Introduction

Thrombin, generated at sites of vascular injury, is both a key enzyme in thrombosis and haemostasis and a mediator of cellular effects that contribute to inflammatory reactions and proliferation of smooth muscle cells (SMC) in the vessel wall. Thrombin is thought to induce cellular actions via activation of three distinct protease-activated receptors (PARs), PAR-1, PAR-3, and PAR-4. PARs belong to a subfamily of putative seven transmembrane domain G-protein-coupled receptors. Activation of PARs involves proteolytical cleavage of their amino terminal exodomain, thereby unmasking a new amino-terminus that binds to the body of the receptor as a tethered ligand to induce intracellular signaling (1-3). Synthetic peptides, representing the sequence of the newly exposed tethered ligand, can directly activate their respective receptor, i.e. independently of receptor cleavage (1, 4). Thus, PAR-selective activating peptides (APs) have proven useful to distinguish between distinct PAR-subtypes that can be activated by thrombin and that are concomitantly expressed in the same cells.

PAR-3, like PAR-1 and PAR-4, has been shown to be a thrombin substrate in transfected COS7 cells at comparable efficiency as that towards PAR-1. In addition, experiments with PAR-3-transfected Xenopus oocytes indicated that thrombin caused a robust calcium signal, whereas other arginine/lysine-specific serine proteases, such as factor Xa, trypsin, factor VIIa, tissue evidence for functionally active protease-activated receptor-3 (PAR-3) in human vascular smooth muscle cells

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Summary
The present study investigates whether vascular smooth muscle cells of the human saphenous vein (SMC) express a functionally active protease-activated receptor-3 (PAR-3). PAR-3 mRNA was detected by RT-PCR. In the presence of thrombin, a rapid and transient increase in PAR-3 mRNA was observed. Stimulation of SMC with thrombin or the synthetic PAR-3-activating peptide, TFRGAP, resulted in transient mobilization of intracellular calcium. After a preceding challenge with thrombin, the calcium signal to TFRGAP was abolished, suggesting cleavage and subsequent desensitization of PAR-3 by thrombin. Activation of PAR-3 by TFRGAP elicited a time-dependent activation of the extracellular-signal-regulated kinase (ERK)-1/2 with a maximum response 10-20 min after stimulation. At 200 µM, TFRGAP increased [3H]-thymidine incorporation into cellular DNA about two-fold. These data indicate that PAR-3 is expressed in human SMC and triggers intracellular signaling. Thus, in the SMC PAR-3 might contribute to thrombin-induced responses.

Keywords
Vascular smooth muscle cells, protease-activated receptor-3, signaling

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Materials and methods

Materials

Synthetic PAR-APs (PAR-1 AP: SFLLRN-NH₂, PAR-3 AP: TFRGAP-NH₂, Biosyntan, Berlin, Germany); phospho-p44/42 MAP kinase (ERK-1/2) monoclonal antibody (New England Biolabs, Beverly, MA, USA); horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); [³H]-thymidine (NEN Life Science Products, Boston, MA, USA); fluo-4 acetoxymethyl ester (Molecular Probes, Leiden, The Netherlands). Purified α-thrombin was kindly provided by Dr. J. Stürzebecher, Zentrum für Vaskuläre Biologie und Medizin Erfurt, Klinikum der Friedrich-Schiller-Universität Jena, Germany. Cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany).

Cell culture

Specimens of saphenous veins were obtained from patients undergoing aortocoronary bypass surgery. SMC were isolated by explant technique and cultured as described previously (14). SMC from passages 4 - 8 were used for the experiments.

Detection of PAR-1 and PAR-3 mRNA

mRNAs of PAR-1 and PAR-3 were identified by RT-PCR and sequences were confirmed. To investigate the effect of thrombin on regulation of PAR-1 and PAR-3 mRNA, SMC were growth-arrested in serum-free culture medium for 24 hours and thereafter stimulated by 100 nM thrombin for different periods of time. Total mRNA was isolated from cultured SMC with Trizol-reagent (peQLab, Erlangen, Germany). RT-PCR was performed with Qiagen® One-Step RT-PCR kit (Hilden, Germany) using 0.25 µg total RNA and the following primers: PAR-1: sense CCA CGCGCATGTGCTGTGTTTG; antisense TAGG CACGGCTCTGTGGTGAAG resulting in a 615 bp fragment; PAR-3 sense TGCAAGACCAA CTTACC; antisense GCCAAGGACGAG AATGGGAGC resulting in a 466 bp fragment. For semiquantitative analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was co-amplified and PAR PCR products were expressed relative to GAPDH PCR product.

The following cycling parameters were used: 1 min 94°C, 1 min 58°C, 1 min 72°C, followed by 28 cycles and 30 cycles for PAR-1 and PAR-3, respectively, and a final elongation step for 15 min at 72°C. Ten µl of each PCR product was separated by agarose gel electrophoresis and visualized using ethidium bromide.

Measurement of intracellular calcium

SMC were grown to confluency on glass cover slides and loaded with fluo-4 acetoxymethyl ester (2 µM) for 30 min at 37°C. After washing with HEPES-buffered salt solution containing NaCl (145 mM), KCl (5 mM), MgSO₄ (1 mM), CaCl₂ (1.5 mM), HEPES (10 mM), glucose (10 mM) and human serum albumin (0.25%) the cells were mounted on a 37°C heated cell culture unit in HEPES-buffer and calcium responses were examined by using an Axiovert 200M inverted microscope equipped with a confocal laser scanning head LSM510, a 20X/0.5 Plan Neofluar objective, and an argon laser (488 nm) (C. Zeiss Jena, Germany). Data for graphs were recorded using LSM510 software.

Phosphorylation of ERK-1/2

Prior to stimulation, confluent SMC were growth-arrested in serum-free culture medium for 24 hours. Phosphorylation of ERK-1/2 was detected in cell lysates by Western blotting. Extracts of SMC were prepared in sodium dodecyl sulphate (SDS) lysis buffer (2% w v⁻¹ SDS; 10% glycerol; 62.5 mM Tris-
HCl, pH 6.8; 50 mM dithiothreitol; 0.1% w v−1 bromphenol blue). Proteins were separated by SDS polyacrylamide (10%) gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bredford, USA). The membranes were blocked in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween) containing 5% w v−1 non-fat dry milk and then incubated with a phospho-specific antibody directed against ERK-1/2 (1:1000) for 24 h at 4°C. After washing three times (15 min each) in TBS-T membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000) for 60 min. After washing three times with TBS-T immunoreactive bands were visualized by chemiluminescence (Roche Diagnostics, Mannheim, Germany).

**Measurement of [3H]-thymidine incorporation**
The increase in DNA synthesis was determined by measuring [3H]-thymidine incorporation. SMC seeded in 24-well plates (4 x 10⁴ cells/ml, 0.5 ml/well) were growth-arrested in serum-free culture medium for 24 h. During the following 24 h cells were stimulated by the indicated agents. Four hours prior to harvesting, SMC were labelled with [3H]-thymidine (2 µCi/ml and [3H]-thymidine incorporation into DNA was determined as described (14).

**Statistical analysis**
The data are expressed as means ± SEM of n independent experiments. Statistical analysis was performed by two-tailed Student t-test. P values of < 0.05 were considered significant. To determine relative PAR mRNA levels PCR products obtained by gel electrophoresis were analyzed densitometrically (ScanPack, version 14.1A27). For unstimulated cells and at each time point after thrombin stimulation the ratio of the PAR mRNA and GAPDH was calculated. For each experiment the ratio in unstimulated cells was related to the ratio in stimulated SMC by use of the Kruskal-Wallis test. ERK-1/2 phosphorylation was quantified densitometrically (Scion Image, NIH).

**Results**

**Expression of PAR-3 mRNA**
mRNAs for PAR-3 and PAR-1 were expressed in both SMC maintained in FCS-containing medium (n = 8, not shown) and in SMC maintained in serum-free medium for 24 hours (n = 4-6) at comparable levels (Fig. 1). Regulation of PAR-1 and PAR-3 mRNAs was analysed in thrombin (100 nM)-stimulated SMC over 24 hours. Figure 1, upper panel, demonstrates a significant transient increase in PAR-3 mRNA above control after 5 min followed by a decrease and a second peak after 3 hours. Under the same conditions, expression of PAR-1 mRNA remained unchanged (Fig. 1, lower panel).

**Mobilization of intracellular calcium**

Stimulation of SMC with thrombin at 10 nM induced a transient rise in [Ca²⁺], (Fig. 2A). When the cells were stimulated for a second time with thrombin, no further calcium signal was observed (not shown). PAR-3-AP TFRGAP (200 µM) also elicited a calcium response. However, calcium signaling to thrombin was maintained after the prior application of TFRGAP (Fig. 2B). In contrast, after a preceding challenge with thrombin
When the cells were stimulated twice with TFRGAP, another though diminished calcium signal was obtained following the second stimulation (Fig. 2C). To ascertain the functionality of intracellular calcium signaling, ionomycin (5 μM)-induced rise in [Ca^{2+}]], was determined at the end of each experiment (not shown).

**Phosphorylation of ERK-1/2**

Stimulation of the SMC with TFRGAP (200 μM) induced a time-dependent activation of ERK-1/2. A maximum response was observed during a time window of 10-20 min reaching near control values after 60 min (Fig. 3A). Interestingly, a maximum response to 10 nM thrombin has been shown to occur after 5-10 min (13). To ascertain that each SMC population was responsive to thrombin, ERK-1/2 phosphorylation by thrombin (10 nM) after a stimulation period of 10 min was determined in parallel. Figure 3B shows quantification of ERK-1/2 phosphorylation 10 min after stimulation with TFRGAP or thrombin as compared to unstimulated control.

**Stimulation of [³H]-thymidine incorporation into DNA**

Stimulation of SMC with TFRGAP (100 μM - 500 μM) caused a concentration-dependent increase in [³H]-thymidine incorporation. At 200 μM, the peptide induced an about two-fold increase in DNA synthesis. There was a further, slight increase after stimulation with 500 μM (n.s.). For comparison, the mitogenic effects of 10 nM thrombin or 200 μM of the PAR-1-activating peptide SFLLRN were also demonstrated (Fig. 4).

**Discussion**

Thrombin, generated at sites of endothelial damage may bind to SMC and has been suggested to be involved in the SMC response to injury through activation of PARs and subsequent intracellular signaling. In SMC of saphenous vein, thrombin has been shown to induce cellular signaling via activation of PAR-1 (15) and PAR-4 (13). The present study demonstrates for the first time that SMC also express PAR-3 and that PAR-3 can trigger transmembrane signaling on its own. This conclusion is based on several lines of evidence: i) PAR-3 mRNA was detected by RT-PCR in SMC; ii) stimulation with the PAR-3 AP TFRGAP resulted in a transient mobilization of intracellular calcium; iii) TFRGAP elicited a time-dependent activation of ERK-1/2; iv) stimulation of the SMC with the PAR-3 AP induced a mitogenic effect.

The desensitization experiments on mobilization of intracellular calcium provided evidence that PAR-3 is a thrombin receptor. Stimulation of the cells with either thrombin (10 nM) or the PAR-3 AP TFRGAP (200 μM) resulted in a transient mobilization of intracellular calcium. The calcium response to thrombin was maintained after a prior application of TFRGAP, indicating that the peptide did not affect PAR-1/PAR-4 activation by thrombin. In contrast, after a preceding challenge with thrombin, the calcium signal to TFRGAP was abolished, suggesting that thrombin-cleaved PAR-3 has now become insensi-
tive for the PAR-3-AP. When the cells were treated twice with thrombin no further calcium response to thrombin was detected after the first application, suggesting homologous receptor desensitization. Interestingly, when TFRGAP was applied for a second time, a further albeit smaller calcium signal was observed. The very short C-terminal tail of PAR-3 may result in potential differences in signaling and desensitization relative to the other PARs (16-17). The irreversible proteolytic mechanism of PAR activation raises the question of how these receptors are shut off. For PAR-1, phosphorylation within the C-terminus has been shown to be important for rapid termination of receptor signaling and internalization, consistent with the action of G-protein receptor kinases (16, 18). PAR-1 is sequestered from the cell surface into coated pits and then into endosomes within 60 s of activation (19). Peptide-activated receptors are also rapidly phosphorylated and follow the same route, suggesting that cleavage of PAR-1 is not required for internalization (20). The details of the subtle distinctions for desensitization/internalization and trafficking properties among the PARs remain to be determined. The ERK-1/2 is known to play a central role in diverse signaling cascades, including those leading to cell proliferation, differentiation, and survival (21). Recently, it has been shown that thrombin can activate ERK-1/2 in SMC concentration-dependently and with distinct kinetics via PAR-1 and PAR-4, respectively. While activation of PAR-1 resulted in a maximum phosphorylation of ERK-1/2 after 5 min, the PAR-4 AP GPYQGV induced a maximum effect only after 60 min (13). TFRGAP was also found to activate ERK-1/2, however, its maximum response was detected 10-20 min after stimulation. These data suggest that the three PARs mediate intracellular signaling with distinct kinetics. The increase in [%H]-thymidine incorporation in TFRGAP-stimulated cells provides compelling evidence that PAR-3 might also contribute to thrombin-induced proliferation of SMC.

Our observation that human PAR-3 can mediate its own signaling in SMC is in agreement with the finding on COS7 cells transfected with human PAR-3 in which thrombin triggered robust phosphoinositide signaling (2). In contrast, COS7 cells, expressing murine PAR-3, did not mediate a response to thrombin (8). Thus, it is suggested that human and murine forms of PAR-3 may have different, species-specific functions (11, 16). The present study indicates that the PAR-3-AP is active. This finding is consistent with a report by Wang et al. (6) that demonstrated that the peptide mimicking the tethered ligand of cleaved PAR-3, TFRGAP, can elicit cellular signaling in rat astrocytes.

From the present data it is concluded that the SMC express all of the three known thrombin receptors, i.e. PAR-1, PAR-3, and PAR-4. A well known example for co-expression of more than one PAR are human platelets. Activation of either PAR-1 or PAR-4 alone was sufficient to trigger platelet secretion and aggregation (12). However, there were differences in activation, signaling and shutoff between the two PARs. Both, PAR-1 and PAR-4, were found to be coupled to Gs but unlike PAR-1, PAR-4 was not coupled to Gi (4). Moreover, PAR-4 activation required higher thrombin concentrations and was shut off less rapidly compared to PAR-1 probably due to differences in receptor phosphorylation (12-13, 22). Thus, it will be of interest to determine the G proteins that couple to PAR-3 in SMC.

The kinetics of PAR-3 activation, signaling and shutoff remain to be elucidated. Nevertheless, the availability of different PARs with cell- and tissue-specific distribution might allow thrombin to trigger distinct signaling pathways with different kinetics as well as differential regulation of receptor levels or function (17). Indeed, the finding that the exposure of SMC to thrombin-induced regulation of PAR-3 but not PAR-1 indicated differences in the regulation of distinct PARs.

In summary, the present study demonstrates that in addition to PAR-1 and PAR-4, SMC from the human saphenous vein express functionally active PAR-3. The biological significance of PAR-3 remains to be determined. However, it is tempting to speculate that during endothelial injury and resulting thrombin generation PAR-3 might be rapidly up-regulated in SMC and subsequently can serve as a receptor reservoir for thrombin signaling. The use of selective PAR-activating agonists and the development of selective antagonists for each of the PARs may provide further insights into the role of PARs in thrombin-induced effects on human SMC and other cells. This, in turn, raises the possibility to develop therapeutics that selectively block thrombotic, inflammatory and proliferative responses to thrombin (2).

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