

Distinct roles for PAR1- and PAR2-mediated vasomotor modulation in human arterial and venous conduits

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Summary. *Background:* Patency rates after coronary artery bypass grafting (CABG) are better if the internal mammary artery (IMA) is used rather than the greater saphenous vein (GSV), and may be related to the endothelial release of vasodilators antagonizing vascular contraction. It has recently been shown that a family of protease-activated receptors (PARs) modulate endothelium-dependent vasodilatation. *Objective and methods:* The aim of this study was to evaluate the presence and functional role of protease-activated receptor 1 (PAR1) and protease-activated receptor 2 (PAR2) in mediating vascular tone in IMAs and GSVs from patients undergoing CABG by means of real time-PCR and isometric tension measurements. *Results:* PAR1 mRNA levels were higher than those of PAR2 mRNA in both vessels. A selective PAR2-activating peptide (PAR2-AP), SLIGKV-NH₂ (0.01–100 μmol L⁻¹), failed to induce vasorelaxation in precontracted IMA and GSV rings, whereas the selective PAR1-AP, TFLLR-NH₂ (0.001 to 10 μmol L⁻¹), caused greater endothelium-dependent relaxation in the IMAs (pD₂ values 7.25 ± 0.6 vs. 7.86 ± 0.42, *P* < 0.05; *E*_{max} values 56.2 ± 17.3% vs. 29.7 ± 13.4%, *P* < 0.001). Preincubation with TNFα (3 nmol L⁻¹) induced vasorelaxation in IMAs in response to PAR2-AP (*P* < 0.05 vs. non-stimulated vessels); the response to PAR1-AP was unchanged. The relaxation induced by both PAR-APs was NO- and endothelium-dependent. *Conclusion:* These data show that functionally active PAR1 and PAR2 are present in IMAs and GSVs, and that inflammatory stimuli selectively enhance endothelium-dependent relaxation to PAR2-AP in IMAs.

Keywords: coronary artery bypass, grafting isometric tension, mammary artery, protease-activated receptors, saphenous vein, TNF-alpha.

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Introduction

Protease-activated receptors (PARs) are a newly discovered class of receptors that control vascular tone and permeability in both physiological and pathophysiological states [1,2]. They are serine protease-sensitive, G-coupled receptors that are widely expressed in different tissue and cell types, but have a single mechanism of activation: serine proteases remove their amino-terminal domain and expose a neo-tethered ligand that binds and activates the receptors to initiate multiple intracellular signalling events. Only serine proteases have been identified as endogenous ligands for PARs, but the existence of other ligands (e.g. endogenous peptides) has been hypothesized [1–3], and the receptors are also activated by synthetic PAR-activating peptides (PAR-APs) without prior proteolysis.

Four PARs have so far been cloned (PAR1–4), of which PAR1 and PAR2 are the best characterized and have been shown to be involved in regulating vascular tone [4]. Thrombin is the selective enzyme activator for PAR1, PAR3 and PAR4, whereas trypsin is the selective activator for PAR2 and PAR4 [2]; others include tryptase, coagulation factors (F) VIIa and Xa, and the TF/FVIIa/Xa complex [5–8].

Coronary artery bypass grafting (CABG) is a surgical procedure used to treat advanced coronary artery disease, but the improvement in symptoms and reduction in long-term mortality is highly influenced by which vascular conduit is adopted [9]. The autologous great saphenous vein (GSV) is still widely used, although its relatively poor long-term patency means that at least 50% of these grafts are occluded 10 years after surgery [10–12], but the use of the internal mammary artery (IMA) has improved long-term patency rates (up to 90% 10 years after surgery) as well as mortality and morbidity, and the better clinical results become increasingly pronounced over 20 postoperative years [12–19]. The difference in patency rates between arterial and venous grafts may be related to the endothelial release of vasodilating substances that antagonize vascular contraction, such as nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF) [20,21].

Over the last few years, considerable efforts have been made to investigate the roles of PARs in controlling vascular tone in animal models, but less information is known about the effects of PARs on human vasculature. Thrombin and selective PAR1-APs cause endothelium-mediated relaxation of precontracted human basilar, pulmonary, coronary and internal mammary arteries [22–26], and contraction in response to thrombin has also been reported [26,27]. Activating PAR2 in human pulmonary and coronary arteries has failed to modify the vasculature response [28], but exposing the coronary arteries to inflammatory stimuli before PAR2-activating peptide (PAR2-APs) led to endothelial-dependent relaxation [28].

The aim of this study was to compare the effects of PAR2 and PAR1 activation using specific activating peptides on human IMAs and GSVs isolated from patients undergoing CABG. To this end, we studied: (i) the presence of PAR1 and PAR2 in IMAs and GSVs; and (ii) the role and regulation of PARs in mediating vascular tone.

Patients and methods

Patient profiles and vessel preparations

Segments of human IMAs ($n = 50$) and SVGs ($n = 30$) were collected from 80 patients undergoing elective coronary artery bypass surgery: 75 males and five females with a mean age of 64 ± 8.8 years (range: 43–84). The patients were receiving standard medication, including ACE inhibitors (36.4%), aspirin (21.2%), β -blockers (62.1%), calcium antagonists (27.3%), diuretics (6.1%), heparin (31.8%), nitrates (57.6%) and statins (4.6%). Permission to use the discarded IMA and GSV tissue was given by the Ethics Committee of the Centro Cardiologico Monzino.

The patients were given heparin (3 mg kg^{-1}) before the securing and transection of the distal end of both vessels, neither of which were predistended. The IMAs were harvested and pedicled by means of low-voltage electrocautery, and the branches were divided between two metallic clips using scissors; the GSVs were harvested by means of a no-touch, atraumatic technique using fine forceps only on the surrounding fascial tissue, and the branches were secured with small vascular clips.

Once collected, the segments of the IMAs (macroscopically free of atheromatous plaque) and GSVs were immediately placed in ice-cold Krebs solution and transferred to the laboratory.

Materials

The acetylcholine and N^G -nitro-L arginine methyl ester (L-NAME) used in the study came from Sigma-Aldrich (St Louis, MO, USA), the U46619 (9,11-dideoxy-9 α ,11 α -epoxymethanoprostaglandin F2 α) from Cayman Chemical Company (Ann Arbor, MI, USA), the indomethacin from Chiesi Farmaceutici S.p.A. (Parma, Italy), and the recombinant human TNF α from R&D Systems (Minneapolis, MN, USA). The specific PAR1-

AP (TFLLRN-NH $_2$) and PAR2-AP (SLIGKV-NH $_2$) tethered ligands (Auspep Pty Ltd, Parkville, Australia) were certified for purity and the absence of endotoxin contamination, and dissolved in 10% methanol/distilled water. The monoclonal antibodies (mAb) against PAR1 (ATAP-2) and PAR2 (SAM 11) came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and the mAb CD31 from Dako A/S (Glostrup, Germany).

RT-PCR and quantitative real-time PCR

Freshly isolated vessel segments ($n = 10$) were snap-frozen in liquid nitrogen and stored at -80°C until total RNA extraction, which was performed as previously described [29]. For the RT-PCR experiments, 200 ng of RNA were reverse transcribed using 20 U reverse transcriptase (Stratascript; Stratagene, La Jolla, CA, USA) and underwent 35 PCR cycles (denaturation at 94°C , 30 s; annealing at 55°C , 30 s; extension at 72°C , 30 s) in a 100- μL reaction mixture containing 2.5 U *Taq* DNA polymerase and 200 nmol L^{-1} sense and antisense PAR1, PAR2, endothelial NO synthase (eNOS) and GAPDH primers [28].

Quantitative real-time PCR was then used to detect PAR1 and PAR2 mRNA in control and stimulated vessels, with 2.5 μL of cDNA being incubated in 25 μL IQ Supermix containing 500 nm of PAR1 or PAR2 primers [28], or 50 nm of 18S primers used for sample normalization, and fluorescence dye SYBR-Green (Bio-Rad Laboratories, Hercules, CA, USA). The optimized two-step protocol consisted of 50 cycles of denaturation at 95°C for 15 s, and annealing/extension for 60 s at 58°C for PAR1 and 60°C for PAR2. The specificity of the amplified products was monitored by means of melting curves at the end of each amplification. Real-time RT-PCR was carried out in triplicate for each sample using an iCycler Optical System (Bio-Rad Laboratories).

Western blotting

The vessels were solubilized in lysis buffer, submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (50 μg protein/line), transferred to nitrocellulose, and incubated with anti-PAR1 and anti-PAR2 mAb. The immunoblotting was developed using enhanced chemiluminescence (ECL).

Immunohistochemistry

The vessels were fixed in 10% buffered formalin, paraffin embedded, and 6 μm thick serial sections were cut and used for the immunohistochemical identification of PAR1 and PAR2. The sections were treated with 10% normal horse serum and endogenous biotin was quenched using a Vector Blocking kit (Vector Laboratories Ltd, Peterborough, UK). The slides were incubated overnight with primary antibodies, which were then detected using a biotin/streptavidine-amplified detection system and developed by diaminobenzidine (Sigma-Aldrich).

Isometric tension measurements

The vessels were dissected from the surrounding fatty tissues and cut into rings of equal length, which were suspended on wires in an organ bath filled with Krebs solution (37 °C, 95% O₂ and 5% CO₂). The rings were then connected to force transducers (Fort 10; World Precision Instruments Inc., Sarasota, FL, USA) in order to record their isometric tension. After 90 min equilibration, functional viability was assessed by means of maximum vasoconstriction to high-potassium (KCl 100 mmol) Krebs solutions (KPSS), which was then replaced with normal Krebs solution and the samples were equilibrated for 45 min. The presence of functional endothelium was checked by verifying vasorelaxation to acetylcholine (ACh, 1 μmol L⁻¹) of vessel rings precontracted with the thromboxane A₂ mimetic U46619 (3–30 nmol L⁻¹).

All of the vessels fulfilling these criteria were used to study the vasorelaxation induced by PAR1-AP and PAR2-AP. In particular, the segments were contracted to ~50% KPSS_{max} with U46619 (3–30 nmol L⁻¹) and cumulative concentrations of the selective PAR1-AP (0.001–10 μmol L⁻¹) and PAR2-AP (0.001–100 μmol L⁻¹) were added to the organ bath. In some experiments, the IMAs or GSVs were preincubated with TNFα (3 nmol L⁻¹) for 14 h at 37 °C (95% O₂ and 5% CO₂). Experiments were also carried out in the absence of endothelium or in the presence of indomethacin (10 μmol L⁻¹, 20 min) or L-NAME (100–300 μmol L⁻¹, 20 min). In additional experiments, the responses to PAR1-AP and PAR2-AP were monitored in non-precontracted vessels from which the endothelium had been removed.

Statistical analysis

The data were analyzed off line using the Data Pad window of the Chart software and then imported into Profit, and the concentration-response curves were fitted to a four-parameter logistic equation using the Levenberg-Marquardt algorithm. All of the data were expressed as mean values ± SD.

The maximum response (E_{max}) and half-maximum effective concentration (EC₅₀) were calculated from the individual concentration-response curves. Sensitivity to the agonists was expressed as the negative logarithm of the EC₅₀ (pD₂).

The data were statistically analyzed using Student's *t*-test or ANOVA, followed by Tukey's *post hoc* test where appropriate. A *P*-value of < 0.05 was considered statistically significant.

Results

PAR1 and PAR2 expression

The RT-PCR experiments using RNA isolated from IMAs led to the amplification of a single fragment of the predicted size for PAR1 and PAR2, thus suggesting that both receptors are expressed in IMAs. The co-amplification of eNOS indicated the presence of endothelium in the analyzed vessels (Fig. 1,

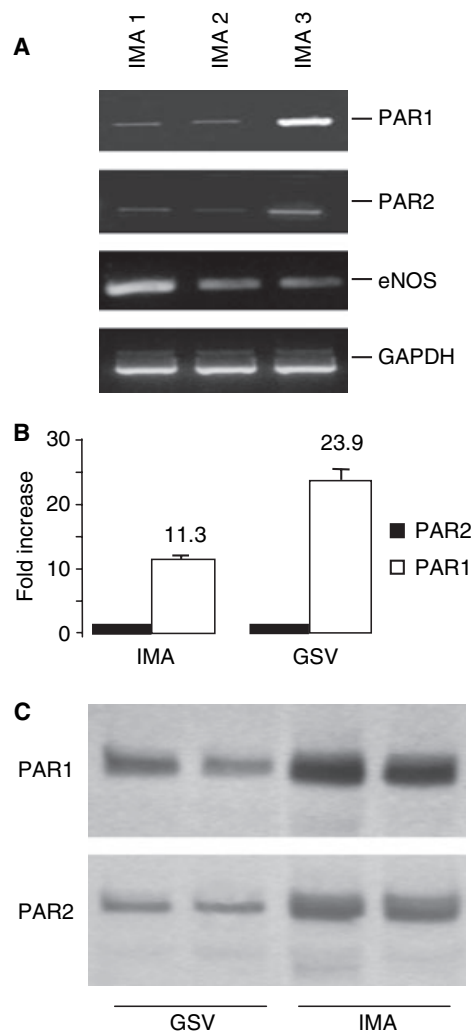


Fig. 1. Protease-activated receptor (PAR) 1 and PAR2 expression in internal mammary artery (IMA) and greater saphenous vein (GSV). Panel A: PAR1, PAR2 and endothelial NO synthase (eNOS) mRNA levels in IMA were determined by means of RT-PCR. The eNOS mRNA was amplified in order to verify the presence of intact endothelium. GAPDH mRNA was used as internal standard. The figure shows a representative experiment using three different vessels. Panel B: Quantitative real-time PCR analysis of PAR1 and PAR2 mRNA levels in IMA and GSV. The numbers above the bars indicate PAR1 mRNA levels in relation to PAR2 in each vessel. Mean values of five experiments performed in triplicate. Panel C: Western blot analysis of PAR1 and PAR2 in IMA and GSV. The proteins were extracted from dissected vessels, solubilized in lysis buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (50 μg protein/line), and revealed as described in Patients and methods. The figure shows a representative experiment using two different vessels.

panel A). Quantitative real-time PCR analysis showed that the levels of PAR1 mRNA were 10–20 times those of PAR2 in IMAs and GSVs ($P < 0.05$; Fig. 1, panel B). Western blotting indicated that PAR1 and PAR2 protein levels mirrored the expression of mRNA (Fig. 1, panel C). Both PAR1 and PAR2 were expressed in endothelial (CD31+) and smooth muscle cells from both vessels (Fig. 2).

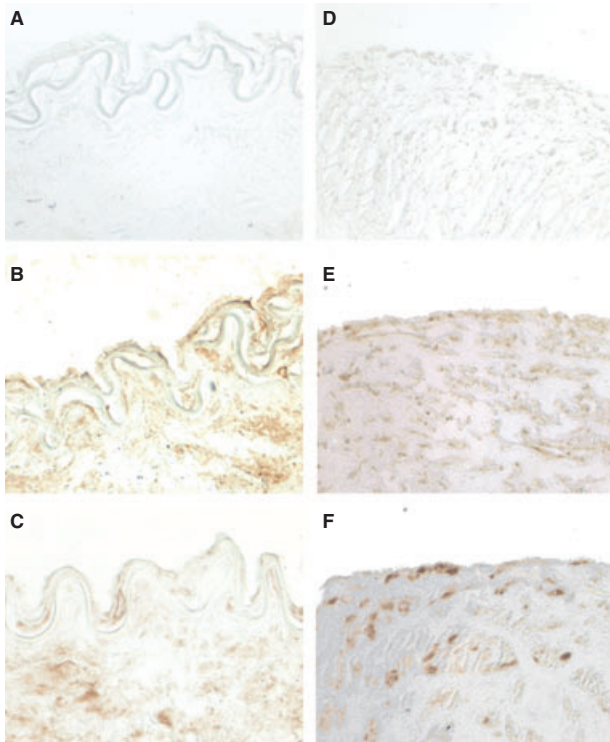


Fig. 2. Immunostaining for protease-activated receptor (PAR) 1 and PAR2 in sections from internal mammary artery (IMA) (A, B, C) and greater saphenous vein (GSV) (D, E, F). PAR1 and PAR2 were respectively detected by monoclonal antibodies ATAP-2 ($4 \mu\text{g mL}^{-1}$; panels B and E) and SAM11 ($4 \mu\text{g mL}^{-1}$; panels C and F). A and D: negative control (no primary antibody) (magnification $400\times$).

Vascular response to PAR2 agonist

PAR2-AP did not evoke the relaxation of IMAs precontracted with U46619 ($n = 6$ from five patients) or GSVs ($n = 4$ from four patients). As it has been previously shown that PAR2-AP induces endothelium-dependent relaxation in coronary arteries after prolonged incubation with proinflammatory cytokines [28], further experiments were carried out in IMAs and GSVs preincubated with $\text{TNF}\alpha$ for 14 h. In this case, PAR2-AP induced consistent relaxation in IMAs, which reached a peak at $100 \mu\text{mol L}^{-1}$ ($50.2 \pm 11.3\%$, $n = 8$ from six patients, $P < 0.001$) (Fig. 3, panel A), but failed to induce any appreciable relaxation in $\text{TNF}\alpha$ -preincubated GSVs ($n = 12$ from six patients). In the $\text{TNF}\alpha$ -preincubated IMAs, the relaxation induced by PAR2-AP was abolished by endothelium removal ($P < 0.001$) and significantly attenuated in the presence of L-NAME $300 \mu\text{mol L}^{-1}$ ($P < 0.05$) (Fig. 3, panel B), but not in the presence of indomethacin $10 \mu\text{mol L}^{-1}$. In quiescent IMAs without endothelium, PAR2-AP had no effect on vascular tone (not shown).

The increase in IMA relaxation by PAR2-AP following preincubation with $\text{TNF}\alpha$ was accompanied by an increase, although not statistically significant, in PAR2 mRNA expression (Fig. 4).

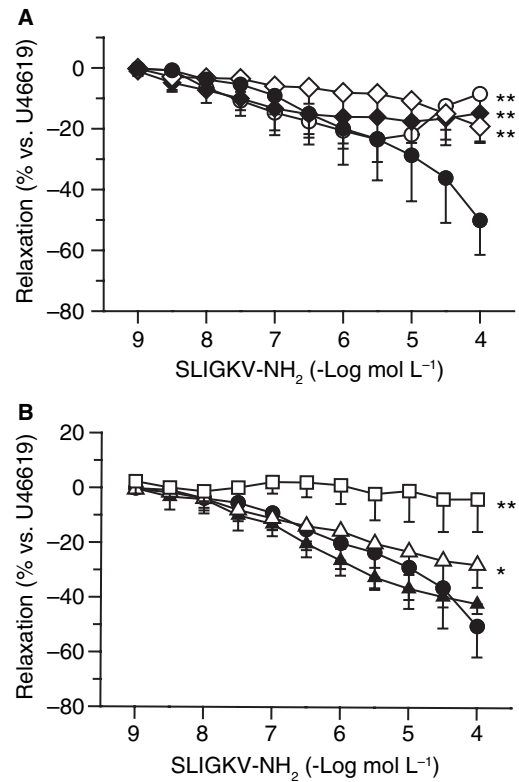


Fig. 3. Panel A. Relaxation response curves to the selective protease-activated receptor 2-activating peptide (PAR2-AP) in U46619-contracted internal mammary artery (IMA) and greater saphenous vein (GSV) rings. Open circles = IMA; filled circles = $\text{TNF}\alpha$ -treated IMA (3 nmol L^{-1}); open diamonds = GSV; filled diamonds = $\text{TNF}\alpha$ -treated GSV. $**P < 0.001$ vs. $\text{TNF}\alpha$ -treated IMA (two-way ANOVA). Panel B. Effect of endothelium denudation and N^G -nitro-L arginine methyl ester (L-NAME) treatment on PAR2-AP in $\text{TNF}\alpha$ -treated IMA. Filled circles = IMA; filled triangle = IMA with L-NAME $100 \mu\text{mol L}^{-1}$; open triangle = IMA with L-NAME $300 \mu\text{mol L}^{-1}$; open square = endothelium-denuded IMA. $*P < 0.05$ L-NAME $300 \mu\text{mol L}^{-1}$ vs. $\text{TNF}\alpha$ -treated IMA. $**P < 0.001$ endothelium-denuded vs. $\text{TNF}\alpha$ -treated IMA (two-way ANOVA). Mean values \pm SD.

Vascular response to PAR1 agonist

PAR1-AP caused greater relaxation in IMAs precontracted with U46619 ($n = 10$ from eight patients) than in GSVs ($n = 8$, from five patients) ($P < 0.001$) (Fig. 5, panel A). The E_{max} values were respectively $56.2 \pm 17.3\%$ and $29.7 \pm 13.4\%$ ($P < 0.001$), and the pD_2 values were also higher in the IMAs than in the GSVs (7.25 ± 0.60 and 7.86 ± 0.42 , respectively; $P < 0.05$).

In the IMAs preincubated with $\text{TNF}\alpha$, the relaxation induced by PAR1-AP was not significantly different from that observed in untreated vessels (E_{max} values $50.4 \pm 16.4\%$ and pD_2 values 6.76 ± 1.08), and the increase in PAR1 mRNA was only 10% ($n = 5$, from five patients) (Fig. 4).

The relaxation response of the IMAs and GSVs to PAR1-AP was abolished by endothelium removal and treatment with L-NAME $100 \mu\text{mol L}^{-1}$ ($P < 0.001$) (Fig. 5, panels B and C), but not by treatment with indomethacin $10 \mu\text{mol L}^{-1}$ (not shown).

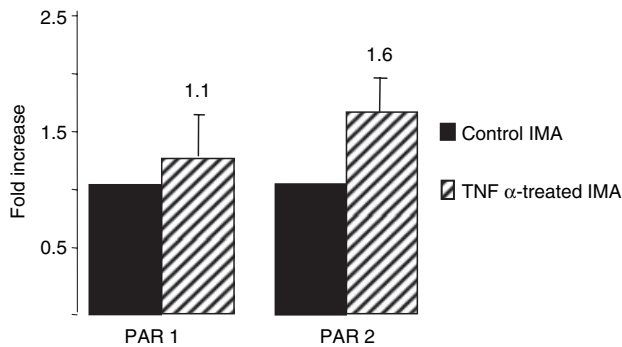


Fig. 4. Protease-activated receptor (PAR) 1 and PAR2 mRNA levels in control and TNF α -stimulated internal mammary artery (IMA) quantified by means of real-time PCR; the data are presented as the relative expression of PAR1 and PAR2 mRNA in stimulated vs. non-stimulated vessels. Mean values of five experiments performed in triplicate.

Even at the highest concentrations used, PAR1-AP failed to cause any significant contraction in the endothelium-free vessels kept at baseline tension.

Discussion

The results of the present study show that IMAs and GSVs express PAR1 and PAR2, but are differentially modulated by PAR-agonists in terms of their endothelial-dependent relaxation. They also show that in TNF α -stimulated IMAs PAR2-AP leads to a marked degree of endothelial-dependent relaxation that is abolished in the presence of inhibitors of NO biosynthesis, whereas it failed to cause any vasorelaxation in TNF α -stimulated GSVs. In line with previously published findings [23–25], the selective PAR2-AP failed to cause the endothelium-dependent relaxation of precontracted and unstimulated human arteries and veins, and did not contract endothelium-free IMAs and GSVs kept at baseline tension. This is in agreement with the findings of Hamilton *et al.* [28], who suggested that the PARs expressed on smooth muscle cells play a limited role in mediating vasoconstriction.

The vasodilatation induced by PAR2-AP (which was only observed in IMAs previously exposed to TNF α) may be at least partially explained by the selective up-regulation of PAR2 over PAR1 mRNA documented by means of quantitative real-time PCR. In line with this, it has been previously reported that the vasodilatation response of human coronary artery to PAR-APs is dependent on the increased expression of PAR2 and PAR4 mRNA after exposure to inflammatory stimuli [28]. Moreover, it has been shown that *in vitro* treatment of human endothelial cells with different cytokines leads to a selective increase in PAR2 but not PAR1 mRNA and cell-surface protein levels [30,31]. These findings suggest that the expression of PAR2 in endothelial cells under physiological conditions is insufficient to allow adequate coupling to G proteins and thus initiate the release of endothelium-derived relaxing factors [32].

This is a very interesting finding and may be pertinent to the better performance of IMAs in CABG. A systemic inflammatory response occurring after coronary cardiopulmonary

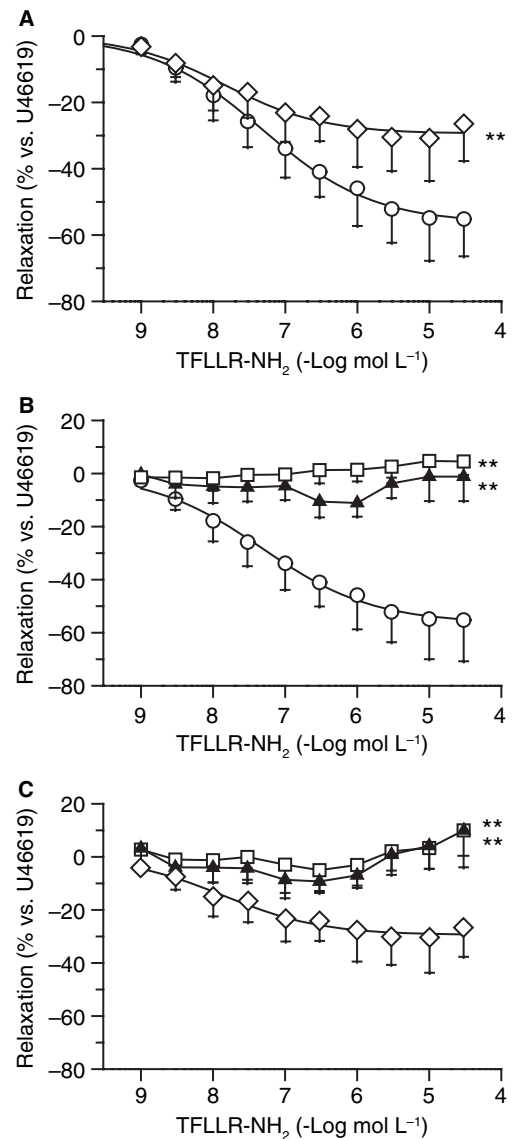


Fig. 5. Panel A. Cumulative concentration-response curves to the selective protease-activated receptor 1-activating peptide (PAR1-AP) in U46619-contracted internal mammary artery (IMA) and greater saphenous vein (GSV) rings. Open circles = IMA; open diamonds = GSV. $**P < 0.001$ vs. IMA (two-way ANOVA). Panel B. Effect of endothelium denudation and treatment with L-NAME 100 $\mu\text{mol L}^{-1}$ on PAR1-AP relaxation in IMA rings. Open circles = IMA; filled triangle = IMA with L-NAME; open square = endothelium-denuded IMA. $**P < 0.001$ vs. IMA (two-way ANOVA). Panel C. Effect of endothelium denudation and treatment with L-NAME on PAR1-AP relaxation in GSV. Open diamonds = GSV; filled triangle = GSV with L-NAME; open square = endothelium-denuded GSV. $**P < 0.001$ vs. IMA (two-way ANOVA). Mean values \pm SD.

bypass surgery that persists for several weeks has been reported [33], and this may not only contribute to adverse postoperative complications, but also represent a protective mechanism similar to that postulated for TNF α in ischemic preconditioning [34]. It is thus possible to hypothesize that the exposure of graft endothelial cells to inflammatory mediators during and after surgery induces a selective up-regulation of PAR2 that is sufficient to induce vasodilatation, and that this may protect

the conduits against the inflammation. In this regard, it is interesting to point out that, under some circumstances, the up-regulation of endothelial PAR2 may counterbalance the organ damage because of tissue injury and the inflammatory state [32]: for example, PAR2-mediated, endothelium-dependent relaxation is preserved in hypertensive rats [35]; PAR2-AP infusion protects against experimental ischemia/reperfusion injury in the heart [36]; and the activation of PAR2 inhibits lipopolysaccharide-induced pulmonary neutrophilia [37]. Moreover, bacterial proteinases activate PAR2, thus suggesting that it may act as one of the first protective mechanisms against inflammation [38].

Although the proteases involved remain to be determined, it is possible that FVIIa and/or FXa play a role in PAR2 activation in patients undergoing coronary revascularization with IMAs, and we have in fact previously reported that the coagulation cascade in such patients is activated for up to 1 month after coronary surgery [39,40].

Our observation that the relaxation of TNF α -stimulated IMA induced by PAR2-AP is abolished by endothelium removal, and significantly attenuated by inhibitors of NO synthesis, suggests that a functionally active endothelium is involved in IMA relaxation. However, we cannot exclude the possibility that NO-independent mechanisms may operate in response to PAR2-AP in TNF α -stimulated mammary arteries, as it has been shown that PAR2 activation causes endothelium-dependent coronary vasodilatation involving the release of EDHF in an animal model of myocardial ischemia [41].

As far as PAR1 is concerned, a specific agonist induced a greater endothelium- and NO-dependent vasodilation in IMAs than in GSVs, which was unaffected by preincubation with TNF α , and it has been found that inflammatory mediators do not induce PAR1 mRNA expression [30,31]. It has been reported that thrombin and selective PAR1 agonists relax precontracted large and small human blood vessels, such as pulmonary and coronary arteries and coronary arterioles [22,24–26], and, under our experimental conditions, the relaxation induced by PAR1-AP was prevented by removing the endothelium or suppressed by inhibiting NO-synthase, in accordance with previously reported data [16,23–25,28]. No contraction was observed under any of the investigated conditions.

In conclusion, the results of this study show the presence of functionally active PAR1 and PAR2 in human arteries and veins. We suggest that, under physiological conditions, PAR1 agonists activate the corresponding receptor to relax the smooth muscle cells of both mammary and saphenous vessels, whereas PAR2 agonists do not contribute to vasodilation because of the low expression of PAR2 receptors. On the other hand, PAR2 may play an important role in pathological conditions accompanied by an inflammatory state, when high levels of proteases from inflammatory cells or activated coagulation factors are accompanied by a higher expression of PAR2 receptors. Indeed, exposure to TNF α electively enhances endothelium-dependent relaxation to PAR2-AP in IMAs. This suggests that the inflammatory state may protect

IMAs rather than GSVs, thus leading to the higher long-term patency rates of the IMAs used in bypass grafts.

The identification and differential activation of PAR1 and PAR2 in a vasculature system that undergoes changes under pathological conditions may offer important insights into the possibility of using PARs as possible therapeutic targets for novel pharmacological approaches with selective agonists.

Study limitations

All of the vessels used in this study were obtained from patients who underwent coronary artery surgery, and so our findings may reflect an adaptation of the vessels to disease rather than a primary physiological process. Nevertheless, they do highlight the importance of examining vascular function in human conduits, especially when assessing the effect of disease.

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Disclosure of Conflict of Interests

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