Differential Expression of Functional Protease-Activated Receptor-2 (PAR-2) in Human Vascular Smooth Muscle Cells

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Abstract—The protease-activated family of G protein–coupled receptors includes PAR-1 and PAR-3, which are activated by thrombin, and PAR-2, which is activated by trypsin and tryptase. PAR-2 has recently been shown to be expressed in human endothelial cells. In the present studies, we have examined the expression of PAR-2 in other cells, particularly vascular smooth muscle, and tested whether the receptors are functional. The results show that PAR-2 is present in human aorta and coronary artery smooth muscle cells, as well as in arteries traversing the walls of the small intestine. It was also detected in human keratinocytes, sweat glands, intestinal smooth muscle, and intestinal epithelium, but not at all in myocardial smooth muscle and only inconsistently in intestinal veins and venules. Activation of aortic smooth muscle cells in culture with PAR-2 peptide agonists caused a transient increase in the cytosolic Ca2+ concentration. In contrast, PAR-2 mRNA could not be detected in saphenous vein smooth muscle cells, and the same cells placed in culture showed little, if any, response to the PAR-2 agonist peptides. These observations show that PAR-2 is widely distributed in human vascular smooth muscle, particularly in arteries. However, this is not a universal finding and at least some venous smooth muscle cells, including those in saphenous veins, apparently do not express the receptor in detectable amounts. (Arterioscler Thromb Vasc Biol. 1998;18:825-832.)

Key Words: vascular smooth muscle ■ protease-activated receptor ■ PAR-2 ■ thrombin ■ trypsin

The protease-activated receptor (PAR) family currently consists of three related proteins: PAR-1,2,3, which can be activated by thrombin and trypsin,1 PAR-2, which is activated by trypsin,4,5 but not thrombin, and the recently-described PAR-3, which is activated by thrombin.5 Like other G protein–coupled receptors, the three PAR family members are comprised of a single polypeptide with seven membrane-spanning domains and an extended extracellular N terminus. Receptor activation occurs when proteolysis at a unique site within the N terminus exposes a new N terminus that serves as a tethered ligand for the receptor.1,3,5 For PAR-1 and PAR-2, synthetic peptides corresponding to the first five or six residues of the new N terminus have been shown to cause receptor activation even in the absence of a protease. Once cleaved, these receptors activate their associated G proteins and signal briefly before uncoupling and desensitization occur, presumably due to receptor phosphorylation.6-11 Since receptor activation by a protease requires an intact N terminus, restoration of signaling requires the replacement of cleaved receptors with intact ones, a process that is supported by the synthesis of new receptors and in some cells, including endothelial cells, mobilization of preformed receptors from a cytoplasmic pool.12-18 In all cases that have been studied, including platelets, endothelial cells, and fibroblasts, PAR-1 activation turns on phospholipase C and causes an increase in the cytosolic Ca2+ concentration. Thrombin can also activate phospholipase A2 and PI 3-kinase, stimulate or inhibit cAMP formation, turn on the MAP kinase pathway, and stimulate mitogenesis and secretion (reviewed in Reference 19). Most of these responses have been ascribed to PAR-1. Less is known about the distribution and function of PAR-2 and PAR-3. However, in endothelial cells, PAR-2 has been shown to evoke responses that are similar to PAR-1,18,20-22 and PAR-3 appears to be responsible for the activation of mouse platelets by thrombin.7 Based on Northern analysis and antibody staining, PAR-1 is widely distributed in vascular and extravascular tissues. Functional PAR-2 has been identified in endothelial cells,18,20,22 human keratinocytes,23 and intestinal epithelial cells.21,24 Northern analysis shows RNA encoding PAR-2 in human and mouse kidney, small intestine, colon, and liver, and to a lesser extent in prostate, heart, and spleen.21,24 Information about protein expression is still limited, in part because antibodies that recognize human PAR-2 have only recently become available. In the few cases in which such information is available, it has been found that some types of
PAR-2 in Vascular Smooth Muscle

cells express only one of these receptors, while others express more than one. Human platelets, for example, express PAR-1, but not PAR-2, while human keratinocytes express predominantly PAR-2. Human umbilical vein endothelial cells (HUVECs) express both. Little is known about PAR-3 distribution in human cells.

In the present studies, we have used Northern blotting and a recently developed monoclonal antibody to examine the expression of PAR-2 in human tissues removed at the time of surgery or organ donation. The emphasis was on vascular tissues, and to complement the expression studies we have also studied the effects of PAR-2 activation in cultured human aortic and saphenous vein smooth muscle cells to determine whether the receptors identified immunologically are functional. The results show that functional PAR-2 is expressed in human aortic smooth muscle cells and endothelial cells. However, RNA encoding PAR-2 could not be detected in saphenous vein smooth muscle cells, and in culture, these cells showed little, if any, response to PAR-2 agonist peptides, suggesting that there are differences in the expression of PAR-2 within arterial and venous smooth muscle cells—an impression that was substantiated by staining human tissue sections with an anti–PAR-2 monoclonal antibody. When studied in vitro, the magnitude of the increase in cytosolic Ca\(^{2+}\) in aortic smooth muscle cells in response to the PAR-2 peptide agonists SLIGKV and SLIGRGL was smaller than the response to thrombin. This finding contrasts with results obtained with endothelial cells in which the responses to the two agonists were similar and suggests that PAR-2 is expressed to a lesser extent than PAR-1 on the smooth muscle cells. Finally, using the anti–PAR-2 antibody, we were also able to detect PAR-2 in situ in human keratinocytes and in human intestinal epithelium and smooth muscle, which is consistent with recent functional studies on human keratinocytes in culture and in situ hybridization on mouse small intestine.

**Methods**

**Materials**

Highly purified \(\alpha\)-thrombin was provided by Dr J. Fenton (New York State Department of Health, Albany). The cDNA for human PAR-2 was generously provided by Dr J. Sundelin (Lund University, Sweden).

**Antibodies**

Antibody SAM11 is an IgG2a monoclonal antibody produced in mice immunized with the peptide SLIGKVQSDSHVTG corresponding to residues 37 to 50 of the human PAR-2 sequence. It was selected by screening against the immunizing peptide, followed by a screen for clones that bind to COS-1 cells expressing human PAR-2 but not to mock-transfected COS-1 cells. Antibody WEDE15 is a previously described IgG1 monoclonal antibody produced in mice immunized with the peptide KYEFPWEDEKINES corresponding to residues 51 to 64 of the human PAR-1. It recognizes both the intact and cleaved forms of the receptor. Normal murine IgG2a was used as the negative control for antibody SAM11. Antibody EHI, a monoclonal IgG1 antibody reactive with the HIV-1 nef protein, was used as the negative control for antibody WEDE15.

**Human Tissue Samples**

Vascular samples were obtained from native human hearts removed at the time of transplantation. Normal small intestine, stomach, kidney, bladder, skin, colon, and liver were obtained from surgical specimens that were not required for pathological examination. Tissue samples for cell culture and RNA extraction were snap-frozen and stored in liquid nitrogen. For immunohistochemistry, 2- to 3-mm sections were fixed by immersing each sample in 10% neutral buffered formalin overnight at 21°C. All samples were then embedded in paraffin, and serial sections of 5 \(\mu\)m were cut on ProbeOn Plus slides for immunohistochemistry.

**Immunohistochemistry**

A modification of the streptavidin-biotin-peroxidase method was performed using capillary action technology and the MicroProbe System (Fisher Scientific). Briefly, sections were deparaffinized, hydrated in 100% and 50% ethanol, washed with 1X automation buffer (Biomeca Corp), and treated with 2% normal horse serum for 20 minutes at room temperature to block nonspecific binding by the antibody. For using the peroxidase system, the endogenous peroxidase activity was quenched with a 2.2% (vol/vol) \(\mathrm{H}_2\mathrm{O}_2\)-methanol solution for 10 minutes before blocking with normal horse serum. The slides were then incubated with primary antibody (overnight at 4°C followed by 1 hour at room temperature) and then washed in 1X automation buffer for 10 minutes. Bound antibody was detected with biotinylated anti-mouse serum (Vector) at a 1:200 dilution incubated for 60 minutes at room temperature. The slides were then incubated for 60 minutes at room temperature with the streptavidin-biotin system (Dako Corp) at a 1:50 combined dilution and developed with 0.05% (vol/vol) 3,3'-diaminobenzidine solution (Sigma) and 0.03% (vol/vol) \(\mathrm{H}_2\mathrm{O}_2\) for 5 minutes. After a final wash, the slides were counterstained with aqueous hematoxylin, dehydrated in ethanol and xylene, and coverslipped with Permount. All samples were reviewed by two investigators. COS-1 cells transiently expressing human PAR-2 cDNA were used to optimize conditions.

**Cell Culture**

Smooth muscle cells were prepared from human aorta or saphenous vein removed at the time of heart transplantation by the explant technique and used at passage numbers 2 to 6 as described previously. Briefly, smooth muscle cells from the media of the vessels were isolated surgically and minced into small pieces with scissors. Using the explant technique, cells were grown to confluence on fibronectin-coated Petri dishes in media containing 10% heat-inactivated fetal calf serum (Hyclone Laboratories), in a 1:1 mixture of Dulbecco’s modified Eagle’s medium with high glucose (JRH Biosciences) and Ham’s nutrient mixture F-12 with L-Glutamine (JRH) with penicillin-streptomycin and fungizone. Greater than 98% of the cells stained positive for actin. Early-passage HUVECs were prepared as previously described. All studies were done with the approval of the University of Pennsylvania’s Institutional Committee on Studies Involving Human Beings.

**RNA Analysis**

Total RNA was isolated with an RNAzol B kit, separated by agarose gel electrophoresis, and analyzed by Northern blotting using \(\sim 25 \text{ ng}\) of RNA. The cDNA labeled using '\(\mathrm{P}\) dCTP (Amersham) and a random-prime labeling kit (Boehringer Mannheim). The filters were incubated at 42°C for 12 to 16 hours with solution containing the labeled and denatured probe and then washed and exposed to Kodak XAR film at \(-70°C\) using intensifier screen.

**Cytosolic Calcium**

Cells were loaded with 5 \(\mu\text{mol/L}\) fura 2-AM (Molecular Probes) in RPMI-1640 medium without phenol red for 1 hour at 37°C, then released from the culture dishes by incubation for 15 minutes at 37°C with phosphate-buffered saline containing 1 \(\text{mmol/L}\) EDTA and 5 \(\text{mmol/L}\) EGTA. The detached cells were then washed, resuspended in RPMI-1640 without phenol red, allowed to equilibrate for 30 minutes at room temperature, washed again and used at \(1 \times 10^6/\text{mL}\). Changes in the cytosolic free Ca\(^{2+}\) concentration were measured with an SLM/Aminco model AB2 fluorescence spectrophotometer.
Transfection

COS-1 cells, cultured in DMEM with 10% fetal calf serum, were transiently transfected, using DEAE-dextran with 0.5 to 1.5 mg/mL cDNA encoding either human PAR-1 or PAR-2 in pRK7 as previously described.31 One day after transfection, the cells were detached from the plates by trypsin/EDTA treatment and seeded into 60-mm tissue-culture dishes. Receptor expression was measured by flow cytometry using antibodies SAM11 and WEDE15 at 10 mg/mL final concentration. After washing with staining buffer (phosphate-buffered saline with 0.02% sodium azide and 0.2% bovine serum albumin) the cells were resuspended in fetal calf serum and incubated with a 1:40 dilution of FITC-labeled goat anti-mouse IgG (BioSource International) and analyzed on a FACscan flow cytometer (Becton Dickinson).

Results

Previous studies have examined the distribution of RNA encoding PAR-2 in human tissues and have shown it to be present in human kidney, small intestine, colon, liver, prostate, heart, and spleen24,27 and in keratinocytes.23,24,27 As a first step in examining PAR-2 expression in human vascular smooth muscle, RNA was extracted from aortic and saphenous vein smooth muscle cells and analyzed with a probe specific for human PAR-2. HUVECs and keratinocytes, both of which are known to express PAR-2, were used as positive controls. PAR-2 message was detectable in aortic smooth muscle cells but was not detectable in saphenous vein smooth muscle cells (Fig 1).

To examine PAR-2 protein expression in human tissues, a monoclonal antibody (SAM11) was prepared by immunizing mice with a peptide corresponding to residues 37 to 50 of the human PAR-2 sequence (SLIGKVDGTSHVTG). This region begins immediately downstream of the trypsin cleavage site in PAR-2 (Arg36-Ser37) and includes the PAR-2 tethered ligand domain, SLIGKV. In analogy to comparable antibodies produced against PAR-1,29 antibody SAM11 would be expected to bind to cleaved as well as intact PAR-2 and, because of sequence differences, would not be expected to recognize human PAR-1 or PAR-3. The studies in Fig 2 show that SAM11 binds to COS-1 cells that had been transfected with PAR-2, but not to mock-transfected cells or cells expressing PAR-1. In contrast, the PAR-1 antibody WEDE15 bound to cells expressing PAR-1, but not to cells expressing PAR-2.

Figs 3 and 4 show results that were obtained when antibody SAM11 was used to detect PAR-2 in human tissue samples by immunohistochemistry. Under the conditions that were used, PAR-2 was detectable in transfected COS-1 cells and in keratinocytes (Fig 3A and 3M). It was also detectable in the epithelial lining of the small intestine and colon (Fig 3K) and in aortic endothelial cells (Fig 3G). Negative results were obtained when the antibody was preabsorbed with its immunizing peptide or when an equal concentration of normal murine IgG2a was substituted for SAM11 (Fig 3B, 3D, 3F, and 3H). Positive staining with SAM11 was also found with longitudinal and circumferential intestinal smooth muscle (Fig 3C) and with smooth muscle cells in the walls of the aorta, a coronary artery, and a medium-size artery traversing the wall of the small intestine (Fig 3C, 3E, and 3G). Finally, strikingly positive SAM11 staining was present in and around sweat glands (Fig 3N).

The tissue sections in Fig 4 focus on the expression of PAR-2 in small arteries, arterioles, small veins, and venules in the heart, colon, and skin. Arterial smooth muscle was consistently reactive with the PAR-2 antibody. Veins and venules were typically, but not always, negative. Myocardial smooth muscle was also negative. Collectively, these results
show that PAR-2 is widely distributed in human tissues, including some, but not all, vascular smooth muscle.

Expression of Functional PAR-2 in Vascular Smooth Muscle

The Northern analysis and immunohistochemistry suggested that PAR-2 is expressed in human arterial vascular smooth muscle. However, reports that rat aortic rings and porcine coronary arteries that have been denuded of endothelium neither relax nor contract in response to PAR-2 agonist peptides imply that the remaining smooth muscle cells in these preparations do not contain functional PAR-2. Therefore, to determine whether the PAR-2 present in human aortic smooth muscle is coupled to intracellular effectors, cultured aortic and saphenous vein smooth muscle cells were loaded with fura 2 to detect intracellular Ca\textsuperscript{2+} transients and stimulated with the PAR-2 agonist peptides SLIGRL and SLIGKV. These peptides activate human PAR-2, but do not activate PAR-1. For comparison, the cells were also stimulated with thrombin, which can cause smooth muscle contraction.

In the aortic smooth muscle cells, SLIGRL and SLIGKV caused the cytosolic Ca\textsuperscript{2+} concentration to increase by approximately 118 ± 25 nmol/L (mean ± SEM, n = 6, Fig 5). Thrombin had a greater effect, increasing the cytosolic Ca\textsuperscript{2+} concentration by 357 ± 92 nmol/L (n = 4). Although extensive dose-response curves were not performed, SFLLRN, which can activate both PAR-1 and PAR-2, had little effect when added after SLIGRL and thrombin (Fig 5A). This finding suggests that both receptors have been fully desensitized at the concentrations of SLIGRL and thrombin that were added and therefore that the maximal PAR-2 response in aortic smooth muscle cells is less than the maximal PAR-1 response. As previously reported, SLIGRL and thrombin also caused a Ca\textsuperscript{2+} transient in HUVECs (Fig 5C and 5D). Here, however, the magnitude of the increase in cytosolic Ca\textsuperscript{2+} by the two agonists was more nearly identical (see also Reference 35).
SFLLRN activates both.\textsuperscript{26,33,34} Pooled results from several such PAR-2 in human tissues removed at the time of surgery or the present studies, we have examined the distribution of respond than it does to broaden the range of responses.\textsuperscript{18} In to broaden the range of proteases to which the cells can activation of each appears to evoke a similar set of responses, which suggests that expression of both receptors serves more about PAR-2 or PAR-3. Tools to detect PAR-2 expression receptors. Considerably more is known about PAR-1 than what is likely to be a larger class of G protein– coupled activation but showed no response to PAR-2 agonist peptides.\textsuperscript{20,22} In comparison, PAR-2 peptides had been shown to cause transient contraction of rat gastric smooth muscle,\textsuperscript{29} and trypsin has recently been shown to cause contraction of denuded rabbit aorta, even after desensitization with thrombin.\textsuperscript{37} These apparently contradictory observations leave open the question of whether functional PAR-2 is present in vascular smooth muscle. We were interested in identifying whether PAR-2 is expressed in human vascular smooth muscle and, if so, determining whether it is functional. Because of the previous reports on the behavior of vascular rings, we were also interested in determining whether there are anatomic differences among vascular smooth muscle cells in the expression of PAR-2.

As a step toward accomplishing these goals, a monoclonal antibody was generated in mice immunized with a peptide corresponding to the sequence of human PAR-2 immediately downstream from the protease cleavage site. Preliminary studies confirmed that this antibody recognizes native PAR-2 and can discriminate between PAR-1 and PAR-2. Because of sequence differences, it would not be expected to recognize PAR-3. Immunohistochemistry showed that PAR-2 is expressed in human aortic smooth muscle, as well as in the smooth muscle cells present in human coronary arteries, colonic muscularis mucosa, dermal arteries, and intramyocardial arterioles. Veins and venules were typically, but not always, negative for PAR-2 expression. Cultured explants from human aorta were used as a source of smooth muscle for functional studies. When stimulated with the PAR-2 agonist peptides SLIGKV and SLIGRL, a transient increase in cytosolic Ca\textsuperscript{2+} was observed. In contrast, saphenous vein smooth muscle cells lacked detectable PAR-2 mRNA and showed little or no increase in cytosolic Ca\textsuperscript{2+} when stimulated with SLIGKV or SLIGRL.

These results demonstrate that functional PAR-2 is present in at least some types of human vascular smooth muscle cells, although apparently not in all types. What might account for the failure in previous studies to see a response from vascular smooth muscle attributable to PAR-2? One possibility is that there is a species difference such that human aorta and coronary artery smooth muscle cells express PAR-2, while their rat and porcine counterparts do not. The evidence for the presence of PAR-2 in the rat and pig cells was indirect and, in the case of the rat aortas in which mRNA was detected, could have been due to contaminating endothelial cells.\textsuperscript{20,32} A second possibility is that PAR-2 is present in the rat and

**Figure 5. Changes in cytosolic Ca\textsuperscript{2+} in response to PAR-1 and PAR-2 activation.** Human aortic and saphenous vein smooth muscle cells (A and B) and human umbilical vein endothelial cells (C and D) were loaded with fura 2 and then stimulated with thrombin (4 U/mL), SLIGRL (100 \(\mu\)mol/L), or SFLLRN (100 \(\mu\)mol/L), as indicated. Note that SLIGRL activates PAR-2, but not PAR-1,\textsuperscript{28} while SFLLRN activates both.\textsuperscript{33,34} Pooled results from several such studies are included in the text.

Thrombin also caused an increase in the cytosolic Ca\textsuperscript{2+} concentration when added to saphenous vein smooth muscle cells (220±50 nmol/L, mean±SEM, \(n=3\)). However, these cells, which did not contain detectable PAR-2 mRNA (Fig 1), showed little, if any, response to the PAR-2 agonist peptides (17±7 nmol/L, \(n=3\)). These results show that at least some human vascular smooth muscle cells express functional PAR-2, but they may not do so in all anatomic locations.

**Discussion**

PAR-1, PAR-2, and PAR-3 are the first three members of what is likely to be a larger class of G protein–coupled receptors. Considerably more is known about PAR-1 than about PAR-2 or PAR-3. Tools to detect PAR-2 expression have become available only recently, and neither antibodies nor selective agonists are yet available to study PAR-3. In endothelial cells, in which PAR-1 and PAR-2 are expressed, activation of each appears to evoke a similar set of responses, which suggests that expression of both receptors serves more to broaden the range of proteases to which the cells can respond than it does to broaden the range of responses.\textsuperscript{18} In the present studies, we have examined the distribution of PAR-2 in human tissues removed at the time of surgery or organ donation, with particular attention to the presence of PAR-2 in vascular smooth muscle. Previous studies had shown that PAR-2 mRNA is present in rat aorta\textsuperscript{32} and that PAR-2 agonists cause vascular relaxation.\textsuperscript{20,22,32,36} However, most, if not all, of this effect appeared to be mediated by endothelium-derived NO.\textsuperscript{22,36} When anesthetized rats were infused with the murine PAR-2 agonist peptide SLIGRL, a sustained drop in blood pressure was observed, which is in contrast to the response to infusing a PAR-1 agonist peptide, which causes a sequential fall in blood pressure due to NO release followed by an increase in pressure attributed to vascular smooth muscle contraction.\textsuperscript{22} Isolated rat aortic rings denuded of endothelium contract in response to PAR-1 activation but showed no response to PAR-2 agonist peptides.\textsuperscript{20,22} In comparison, PAR-2 peptides had been shown to cause transient contraction of rat gastric smooth muscle,\textsuperscript{29} and trypsin has recently been shown to cause contraction of denuded rabbit aorta, even after desensitization with thrombin.\textsuperscript{37} These apparently contradictory observations leave open the question of whether functional PAR-2 is present in vascular smooth muscle. We were interested in identifying whether PAR-2 is expressed in human vascular smooth muscle and, if so, determining whether it is functional. Because of the previous reports on the behavior of vascular rings, we were also interested in determining whether there are anatomic differences among vascular smooth muscle cells in the expression of PAR-2.

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porcine arteries that were studied, but at levels too low to be detected. For PAR-1, it has been shown that the magnitude of the response to thrombin is determined in part by the number of receptors that are cleaved in a short period of time.\(^2\)\(^3\) The observation that the response of the human aortic smooth muscle cells to SLIGRL was smaller than the response of the same cells to thrombin (Fig 4) suggests that PAR-2 expression is less than the expression of PAR-1 and, potentially, PAR-3—although this hypothesis has not been tested directly with formal binding studies. A final possibility is that PAR-2 activation in vascular smooth muscle cells is needed to evoke responses other than contraction or relaxation. In HUVECs, PAR-1 and PAR-2 agonists appear to evoke the same repertoire of responses. This need not be the case in smooth muscle, although Al-Ani and coworkers\(^20\) have shown that rat gastric smooth muscle preparations contract when stimulated with PAR-2 agonist peptides. PAR-2 has been shown to be activatable by tryptase released from mast cell secretory granules.\(^4\)\(^–\)\(^6\) Given the presence of mast cells in arterial walls, especially in and around atherosclerotic plaques, it is reasonable to speculate that secreted tryptase may interact with PAR-2 on smooth muscle as well as endothelial cells in this setting.

Finally, in addition to demonstrating the presence of functional PAR-2 in human aortic smooth muscle cells, the present studies also confirm that PAR-2 is expressed in human keratinocytes\(^23\) and show that PAR-2 can be detected in sweat glands and in intestinal epithelium and smooth muscle, as it has been in mice.\(^24\) The role of PAR-2 in the skin is unknown. Its role in the intestinal tract is also unknown, but the observation that PAR-2 agonists contract rat gastric smooth muscle\(^25\) and the presence of a known PAR-2 activator (trypsin) in pancreatic secretions suggest at least a possible role in intestinal motility. The role played by the PAR-2 detected immunologically in intestinal epithelium in the present study and by in situ hybridization in mouse intestinal epithelium in a previous study\(^26\) awaits further investigation.

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