaction. Similar IL-1α treatment did not affect TFLLR-induced relaxations but revealed significant endothelium-dependent relaxations to SLIGKV (100 μmol/L, 61.4 ± 6.7%) and GYPGQV (100 μmol/L, 34.8 ± 6.4%). These studies are the first to demonstrate functional PAR2 and PAR4 in human arteries in situ. The selective upregulation of PAR2 and PAR4 expression and the increased vascular response in HCAs after exposure to inflammatory stimuli suggest a role for these endothelial receptors during inflammation. (Circ Res. 2001;89:92-98.)

Key Words: endothelium ■ human coronary arteries ■ protease-activated receptors

Protease-activated receptors (PARs) located on vascular smooth muscle and/or endothelial cells have been implicated in the control of vascular tone and hemostasis, particularly during inflammatory states (see reviews1–3). PARs are G-protein–coupled receptors activated by particular serine proteases via a unique mechanism as follows: site-specific proteolysis of the amino-terminal exodomain of the receptor exposes a neo-N-terminal, which acts as a self-activating “tethered” ligand.4–5 Four PARs have been cloned. PAR14 and PAR36 are essentially thrombin receptors. PAR27 is activated most sensitively by trypsin and can be activated to a lesser extent by blood-borne enzymes, such as mast cell tryptase8,9 and coagulation factor VIIa.10 The known enzyme activators of PAR4 include thrombin, trypsin, and neutrophil-derived cathepsin G.11,12 Intriguingly, at least for mouse receptors expressed in COS-7 cells, activation of PAR4 by thrombin requires PAR3 as a thrombin-binding cofactor.13 Because each cloned PAR is activated by trypsin (PAR2) or both thrombin and trypsin (PAR1, PAR3, and PAR4), it is fortunate that PARs, with the exception of PAR3,6 can be selectively activated by synthetic peptides corresponding to the distinct tethered ligand sequences of each receptor.4,6,7,12

Emerging literature suggests that endothelial PARs play important roles during vascular inflammation and tissue injury.1,2,14–16 PAR1 and PAR2 are present on smooth muscle and/or endothelial cells, where they mediate direct contraction and NO-mediated endothelium-dependent relaxation, respectively, depending on the preparation, species, and disease state of the tissue. For example, activation of PAR1 (with thrombin or the PAR1-activating peptide, SFLLRN) or PAR2 (with trypsin or the PAR2-activating peptide, SLIGRL) induces endothelium-dependent relaxation of the pig coronary artery17 and rat aorta.18 However, in endothelium-denuded pig coronary arteries, none of these PAR agonists alters vascular tension.17 By contrast, activators of PAR1, but not PAR2, cause contraction of the endothelium-denuded rat aorta.18 This differs again from the mouse renal artery, in which activators of PAR2 not only cause typical endothel-
um-dependent relaxation in endothelium-intact vessels but also induce smooth muscle contraction of endothelium-denuded preparations. In addition to PAR1 and PAR2, functional PAR3 and PAR4 have been reported on endothelial cells of rat brain capillaries and rat aorta, respectively.

Although our insight into the roles of vascular PARs in animal models is progressing, little is known about PARs in human vasculature. Endothelial localization of PAR1 mRNA via in situ hybridization has been described in nonatherosclerotic hepatic arteries and in both endothelial and smooth muscle cells of atherosclerotic arteries. This finding is supported by functional studies on human isolated coronary arteries (HCAs), which demonstrated PAR1-mediated endothelium-dependent relaxation of nonatherosclerotic arteries and a lack of contraction to PAR1 agonists in artery preparations with or without functional endothelium but an increased contraction associated with an increased degree of atheroma in endothelium-denuded artery segments. For PAR2, immunoreactivity has been reported in the endothelium and smooth muscle of HCAs. Functionally, PAR2-activating peptides induce calcium fluxes in human umbilical vein endothelial cells (HUVECs) and mitogenic responses in human aortic smooth muscle cells in culture. However, PAR2 agonists cause neither contraction nor relaxation of HCAs.

We wondered whether the discrepancy between PAR2 expression and the ability of PAR2 agonists to cause changes in human vascular tone is due to low receptor number. Because the inflammatory cytokines interleukin (IL)-1α and tumor necrosis factor (TNF)-α increase PAR2 expression in HUVECs and because similar inflammatory stimuli ameliorate the hypotensive response to PAR2 activation in the rat in vivo, the present study investigated whether exposure of HCAs to inflammatory stimuli, in vitro, would unveil an endothelium-dependent relaxation to PAR2 activation in this tissue. Also, because there are no reports of PAR3 or PAR4 in human arteries in situ, we investigated the presence of these receptors in HCAs.

The present study detected PAR1, PAR2, and PAR4 mRNAs in HCAs by polymerase chain reaction (PCR) and localized immunoreactivities for PAR1 and PAR2 to HCA endothelial cells. Despite the expression of three PARs, only agonists of PAR1 induced changes in vascular tone in HCAs in the absence of inflammatory stimuli. Exposure of HCAs to either IL-1α or TNF-α significantly increased the expression of both PAR2 and PAR4, as determined by quantitative PCR, which unveiled endothelium-dependent relaxations in response to agonists of these receptors. The present study provides the first evidence of functional PAR2 and PAR4 in human arteries in situ and suggests a role for these endothelial cell receptors during vascular inflammation.

### Materials and Methods

#### Materials

PAR1 and PAR2 antisera were gifts from Prof Lawrence Brass (University of Pennsylvania, Philadelphia). Anti-mouse antisera and FITC-streptavidin were from Amersham Pharmacia Biotech; U46619 and nifedipine, from Sapphire Bioscience; (-)-isoprenaline, substance P (acetate salt), and α-thrombin (bovine serum), from Sigma Chemical Co; IL-1α and TNF-α (both recombinant, human), from Endogen; and trypsin (bovine pancreas), from Worthington Biochemical. TFLR, SLIGKV, and GYQQGV were synthesized with carboxyl-terminal amidation and purified to 95% via high-performance liquid chromatography by Auspep.

#### Human Coronary Arteries

With informed patient consent and ethics approval from The Alfred Hospital and University of Melbourne Ethics Committees, epicardial coronary arteries were dissected from explanted hearts of 12 patients (9 men and 3 women, aged 45.3±5.1 years, range 28 to 61 years) undergoing transplantation at The Alfred Hospital (Melbourne, Australia) because of dilated congestive cardiomyopathy (n=6), restrictive cardiomyopathy (n=3), ischemic heart disease (n=2), or septic defects (n=1). Three-millimeter-long arterial ring segments that were macroscopically free of atheromatous plaques (endothelium was mechanically removed in some) were prepared and (1) frozen in liquid nitrogen and stored at −70°C for RNA extraction, (2) fixed in 4% paraformaldehyde (4°C overnight) for immunohistochemistry, (3) incubated at 37°C/5% CO2 for 12 hours (CO2 incubator, Forma Scientific) in 5 mL Krebs solution in the absence or presence of IL-1α (1 ng/mL), or TNF-α (3 mM/L), or (4) immediately mounted in organ chambers for isometric tension measurement.

#### Polymerase Chain Reaction

Total RNA was isolated and subjected to DNase digestion by using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed at 42°C for 1 hour with 100 ng total RNA, 1 μM/L poly(dT)12-16 primers, and 200 U Moloney murine leukemia virus reverse transcriptase with use of the First Strand Synthesis Kit (Promega). For conventional PCR, cDNA was amplified by a “touchdown” PCR method to detect transcripts under high-stringency hybridization conditions, with the use of 5 ng cDNA, 0.5 μM/L specific primers (Table 1), 0.2 mM/L dNTPs, 2.5 U Tag DNA polymerase, 10 mM/L Tris HCl (pH 8.3), 50 mM/L KCl, and 1.5 mM/L MgCl2, and with heating to 94°C for 3 minutes, followed by 5 cycles of 94°C, 70°C, and 72°C (each for 30 seconds) and then 35 cycles of 94°C, 55°C, and 72°C (each for 30 seconds) and, finally, 72°C for 5 minutes. Quantitative PCR was performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. Specific primer sequences were used in combination with internal probes, each labeled at the 5’ end with the reporter dye 6-carboxyfluorescein and at the 3’ end with the quencher dye 6-carboxytetramethylrhodamine (Table 2). Amplification was performed for 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

### Table 1. Gene-Specific Primer Sequences Used in Conventional PCR Amplifications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>PCR Product Size, bp</th>
</tr>
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<tbody>
<tr>
<td>PAR1</td>
<td>Sense: 5'-TGTGACTGTCACTGTATTAAG-3'; Anti-sense: 5'-TGGTAGATGTAATGAGTAAGT-3'</td>
<td>708</td>
</tr>
<tr>
<td>PAR2</td>
<td>Sense: 5'-GAGGTTAGAGCGCTATTTGTT-3'; Anti-sense: 5'-TGATGTAATGAGTAAGT-3'</td>
<td>334</td>
</tr>
<tr>
<td>PAR3</td>
<td>Sense: 5'-GAAGGGCTCTATCTTGAGG-3'; Anti-sense: 5'-GAGTTGAAGGATGAGGACG-3'</td>
<td>599</td>
</tr>
<tr>
<td>PAR4</td>
<td>Sense: 5'-GGAACCTCTATGTGGGCTTA-3'; Anti-sense: 5'-TTGACCGATGACGCCT-3'</td>
<td>244</td>
</tr>
<tr>
<td>ecNOS</td>
<td>Sense: 5'-ACCCCTACGGCCATCACATC-3'; Anti-sense: 5'-AGATGCTTGAAGGAGAT-3'</td>
<td>744</td>
</tr>
</tbody>
</table>
TABLE 2. Gene-Specific Primer Sequences and Internal Probes Used in Quantitative Real-Time PCR Amplifications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/Probe Sequence* (5′−3′)</th>
</tr>
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<tbody>
<tr>
<td>PAR1</td>
<td>Sense 237TAGGCTATTCCTGAGAGCCTGAT2503</td>
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<tr>
<td></td>
<td>Antisense 248ATGCCCTGCTGATGCT2456</td>
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<tr>
<td></td>
<td>Probe† 246TGCTCCCGCCCAGATGAGC2536</td>
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<tr>
<td>PAR2</td>
<td>Sense 45GCAAGGTAGAGGCTGACCTT112</td>
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<td></td>
<td>Antisense 127GGACCGGATTGAAACT120</td>
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<tr>
<td></td>
<td>Probe† 105CTCCGTTCTGACCTGAGACTC108</td>
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<tr>
<td>PAR4</td>
<td>Sense 1216GAGGTTGGTACACCCCTTTCC1242</td>
</tr>
<tr>
<td></td>
<td>Antisense 1220GACAGTTGTAACAACCCTATTTCCAAA1242</td>
</tr>
<tr>
<td></td>
<td>Probe† 2472TTCCCTGGGACCTCAGAATGTGACC2500</td>
</tr>
</tbody>
</table>

*Numbers refer to base positions on the known human gene sequence. †PAR probes were labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine.

Immunohistochemistry
Paraformaldehyde-fixed HCA was washed in PBS, placed in sucrose solution (15% [wt/vol] in PBS) at 4°C overnight, embedded in OCT compound (Sakura Finetek), and frozen in liquid nitrogen. PAR1 and PAR2 were localized by using previously characterized monoclonal antibodies produced in mice immunized with peptides corresponding to the amino-terminus of human PAR1 (S42FLLRNPNDKYEP25, 1:100) and PAR2 (S37LIGKVDGTSHVTG20, 1:20) (see Molino et al8). Primary antisera were labeled with a biotinylated sheep anti-mouse antiserum (1:100, 1 hour) and FITC-conjugated streptavidin (1:20, 1 hour).

Isometric Tension Measurements
HCAs were mounted for isometric tension measurement as previously described.24 After a standard equilibration procedure,24 including treatment with nifedipine (0.3 μmol/L) to inhibit characteristic spontaneous contractile activity,9 HCAs were contracted to ∼50% of their maximum contraction to 125 mmol/L KCl with U46619, and cumulative half-log increases in the concentration of PAR agonists were added. The maximum endothelium-dependent and -independent relaxations were determined by adding substance P (3 nmol/L) and isoprenaline (1 mmol/L), respectively.

Statistical Analysis
Changes in isometric tension were normalized as percent tissue response to 1 μmol/L isoprenaline. pEC_{50} values represent the negative logarithm of the molar concentration that caused 50% of the maximum response. mRNA levels are expressed as fold increase in PAR mRNA normalized against 18S rRNA as the within-tissue molecular weight markers (M) and PCR products for PAR1, PAR2, PAR3 (absent), PAR4, and ecNOS in endothelium-intact and endothelium-denuded HCAs (A) and molecular weight markers (M) and PCR products for PAR3 and PAR4 in human prostate and cardiac muscle (B). Numbers represent product sizes (bp).

Results
Polymerase Chain Reaction
Conventional PCR analyses on mRNA extracted from endothelium-intact HCAs resulted in amplification of a single fragment of predicted size (Table 1) for PAR1, PAR2, and PAR4 (n=4 artery segments, from four patients) (Figure 1). By contrast, no PCR fragment was detected for PAR3 in these tissues (n=4 artery segments, from four patients) (Figure 1). When human cardiac muscle was used as a positive control for PAR3 expression,a a single PCR product of predicted size was detected (n=2 artery segments, from two patients) (Figure 1).

Figure 1. Shade-inverted digitized photographs of UV-illuminated agarose gels (1.5%, plus 0.5 μg/mL ethidium bromide) showing molecular weight markers (M) and PCR products for PAR1, PAR2, PAR3 (absent), PAR4, and ecNOS in endothelium-intact and endothelium-denuded HCAs (A) and molecular weight markers (M) and PCR products for PAR3 and PAR4 in human prostate and cardiac muscle (B). Numbers represent product sizes (bp).

In similar PCR analyses on mRNA extracted from HCA preparations from which the endothelium had been removed, we again observed the expected PCR products for PAR1 and PAR4 (n=2 artery segments, from two patients) (Figure 1). By contrast, the PAR2 PCR fragment detected in endothelium-intact HCAs was no longer evident (n=2 artery segments, from two patients) (Figure 1). We confirmed the effective removal of endothelial cells in these “endothelium-denuded” HCA segments by using specific primers for endothelial constitutive NO synthase (ecNOS), which amplified a product of the expected size in endothelium-intact HCA segments but not in endothelium-denuded HCA segments (n=2 artery segments, from two patients) (Figure 1).

Because PAR1, PAR2, and PAR4 are expressed in the vasculature, it was difficult to obtain negative tissue controls for the expression of these receptors. Thus, PCR products for PAR1, PAR2, and PAR4 were detected in vascularized human tissues, such as testis, thyroid, and muscle of cardiac, ocular, and abdominal origin (data not shown). However, no products were observed if cDNA from rat heart was used in the PCR reaction (data not shown). The possibility of contamination by genomic DNA was unlikely because isolated RNA was subjected to DNase treatment before transcription. Furthermore, no PCR products were detected when PCR was performed on untranscribed RNA (data not shown).
Immunohistochemistry
Punctate PAR1 and PAR2 immunoreactivities were localized to the endothelium with little, if any, staining with either antisera detectable in the smooth muscle (Figure 2).

Quantitative PCR
Treatment of endothelium-intact HCA segments with IL-1\(\alpha\) (1 ng/mL, 12 hours) caused a significant (\(P<0.05\)) 
5- and 4-fold increase in mRNA expression for PAR2 and PAR4, respectively, as measured by real-time quantitative PCR (n=3 artery segments, from three patients) (Figure 3). By contrast, mRNA levels for PAR1 were virtually unaffected (1.3-fold increase) by similar treatment with IL-1\(\alpha\) (n=3 artery segments, from three patients) (Figure 3). Treatment of HCAs with TNF-\(\alpha\) (3 nmol/L) caused a similar increase in PAR2 (4-fold) and PAR4 (3-fold) mRNA levels but, again, had little effect on PAR1 expression (1.3-fold increase in mRNA; n=3 artery segments, from three patients) (Figure 3). The effects of IL-1\(\alpha\) and TNF-\(\alpha\) were likely specific because boiling these cytokines before treatment of HCAs abolished their effect (data not shown).

Isometric Tension Measurements
In endothelium-intact HCAs mounted for isometric tension measurement immediately after dissection, the selective PAR2-activating peptide, SLIGKV, failed to cause relaxation of contracted preparations at concentrations of up to 100 \(\mu\text{mol/L}\) (data not shown). In similar endothelium-intact HCA preparations incubated in the presence of IL-1\(\alpha\) (1 ng/mL, 12 hours), SLIGKV induced significant relaxations, with a threshold at \(3 \mu\text{mol/L}\) and a maximum at 100 \(\mu\text{mol/L}\) of 61.4\%\(\pm\)6.7\% (n=10 artery segments, from five patients) (Figure 4). Almost no relaxation to SLIGKV was observed in endothelium-intact HCA preparations incubated under similar conditions but in the absence of IL-1\(\alpha\) (Figure 4). Also, SLIGKV-induced relaxations were abolished by removal of the endothelium immediately after the IL-1\(\alpha\) treatment period (Figure 4). Similar to the results obtained with SLIGKV, the selective PAR4-activating peptide, GYPGQV (up to 100 \(\mu\text{mol/L}\)), failed to relax HCAs mounted for isometric tension measurement immediately after dissection (data not shown). GYPGQV also caused no relaxation of endothelium-intact HCAs incubated for 12 hours in the absence of IL-1\(\alpha\) but...
caused significant relaxations in tissues incubated under the same conditions but in the presence of IL-1α (1 ng/mL, 34.8 ± 6.4% at 100 μmol/L; n = 4 artery segments, from two patients) (Figure 4). GYPGQV-induced relaxations were abolished by removal of the endothelium after the incubation period (Figure 4).

In contrast to SLIGKV- and GYPGQV-induced responses, relaxations caused by the selective PAR1-activating peptide, TFLLR, were not different between endothelium-intact HCAs incubated in the absence (pEC50 6.4 ± 0.2, maximal relaxation 89.0 ± 2.6%) and presence (pEC50 6.3 ± 0.1, maximal relaxation 87.8 ± 2.5%) of IL-1α (n = 8 artery segments, from four patients) (Figure 4).

A similar upregulation of endothelium-dependent relaxations to SLIGKV and GYPGQV, but not TFLLR, was observed after treatment of HCAs with TNF-α (3 μmol/L) (n = 4 artery segments, from two patients) (data not shown).

Relaxations to both SLIGKV and GYPGQV in IL-1α-treated HCAs were abolished by combined treatment with the ecNOS inhibitor, Nω-nitro-L-arginine (L-NOARG, 100 μmol/L), and the cyclooxygenase inhibitor, indomethacin (3 μmol/L) (n = 4 artery segments, from two patients) (Figure 4). Also, TFLLR, SLIGKV, and GYPGQV all failed to cause relaxation of endothelium-denuded HCAs incubated with either IL-1α or TNF-α (data not shown).

Finally, TFLLR, SLIGKV, and GYPGQV caused no significant contraction under any of the conditions investigated in the present study. Thus, none of the PAR-activating peptides, at concentrations of up to 100 μmol/L, contracted endothelium-intact or -denuded HCAs incubated with or without IL-1α or TNF-α, either in the absence or presence of U46619-induced tone (data not shown). Furthermore, the failure of TFLLR, SLIGKV, and GYPGQV to contract HCAs persisted in the absence of nifedipine (data not shown).

Discussion

The main finding of the present study was that exposure of HCAs to inflammatory stimuli selectively increased PAR2 and PAR4 expression, which unveiled endothelium-dependent relaxations to agonists of these receptors. To our knowledge, the present study is the first to report functional PAR2- and PAR4-dependent relaxations to agonists of these receptors in HCA preparations.

We previously reported endothelium-dependent relaxation of HCAs by thrombin and the PAR1-activating peptide, SFLLRN.24 The present study first confirmed that the more selective PAR1-activating peptide, TFLLR,31 caused similar relaxation of HCAs. By contrast with TFLLR, the selective PAR2-activating peptide, SLIGKV, failed to relax contracted HCAs, a finding that we have previously reported.24 Despite this, two findings from our present study indicated that PAR2 was expressed on HCA endothelium. First, PAR2 immunoreactivity was localized specifically to the endothelial cell layer. Second, PAR2 mRNA was readily detected in endothelium-intact HCAs but not in segments of artery from which the endothelium had been removed. Importantly, we used PCR for ecNOS, the expression of which is limited to endothelial cells in the vasculature,32 to confirm the absence of contaminating endothelium in HCA segments from which the endothelium was presumed to have been effectively removed. Our finding that PAR2 is expressed only in the endothelium of HCAs differs from the study of Molino et al,25 who reported PAR2 immunoreactivity on smooth muscle as well as endothelial cells in this tissue. The reason for the discrepancy between the present study and that of Molino et al is unclear, although one possibility may involve differences in the disease state of the tissue used in each study. For example, disease-related differences in receptor expression have been reported for PAR1. Thus, PAR1 expression was limited to the endothelium of nonatherosclerotic arteries but was observed in both endothelial and smooth muscle cells in atheromatous arteries.22 Regardless of these differences in the detection of PAR2 expression on smooth muscle, the present study and that of Molino et al have clearly shown that unlike the expression in arteries of other species, the expression of PAR2 on the endothelium of HCAs does not necessarily translate to PAR2-mediated endothelium-dependent relaxation of this tissue.

In an attempt to reconcile the apparent discrepancy between PAR2 expression and function on HCA endothelial cells, we sought to investigate the effects of inflammatory stimuli on this receptor. Previously, Nystedt et al27 showed that treatment of HUVECs with IL-1α, TNF-α, or lipopolysaccharide increased mRNA and cell-surface protein levels for PAR2, but not PAR1. In addition, Cicala et al28 reported that treatment of rats in vivo with lipopolysaccharide similarly increased PAR2 expression in the aorta and jugular vein, which was manifested as an increased sensitivity to the dilator response of PAR2 agonists in rat vasculature in vitro and in vivo. Again, this upregulation in response to an inflammatory stimulus was selective for PAR2 over PAR1.28 A similar selective upregulation of PAR2 mRNA in HCAs treated with either IL-1α or TNF-α was observed in the present study with the use of quantitative PCR. The 4- to 5-fold increase in PAR2 mRNA observed in the present study occurred at the same concentrations of the two cytokines (3 nmol/L and 1 ng/mL for TNF-α and IL-1α, respectively) and within the same time frame (12 hours) that gave an ≈3-fold increase in PAR2 mRNA in the study of Nystedt et al. Although Nystedt et al found that 1 ng/mL IL-1α was sufficient to cause maximum increase in PAR2 mRNA (≈13-fold increase), this response occurred after 48 hours of IL-1α treatment. Unfortunately, such an incubation period was not possible in the present study, inasmuch as preliminary experiments determined that endothelium-dependent relaxation of HCA ring preparations declined with incubations beyond 12 hours. This is an important experimental limitation of incubating isolated tissues, because we also observed that a 10-fold increase in IL-1α concentration (to 10 ng/mL) caused no greater induction of PAR2 mRNA expression or SLIGKV-induced vascular relaxation (not shown). Therefore, the ≈60% relaxation caused by 100 μmol/L SLIGKV appears to be the maximum response that we can induce in this tissue with IL-1α. However, on the basis of the time course studies of Nystedt et al in cultured HUVECs, it seems likely that prolonged (ie, >12-hour) exposure to inflammatory cytokines, such as that occurring during patho-
physiological conditions in vivo, will further increase the sensitivity to PAR2 agonists.

A pattern of vascular reactivity similar to that observed for PAR2 was seen with PAR4. As was the case with SLIGKV, the selective PAR4-activating peptide, GYPGQV, failed to cause relaxation of contracted HCAs at concentrations of up to 100 μmol/L. As with PAR2, PAR4 mRNA was readily detected in HCAs, although in contrast to PAR2, PAR4 mRNA was also detected in endothelium-denuded HCA segments. Because antibodies directed against PAR4 were not available at the time of the present study, definitive evidence for PAR4 expression on HCA endothelial cells could not be provided. However, our finding (ie, that the upregulation of vascular relaxation to GYPGQV after exposure of HCAs to IL-1α or TNF-α was entirely dependent on the presence of functional endothelium) suggests that like PAR2, PAR4 was expressed in HCA endothelial cells. To our knowledge, this is the first demonstration of the in situ localization and functional description of PAR4 in the human vasculature. This result supports an earlier study in which an NO-mediated endothelium-dependent relaxation was observed in rat aortas in response to PAR4-activating peptides.21

Our hypothesis that relaxations of HCAs by agonists of PAR2 or PAR4 are due to upregulation of these receptors on endothelial cells is supported by our observations that both SLIGKV- and GYPGQV-induced relaxations were entirely dependent on an intact endothelium and were abolished in the presence of inhibitors of the synthesis of NO (L-NOARG) and prostacyclin (indo-methacin), the primary mediators of endothelium-dependent relaxation in human arteries.30 In addition, the effects of IL-1α and TNF-α are likely specific because they were (1) blocked by boiling the cytokines before the treatment period, (2) concentration-dependent and occurred at the same concentration previously reported to cause similar effects, (3) dependent on the presence of an intact endothelial cell layer, and (4) selective for SLIGKV and GYPGQV, inasmuch as relaxations to TFLLR, thrombin, and substance P were unaffected by similar treatment regimes (not shown).

Upregulation of endothelial cell PAR2 in response to inflammatory stimuli has been taken to indicate that PAR2 is involved in the progression of vascular inflammatory diseases.3 However, as we have previously pointed out,1 the opposite could apply. Thus, PAR2 (and PAR4) upregulation may increase the protective barrier function of the endothelium and limit, for example, clotting and disseminating intravascular coagulation in proinflammatory conditions, such as sepsis and hypertension. It is interesting to note that in hypertension, although most other endothelium-dependent vasodilator function is diminished33 and although there is an increased potential for thrombosis,34 PAR2-mediated vasodilatation is preserved.55 Further evidence that endothelial PAR2 is anti-inflammatory, albeit indirect, comes from our earlier observation in porcine isolated coronary arteries. We found that proinflammatory enzymes, such as bacterial thermolysin, which disable PAR2 without causing receptor activation, trigger a novel translation-dependent mechanism for replenishment of the cell surface receptor, which is rapid and specific for PAR2 over PAR1.17 Therefore, vascular endothelial PAR2 (and possibly PAR4) is not only upregulated in inflammation, but novel mechanisms appear to exist to maintain PAR2 responsiveness during such conditions.

The inability of agonists of PAR1, PAR2, or PAR4 to induce contraction of HCAs under any of the conditions investigated in the present study suggests that smooth muscle cell PARs play a limited role in contracting human arteries. Although PAR1-mediated contractions occur in HCAs displaying signs of atheroma,29 smooth muscle cell PARs may be involved in functions other than the regulation of vascular tone, such as smooth muscle growth and differentiation,36 in the absence of atheroma.

Finally, the present study raises the possibility of a lack of PAR3 in the human vasculature. The inability of synthetic PAR3-activating peptides to activate this receptor30 prohibits us from investigating PAR3 function in this way. However, we were unable to detect PAR3 mRNA in HCAs. Furthermore, desensitization to the PAR1-activating peptides, SFLLRN and TFLLR (which cannot activate PAR3 at the concentrations used in the present study6,12), abolished subsequent responses to thrombin (the most potent known activator of PAR3) (J.R. Hamilton, T.M. Cocks, unpublished data, 2000). These findings contrast with those of previous reports that showed functional PAR3 in HUVECs via measurement of intracellular calcium concentration changes.37 The discrepancy between our results and those obtained in HUVECs remains unexplained but may simply reflect differences between HUVEC and native human endothelial cells or differences in PAR3 expression between venous and arterial endothelial cells, such as that reported for other PARs.25

In conclusion, the present study is the first to demonstrate functional PAR2 and PAR4 in human vasculature in situ. We suggest that under physiological conditions, circulating thrombin activates PAR1 to relax underlying smooth muscle. In contrast with PAR1, endothelial PAR2 and PAR4 are expressed in low levels and do not contribute to serine protease regulation of vasodilator tone in the coronary bed. However, in pathological conditions in which inflammatory cytokines are released, such as ischemia/reperfusion injury,58 atherosclerosis,59 and, perhaps, hypertension,60 PAR2 and PAR4 are upregulated and may now respond to (patho)physiological concentrations of activating enzymes and contribute to endothelium-dependent vasodilatation. PAR2 is insensitive to thrombin, and at least for the mouse receptor, PAR4 cannot be activated by thrombin in the absence of PAR3.13 Because PAR3 was not expressed in HCAs, PAR2 and PAR4 are likely nonthrombin PARs in this tissue. On this assumption, we suggest that vascular inflammation promotes the switching of endothelial cell PARs from being thrombin sensitive under physiological conditions to being thrombin insensitive during vascular inflammation. As such, the inflammatory stimuli–induced upregulation of PAR2 and PAR4 in HCAs may indicate that these receptors are protective when expressed on endothelial cells and that they regulate vascular tone in inflammation and/or vascular injury.

Acknowledgments
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References

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