Upregulation of proteinase-activated receptors and hypercontractile responses precede development of arterial lesions after balloon injury

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Upregulation of proteinase-activated receptors and hypercontractile responses precede development of arterial lesions after balloon injury. Am J Physiol Heart Circ Physiol 291: H2388–H2395, 2006. First published July 14, 2006; doi:10.1152/ajpheart.01313.2005.—Thrombin and other proteinases exert vascular effects by activating the proteinase-activated receptors (PARs). The expression of PARs has been shown to be upregulated after balloon injury and in human arteriosclerosis. However, the relationship between the receptor upregulation and the alteration of vasomotor function remains to be elucidated. We herein demonstrated that the contractile responses to the PAR-1 and PAR-2 agonist were markedly enhanced in the rabbit femoral arteries after balloon injury. Neointimal thickening was established 4 wk after the injury. No histological change was observed in the sham operation, where the saphenous artery was ligated without balloon injury. The contractile response to K+ depolarization was significantly attenuated 1 wk after the injury and then partly recovered after 4 wk. Thrombin, PAR-1-activating peptide, trypsin, and PAR-2-activating peptide induced no significant contraction in the control. All these stimuli induced enhanced responses 1 wk after balloon injury. Such enhanced responses were seen 4 wk after the injury, except for thrombin. There was no change in the Ca2+ sensitivity of the contractile apparatus as evaluated in the permeabilized preparations. PAR-1-activating peptide (100 μmol/l), but no other stimuli, induced an enhanced contraction in the sham operation. The expression of PAR-1 and PAR-2 slightly increased after the sham operation, whereas PAR-1, PAR-2, and PAR-4 serve as receptors for thrombin, whereas PAR-1, PAR-2, and PAR-4 serve as receptors for trypsin (4, 9). Unlike other receptors, PAR activation involves the proteolytic unmasking of a cryptic NH2-terminal sequence, which remains tethered and acts as a ligand binding to the extracellular domain (6, 9, 23). The expression of PARs has been reported to be either upregulated or downregulated in response to various types of stimulation and pathological situations (22). PAR-1 and PAR-2 have been reported to be upregulated in the vascular lesions after balloon angioplasty in the rat or baboon (8, 47) and in human advanced atherosclerotic plaques (37). It is thus suggested that the upregulation of PAR plays an important role in the pathogenesis of vascular diseases. Previous studies have observed the receptor upregulation in the area of the active cell proliferation and the neointima (8, 37, 47). Such receptor upregulation is thus suggested to contribute to the development of proliferative vascular lesion. The hypercontractile state and vasospastic activity are also associated with vascular lesions. However, the link between the upregulation of PARs and the alteration of the vasomotor function still remains to be elucidated.

Thrombin and trypsin have been shown to induce endothelium-dependent relaxation, endothelium-dependent contraction, and direct smooth muscle contraction, depending on the type of blood vessels (21, 45). Under physiological conditions, PAR-1 and PAR-2 are considered to mainly mediate endothelium-dependent relaxation in many types of blood vessels. The direct contractile effect has been reported with certain type of vessels, such as the guinea pig aorta, rabbit aorta, and canine coronary artery, under physiological conditions (18, 21, 25, 27, 35). It may thus play a more important role under pathological conditions, partly because PAR-1 and PAR-2 were upregulated in vascular smooth muscle under pathological conditions (8, 21, 22, 37, 47). In fact, the correlation between the direct contractile response and the severity of atherosclerotic lesion has been reported in the human coronary artery (26). In the

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normal artery, however, thrombin induced only an endothelium-dependent relaxation. Such a relaxation response was attenuated with an increase in the severity of the lesion. Eventually, a relaxation response disappeared in the artery with severe intimal proliferation, whereas PAR-1 stimulation induced a direct contractile response. However, the temporal relationship between the enhancement of the direct contractile response and the development of the vascular lesion remains to be elucidated.

In the present study, utilizing a vascular balloon injury model in rabbit, we investigated the alteration of the contractile responses toward the PAR agonists thrombin, trypsin, PAR-1-activating peptide (PAR-1AP), PAR-2AP, and PAR-4AP and evaluated the relationship between the contractile response and the development of the vascular lesion. We also examined the expression of PAR-1 and PAR-2 with immunoblot analysis. The balloon injury has been shown to cause deendothelialization of the injury sites (32). Subsequently, the injury sites are reendothelialized, but it remains incomplete by 1 wk after injury. Therefore, the status of endothelial function varies with time after balloon injury. In the present study, we thus focused on the alteration of the smooth muscle contractility and utilized the deendothelialized strips for evaluation. The present study demonstrated for the first time the correlation between the upregulation of the expression of PAR-1 and PAR-2 and the enhancement of the contractile responses to PAR-1 and PAR-2 agonists. Notably, we observed such enhanced vasomotor response before the establishment of the proliferative vascular lesion after balloon injury.

MATERIALS AND METHODS

Balloon injury model. Male Japanese white rabbits (2.5 to 3.0 kg body wt; Kyudo, Saga, Japan) were anesthetized with an intramuscular injection of xylazine (10 mg/kg) and ketamine hydrochloride (25 mg/kg). Under sufficient anesthesia, a longitudinal medial incision was made in the lower thigh, and the saphenous artery was exposed. From the saphenous artery, a 2-Fr Fogarty balloon embolectomy catheter was inserted into the femoral artery. The balloon was then inflated, and the catheter was retracted. This procedure was repeated three times to induce vascular injury. After the catheter was removed, the saphenous artery was ligated, and the wound was closed. Sham-operated rabbits underwent the same operation, except that the balloon catheter was not inserted into any arteries; however, the saphenous artery was ligated. This experimental protocol has been approved by the Committee of Ethics on Animal Experiments in the Graduate School of Medical Sciences, Kyushu University.

Histological evaluation. One week or 4 wk after the operation, the animals were euthanized with intravenous injection of a lethal dosage of pentobarbital sodium. The femoral artery was excised en block and perfusion fixed with 10% formaldehyde at 100 mmHg for 15 min. The arteries were then immersed in neutralized 10% formaldehyde overnight at room temperature. Perfusion-fixed arteries were mounted in paraffin, and 5-μm-thick tissue sections were subjected to hematoxylin-eosin staining and elastica van Gieson staining.

Direct assessment of blood flow using an ultrasonic transit-time flowmeter. The blood flow rate of the femoral artery was monitored as previously described (44). In brief, the femoral artery was surgically exposed, and a 5-mm flow probe (Transonic Flow Probe 0.5 V; Transonic Systems, Ithaca, NY), which was connected to an ultrasonic transit-time flowmeter (USTF, Transonic T201; Transonic Systems), was applied directly onto the femoral artery. The flow waveforms were then recorded, and the traces were loaded into a personal computer with a digitizer (K-150; Kanto Denshi, Tokyo, Japan). The mean flow rate was then calculated during the 5-min recording.

Tissue preparation for tension study. One week or 4 wk after the operation, the animals were heparinized with an intravenous administration of 200 IU heparin/kg wt and euthanized by an intravenous injection of a lethal dose of pentobarbital sodium; the femoral artery was then isolated. Under a binocular microscope, the adventitia was trimmed away and the arterial segments were cut open longitudinally. The endothelium was removed with a cotton swab. The medial segments were then cut in a circular direction to obtain strips measuring 2 mm in length and 1 mm in width. These strips were equilibrated in normal physiological saline solution (PSS) aerated with 5% CO2-95% O2.

Tension measurement in intact strips. The 1-mm-long slit was made in the center of the strip, and the strips were then mounted between two tungsten wires under microscopy by passing the wires through the slit. One of the wires was fixed, whereas the other was attached to a force transducer (UL2; Minebea, Osaka, Japan). The strips were stimulated with 118 mmol/l K+ PSS repeatedly, and the resting tension was increased stepwise to obtain the maximal force development by 118 mmol/l K+. The developed tension was expressed in either an absolute value or a relative value (percentage). When expressed in relative values, the values at rest in normal PSS (5.9 mmol/l K+) and those obtained at a steady state of contraction induced by 118 mmol/l K+ PSS were assigned to be 0% and 100%, respectively.

Tension measurement in α-toxin-permeabilized strips. The permeabilization of the strips with α-toxin was performed according to previously described methods with minor modifications (38). In brief, the strips were permeabilized in relaxing solution (100 mmol/l potassium methansulfonate, 2.2 mmol/l Na2 adenosine triphosphate, 3.38 mmol/l MgCl2, 10 mmol/l EGTA, 10 mmol/l creatine phosphate, and 20 mmol/l Tris-maleate, pH 6.8) containing 5,000 U/ml Staphylococcus aureus α-toxin for 60 min at room temperature. The composition of Ca2+- solution (activating solution) was the same as the relaxing solution, except that it contained the indicated concentration of free Ca2+ buffered by 10 mmol/l EGTA, according to the EGTA-Ca2+-free solution, and that obtained at a steady state of contraction induced by 10 μmol/l Ca2+ solution were assigned to be 0% and 100%, respectively. All experiments were performed at room temperature.

Western blot analysis of expression of PARs. Tissue samples were kept frozen at −80°C until use. They were thawed and lysed in the extraction buffer (50 mmol/l Tris·HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 500 mmol/l NaCl, 10 mmol/l MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μmol/l 4-aminophenylmethylsulfonyl fluoride). Twenty micrograms of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were incubated with the primary antibodies diluted ≥1000 in an immunoreaction enhancer solution named Can-Get-Signal (Toyobo, Osaka, Japan), followed by appropriate secondary antibodies conjugated with horseradish peroxidase. The immune complex was detected by an ECL plus detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The luminescence signal was detected and analyzed with the ChemiDoc XRS-1 image analysis system (Bio-Rad, Tokyo, Japan).

Solutions and drugs. Normal PSS was of the following composition (in mmol/l): 123 NaCl, 4.7 KCl, 15.5 NaHCO3, 1.2 KH2PO4, 1.2 MgCl2, 1.25 CaCl2, and 11.5 glucose. PSS containing 118 mmol/l K+ was prepared by replacing NaCl with equimolar KCl. PSS was bubbled with a 95% O2-5% CO2 mixture, with a resulting pH 7.4. Antibodies against PAR-1 (sc-5605, raised against the recombinant protein corresponding to amino acids 1–111 of human PAR-1) and
PAR-2 (sc-5597, raised against the recombinant protein corresponding to amino acids 230–328 of human PAR-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Thrombin (bovine plasma, 1,880 NIH U/mg protein), trypsin (bovine pancreas, 12,000 U/mg protein), heparin (mol wt 3,000) and α-toxin were purchased from Sigma (St. Louis, MO). EGTA was obtained from Dojindo Laboratories (Kumamoto, Japan). PAR-1AP (TFLLR-NH₂) and PAR-2AP (SLIGRL) were obtained from Bachem (Budendorf, Switzerland). PAR-4AP (AYPGKF-NH₂), PAR-1 inactive peptide (FTLLR-NH₂), and PAR-2 inactive peptide (LSIGRL-NH₂) were synthesized by Rapid Multiple Peptide Synthesis Service, University of Calgary (Calgary, AB, Canada).

Data analysis. All data are expressed as means ± SE. One strip obtained from one animal was used for each experiment, and, therefore, the number of experiments indicates the number of animals. The statistical significance was evaluated with ANOVA and Fisher protected least significant difference test. P < 0.05 was considered to be significant. Data were collected using a computerized data acquisition system (MacLab, AD Instruments, Castle Hill, NSW, Australia) running on an Apple Macintosh computer.

RESULTS

Histological changes after balloon injury. The section from the sham operation showed no significant change in comparison with the control (Fig. 1, Aa–Ac). However, the section from 1 wk after balloon injury showed slight intimal hyperplasia (Fig. 1Ad) and the partial fragmentation of the internal elastic layers in the elastic van Gieson staining (data not shown). The section from 4 wk after balloon injury showed a significant degree of intimal hyperplasia and some slight inflammatory infiltration (Fig. 1Ae).

Changes in blood flow rate after sham operation and balloon injury. The femoral artery flow rate significantly (P < 0.05) decreased 1 wk after balloon injury (10.6 ± 1.4 ml/min) and sham operation (8.73 ± 0.30 ml/min) in comparison with the control situation (15.8 ± 0.78 ml/min) (Fig. 1B).

Attenuation of contractile response to high K⁺ depolarization after balloon injury. The application of 118 mmol/l K⁺ induced a sustained contraction in the control artery. The extent of the developed tension during the steady state of contraction seen in the sham-operated arteries did not significantly differ from that seen in the control (Fig. 2). However, the contractile response to 118 mmol/l K⁺ was markedly attenuated 1 wk after balloon injury, and it partly but significantly recovered 4 wk after balloon injury (Fig. 2).

Enhanced contractile responses to thrombin and PAR-1AP after balloon injury. In the control arteries and sham-operated arteries, 10 U/ml thrombin induced almost no contractile responses (Fig. 3). However, 10 U/ml thrombin induced a sustained contraction (249.6 ± 79.7% and 0.22 ± 0.06 g; P < 0.01 vs. control, n = 5) in the arteries obtained 1 wk after balloon injury, whereas it induced no significant contraction 4 wk after balloon injury (Fig. 3 and Fig. 4, A and B). The lower concentration of thrombin (1 U/ml) induced no significant contraction in any experimental groups (Fig. 4A). Thrombin can activate the intracellular signaling via PAR-1 and PAR-4. We thus examined the contractile responses to the activating peptides for PAR-1 and PAR-4.

The stimulation with 10 μmol/l TFLLR-NH₂, PAR-1AP, induced a significant contraction only in the arteries obtained 1 wk after balloon injury, whereas it induced no significant response under other conditions (Fig. 4C). Such changes in the responsiveness seen with 10 μmol/l TFLLR-NH₂ were consistent with those seen with 10 U/ml thrombin. However, at a higher concentration (100 μmol/l), TFLLR-NH₂ induced a significant contraction not only in the arteries obtained 1 wk after balloon injury but also in those obtained 4 wk after balloon injury (Fig. 3). The arteries obtained 1 and 4 wk after the sham operation also responded to 100 μmol/l TFLLR-NH₂ (Fig. 3). The extent of TFLLR-NH₂-induced contractions seen in the arteries obtained 4 wk after balloon injury and those obtained with the sham operation was significantly smaller than that seen 1 wk after balloon injury, when it is expressed as a relative value (Fig. 4C). However, when evaluated according
to the absolute values, the extent of contraction was similar among all these four conditions (Fig. 4D). On the other hand, PAR-1-inactive peptide induced no contraction in the arteries obtained 1 wk after balloon injury (Fig. 3B), and PAR-4AP (AYPGKF-NH₂; up to 100 μmol/l) induced no contraction under any experimental conditions (data not shown).

**Enhanced contractile responses to trypsin and PAR-2AP after balloon injury.** Trypsin, at 1 μmol/l, induced no significant contraction in the control at 1 and 4 wk after the sham operation (Fig. 5A). At 10 μmol/l, trypsin induced only a slight development of tension under these conditions. On the other hand, 1 wk after balloon injury, trypsin induced a large contraction even at 1 μmol/l, and 10 μmol/l trypsin induced a similar contraction to that seen with 1 μmol/l (Fig. 5A). These contractile responses to trypsin were significantly attenuated 4 wk after balloon injury in comparison with those seen 1 wk after balloon injury, when evaluated with a relative value. However, when evaluated with an absolute value, the arteries obtained 4 wk after balloon injury exhibited similar or slightly attenuated responses to both 1 and 100 μmol/l trypsin (Fig. 5B).

The changes in the responsiveness to SLIGRL, PAR-2AP, after balloon injury were similar to those observed with trypsin (Fig. 5). The control arteries and those obtained with the sham operation slightly contracted in response to 100 μmol/l SLIGRL, whereas they did not respond to 10 μmol/l SLIGRL (Fig. 5C). On the other hand, the arteries obtained 1 and 4 wk after balloon injury exhibited enhanced contractile responses to both 10 and 100 μmol/l SLIGRL (Fig. 5C), whereas PAR-2 inactive peptide (LSIGRL-NH₂) induced no contraction (data not shown). The extent of contraction seen 4 wk after balloon injury was significantly smaller than that seen 1 wk after balloon injury, when evaluated with a relative value. However, the absolute value of tension induced by SLIGRL 4 wk after balloon injury was similar to that seen 1 wk after balloon injury.

**Changes in contractile responses induced by Ca²⁺ and GTPγS in α-toxin-permeabilized arteries.** In the α-toxin-permeabilized strips, a graded increment of Ca²⁺ concentrations induced a stepwise increase in tension. There was no significant difference in the concentration-response curves of the Ca²⁺-induced contraction among the five groups (Fig. 6A). The addition of 100 μmol/l GTPγS during the 180 mmol/l Ca²⁺-induced contraction induced an additional development of tension to 78.24 ± 3.02% of 10 μmol/l Ca²⁺-induced contraction (n = 4) in the control femoral arteries. There was no significant difference in such 100 μmol/l GTPγS-induced development of tension among the five groups (Fig. 6B).

**Upregulation of expression of PAR-1 and PAR-2 after balloon injury.** Western blot analysis of PAR-1 and PAR-2 detected a major band of ~50 kDa and a minor band of ~83 kDa in the control artery (Fig. 7). The level of the lower bands increased in both sham operation and balloon injury, whereas the upper bands disappeared. The lower bands are thus sug-
gested to represent PAR-1 and PAR-2 in the femoral artery. The level of both PAR-1 (2.3 ± 1.0 fold of the control level, \( n = 3 \)) and PAR-2 (1.7 ± 0.5 fold, \( n = 3 \)) seen 1 wk after the sham operation was similar to the control level (Fig. 7). Four weeks after the sham operation, level of PAR-1 (4.0 ± 2.3-fold, \( n = 3 \)) and PAR-2 (3.6 ± 0.7 fold, \( n = 3 \)) was slightly elevated. However, such elevations were not statistically significant (Fig. 7). The balloon injury induced much greater and significant increases in the level of PAR-1 and PAR-2. The level of the PAR-1 expression reached 7.8 ± 3.6-fold (\( n = 4 \)) and 11.1 ± 2.8-fold (\( n = 4 \)) of the control level, and that of the PAR-2 expression reached 5.4 ± 1.4-fold (\( n = 4 \)) and 5.3 ± 1.0-fold (\( n = 4 \)) 1 wk and 4 wk after balloon injury, respectively.

**DISCUSSION**

We herein demonstrated, for the first time, that the contractile responses mediated by PAR-1 and PAR-2 were markedly enhanced after balloon injury in the rabbit femoral artery. Such an enhancement of the contractile response was associated with an increase in the expression of PAR-1 and PAR-2. Upregulation of PAR-1 and PAR-2 has already been reported in rat carotid artery after balloon injury (8, 47) or in human athero-

![Fig. 4. Enhancement of contractile response to thrombin and PAR-1AP after balloon injury.](image)

![Fig. 5. Enhancement of contractile responses to trypsin and PAR-2AP after balloon injury.](image)
sclerotic lesions (37). Because the receptor upregulation was observed in the area of the active cell proliferation and the neointima, it is thus conceivable that such receptor upregulation is linked to the development of the proliferative vascular lesion (8, 37, 47). Indeed, the stimulation of PAR-1 and PAR-2 has been reported to activate the migration and proliferation of smooth muscle cells and the smooth muscle production of the extracellular matrix (7, 14, 34). However, studies of balloon injury also demonstrated receptor upregulation before the establishment of proliferative vascular lesions, as we observed in the present study. The functional relevance of such receptor upregulation as that seen before the establishment of vascular lesions remains to be elucidated. The present study thus provides the first evidence of the link between the receptor upregulation and the enhancement of the contractile response in the early phase after balloon injury.

The enhancement of the contractile response can be achieved simply by altering the composition or responsiveness of the contractile apparatus. However, the contractile response to high K⁺ depolarization was attenuated after balloon injury, in contrast to the enhancement of the contractile response to thrombin and trypsin. The responsiveness to high K⁺ partly recovered 4 wk after balloon injury in comparison with that seen 1 wk after balloon injury. Such transient attenuation of the high K⁺-induced contraction was consistent with the previous report (40). We previously reported that mitogenic stimulation suppresses the expression of the L-type voltage-operated Ca²⁺ channels in the cultured vascular smooth muscle cells (24, 28, 29). Such a mechanism may contribute to the transient suppression of high K⁺-induced contraction. Furthermore, the observations with the permeabilized preparation showed no significant changes in the Ca²⁺ sensitivity of the contractile apparatus. Our findings thus suggest that the alteration of the contractile apparatus does not play a major role in the enhancement of the contractile responses to thrombin and trypsin. Such enhancement of the contractile response is thus considered to be due to the receptor upregulation. Because PAR-4AP induced no contractile response under any conditions, the contractile response to thrombin is mainly mediated by PAR-1. However, SCH-79797, which was widely used as a PAR-1 antagonist (30), had no significant effects on the contractile responses to both thrombin and PAR-1AP in the arteries obtained 1 wk after balloon injury (data not shown). The reason why this antagonist failed to exert an expected antagonizing effect remains unclear. Although SCH-79797 was originally developed to block human PAR-1, it may not block the rabbit PAR-1. Trypsin has been reported to activate both PAR-1 and PAR-2 (9, 23, 31). However, trypsin induced a significant contraction in the artery obtained 4 wk after balloon injury, where thrombin induced no significant contraction. Furthermore, the changes in the contractile response to trypsin after balloon injury were consistent with those seen with PAR-2. These observations thus suggest that the contractile response to trypsin is mainly mediated by PAR-2.

Precisely how balloon injury upregulates the expression of PAR-1 and PAR-2 remains an important question. The balloon injury could cause activation of the coagulation cascade and platelets (2, 11, 20). The concentration of thrombin at the site of vascular injury has been reported to be significantly elevated (19). Various growth factors, including platelet-derived growth factor, could contribute to the receptor upregulation (22). Thrombin has also been shown to induce the transcriptional upregulation of PAR-1 in the cultured endothelial cells (12). However, the daily treatment with a subcutaneous injection of heparin failed to prevent an enhancement of the contractile response to thrombin (data not shown), thus ruling out a major contribution of thrombin or any other heparin-sensitive proteases to the receptor upregulation. Inflammatory cytokines can also be involved in the upregulation of PAR (22). In this regard, it is intriguing that the expression of PAR-1 and PAR-2 slightly increased 4 wk after the sham operation. In the sham

Fig. 6. Changes in contractile responses after balloon injury in α-toxin-permeabilized arteries. A: concentration-response curves for contractions induced by increasing Ca²⁺ concentrations in α-toxin-permeabilized arteries. B: contractile responses induced by 100 μmol/l GTPγS in α-toxin-permeabilized arteries. Tension development was expressed as a relative value, whereas values obtained in Ca²⁺-free solution and those obtained with 10 μmol/l Ca²⁺ were 0% and 100%, respectively. Data are means ± SE (n = 3–4).

Fig. 7. Upregulation of PAR-1 and PAR-2 after balloon injury. Representative immunoblots and summary of analysis of PAR-1 (A) and PAR-2 (B) expression in control artery and in arteries obtained 1 and 4 wk after either balloon injury or sham operation. Actin was detected by amidoblack staining. Molecular size is indicated at the right. Levels of PAR-1 and PAR-2 were evaluated by the ratio to the density of actin while assigning control value to be 1. Data are means ± SE (n = 3–4). **P < 0.01 compared with control situation; *P < 0.05.
operation, we did not insert the balloon catheters into any arteries, but we did make an incision and ligated the saphenous artery as in the balloon injury model. The contralateral arteries in the sham operation and in the balloon injury exhibited no enhanced responsiveness to thrombin or trypsin. These observations thus suggest that the local inflammation contributed to the receptor upregulation seen in the sham operation. Another possible mechanism for upregulation of PARs, which was operable in a sham operation, is a reduction of blood flow and the resulting tissue ischemia (36). The ultrasonic transit-time flowmeter analysis revealed that the blood flow rate of the femoral arteries was significantly decreased to an extent similar to that after a sham operation and balloon injury. The ischemia or reperfusion may, directly or by inducing cytokine production, upregulate the expression of PAR-1 and PAR-2. However, the balloon injury increased the expression of PAR-1 and PAR-2 to the level much higher than that seen in the sham operation. It is thus conceivable that some additional factors, which are specific to the balloon injury, contributed to the receptor upregulation seen with the balloon injury. Such factors and the precise mechanism of the upregulation of PAR-1 and PAR-2 all remain to be elucidated in a future study.

There is some inconsistency remaining between the level of the PAR-1 expression and the degree of the contractile response to thrombin in balloon injury. The expression of PAR-1 observed 4 wk after balloon injury was either similar to or slightly higher than that observed 1 wk after balloon injury. However, thrombin induced contraction only 1 wk after balloon injury. On the other hand, PAR-1AP induced a significant contraction both 1 and 4 wk after balloon injury. When evaluated with the relative value of tension, all responses to thrombin, PAR-1AP, trypsin, and PAR-2AP seen 4 wk after balloon injury were significantly smaller than those seen 1 wk after balloon injury. Such attenuation appeared to be inconsistent with the sustained upregulation of both PAR-1 and PAR-2. However, some specificity of the contractile effects seen with PAR-1AP and PAR-2AP was supported by the observation that their inactive derivatives had no contractile effect. In the present study, the neointimal and medial thickening became obvious 4 wk after balloon injury. PAR-1 and PAR-2 have been reported to be expressed in both the neointimal and medial regions (8, 37, 47). The receptors expressed in the neointimal region may not contribute to the contraction. Therefore, an increase in such nonfunctional receptors might contribute to the attenuation of the relative responsiveness despite the sustained expression of the receptors. However, such an apparent inconsistency may be mostly caused by a recovery of the contractile response to high K\(^+\) depolarization 4 wk after balloon injury. Indeed, when evaluated with an absolute value, the extent of the contractions induced by PAR-1AP, trypsin, and PAR-2AP seen 4 wk after injury was similar to those seen 1 wk after injury. Such responsiveness was thus consistent with the level of PAR-1 and PAR-2. However, this was not the case with thrombin. The responsiveness of PARs to the agonist proteinases can be inactivated by the preceding proteolytic cleavage of the receptors, whereas the responsiveness to the peptide agonists remains intact (21, 23). The activity of such proteinases as matrix metalloproteinase has been reported to increase in the vascular lesions after balloon injury (10). It is thus speculated that PAR-1 obtained 4 wk after balloon injury might have been proteolytically cleaved in situ by some proteinases that became active during the reparative process. In such a case, PAR-1 plays a significant role only in the early phase of the balloon injury, whereas PAR-2 plays a significant role in both early and late phases of the balloon injury. Such an enhancement in contractility may explain why high concentrations of thrombin (10 U/ml) were required to induce a significant contraction in the present study. Another possibility is that thrombin inhibitor or serine proteinase inhibitors (serpins) were generated in the early phase of injury, especially at 4 wk after balloon injury, thereby blocking the contractile effect of thrombin but not PAR-1AP. Indeed, protease nexin-1, one of the serpins, has been reported to inhibit responses to thrombin but not PAR-1AP in vascular smooth muscle cells (41), whereas its expression was shown to be upregulated by ischemia in the rat brain (39).

There is also some apparent inconsistency between the level of the receptor expression and the degree of the contractile response in the sham operation. Four weeks after the sham operation, the level of PAR-2 was slightly elevated, whereas trypsin or PAR-2AP induced no significant contraction. However, the elevation of the level of PAR-2 seen 4 wk after the sham operation was not found to be statistically significant. The level of PAR-2 may thus not be high enough to induce the contractile effect. In this case, the observation that 100 μmol/l PAR-1AP induced a significant contractile effect both 1 and 4 wk after the sham operation is apparently inconsistent with the observed effects obtained with PAR-2AP. However, the lower concentration (10 μmol/l) of PAR-1AP induced a contractile effect only in balloon injury. A high concentration of PAR-1AP might have induced a contractile response via mechanisms other than PAR-1.

In conclusion, the present study demonstrated, for the first time, that the balloon injury induced an enhanced contractile response to thrombin and trypsin by upregulating the expression of PAR-1 and PAR-2 in the rabbit femoral artery. Importantly, such an enhancement of the contractile response was observed far before the establishment of the proliferative vascular lesion, thus contributing to an increase in the vascular tone or vasospasm seen without any obvious organic lesions.

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