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Inactivation of protease-activated receptor-1 by proteolytic removal of the ligand region in vascular endothelial cells

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Abstract

Proteolysis plays an important role in inactivating protease-activated receptor-1 (PAR1). We aimed to determine the cleavage site(s) responsive for the proteolytic inactivation of PAR1 in human umbilical vein endothelial cells. Fura-2 fluorometry revealed that the preceding stimulation with trypsin abolished the subsequent $[Ca^{2+}]_i$ response to thrombin, while the responses to PAR1-activating peptides remained intact. On the other hand, thrombin had no effect on the subsequent response to trypsin. The immunostaining with antibodies against the residues 35–46 (SPAN12) and 51–64 (WEDE15) revealed the broad boundaries of cleavage. Trypsin removed both epitopes from the cell surface within 3 min, while thrombin removed the epitope of SPAN12. The longer incubation with thrombin removed the epitope of WEDE15. However, PAR1-activating peptides thereafter induced an attenuated but significant elevation of $[Ca^{2+}]_i$. Not only the receptor internalization as observed with a confocal microscope, but also an additional cleavage was thus suggested to contribute to the thrombin-induced removal of the epitope of WEDE15. The analyses of the PAR1 mutants identified three cleavage sites for trypsin; residues 41–42, 70–71 and 82–83. The cleavage at the latter two sites was suggested to dominate that at the former, and thus remove the ligand region (residues 42–47). The inactivation of PAR1 due to proteolytic removal of the ligand region may contribute not only to the inactivation of PAR1 by proteases such as trypsin, but also to the termination of the intracellular signaling initiated by thrombin in the vascular endothelial cells.

Keywords: Endothelial cells; Receptor; Thrombin; Trypsin; Cytosolic Ca^{2+} signal; Proteolysis

1. Introduction

Protease-activated receptors (PARs) are known to be activated by the proteolytic cleavage of their N-terminal extracellular domain at a specific site [1,2]. A new N-terminus acts as a tethered ligand and initiates intracellular signaling [3]. PARs belong to a family of the G-protein coupled receptors [1,3], and four members of PARs, PAR1, PAR2, PAR3 and PAR4, have been identified [4–7]. The activating cleavage sites of PAR1, PAR2, PAR3 and PAR4 are the residues 41–42, 36–37, 38–39 and 47–48 in humans [1,3]. On the other hand, the synthetic peptides corresponding to the tethered ligand sequences have been shown to activate PARs independent of proteolysis, except for PAR3 [8]. These PAR-APs may serve as a useful tool for investigating the regulatory mechanism of PARs. Since the proteolytic activation process is irreversible, the receptor internalization, association with arrestin-like molecules and an additional cleavage are thus considered to terminate

Abbreviations: PARs, protease-activated receptors; PAR-APs, PAR-activating peptides; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; HUVECs, human umbilical vein endothelial cells; DMEM, Dulbecco's modified Eagle medium

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the intracellular signaling which originates from the proteolytically activated PARs [1,3,9–11].

Thrombin and trypsin exert cellular effects such as platelet aggregation, endothelium-dependent relaxation and smooth muscle contraction and proliferation by activating PARs [3,12–14]. PAR1, PAR3 and PAR4 are considered to serve as receptors for thrombin, while PAR1, PAR2 and PAR4 are considered to serve as receptors for trypsin [1–3,15]. Trypsin has been suggested to cleave PAR1 at the activating cleavage site, residues 41–42 [1,2,16]. However, in vitro study with the recombinant N-terminal fragments demonstrated trypsin to cleave PAR1 at residues 70–71 and 82–83 with similar kinetics to that observed with the cleavage at residues 41–42 [16]. The functional relevance of trypsin cleavage of PAR1 thus remains to be investigated. We previously reported that the preceding stimulation with trypsin completely abolished the response to the subsequent stimulation with thrombin, while the preceding stimulation with thrombin had no effect on the subsequent response to trypsin in rat myometrium [14] and vascular endothelial cells [17]. Our findings thus suggested that trypsin cleaves PAR1, but does not activate it. Moreover, we proposed that the trypsin-cleaved PAR1 is no longer responsive to thrombin. However, the cleavage site(s) responsible for the trypsin-induced inactivation of PAR1 remains to be determined.

In the present study, we aimed to determine the site(s) responsible for the proteolytic inactivation of PAR1 in HUVECs. HUVECs were selected because the anti-human PAR1 antibodies are available. Front-surface fura-2 fluorometry of $[Ca^{2+}]_i$ [18] was used to examine the responsiveness to thrombin, trypsin and PAR1-activating peptides (PAR1-APs) [TRAP7 (SFLLRNP) and a high affinity PAR1-AP (haPAR1-AP; Ala-Phe(pF)-Arg-Cha-homoArg-Tyr-NH_2)] [19]. The broad boundaries of PAR1 cleavage were determined by immunofluorescnce staining with two antibodies, SPAN12 and WEDE15. The responsiveness of the proteolysis-resistant mutants of PAR1 was examined to determine the sites responsible for inactivation of PAR1. Furthermore, we also examined the thrombin-induced internalization of PAR1 with confocal microscopy. We herein provide the first physiological evidence that trypsin cleaves PAR1 at three sites, and suggest that the cleavage at sites 70–71 and 82–83 dominates the cleavage at site 41–42, thereby inactivating the responsiveness of PAR1 to thrombin. We also suggest that the similar mode of inactivation of receptor responsiveness is operable in terminating the intracellular signaling initiated by thrombin.

2. Materials and methods

2.1. Cell culture of HUVECs and HeLa cells

The human umbilical cords were kindly provided from the Fukuoka Municipal Hospital. HUVECs were isolated by treatment with trypsin in the EDTA-containing PBS as previously described [20], and plated in 35 mm culture dishes and cultured to confluence in EBM-2 media supplemented with EGM-2 Bred kit (Sankyo Junyaku). After achieving a confluent monolayer of endothelial cells, the medium was changed to RPMI-1640 media (Life Technologies) containing 10% fetal bovine serum, and the cells were further incubated for 48 h until experimental use. The cells were used in the experiment within the second passage. When the cells were subcultured, they were harvested by treatment with trypsin as previously described [21]. The HUVECs at confluence showed a typical cobblestone monolayer morphology under a phase contract microscope. HeLa cells were cultured in DMEM containing 10% fetal bovine serum as previously described [22].

2.2. Measurement of $[Ca^{2+}]_i$ in endothelial cells

HUVECs were loaded with fura-2 by incubating in DMEM containing 10 μM fura-2 acetoxymethyl ester for 1 h at 37 °C as previously described [23]. After fura-2 loading, the cells were washed and equilibrated in HEPES-buffered saline (HBS; 10 mM Hepes, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 5.5 mM D-glucose) for at least 30 min at room temperature before starting the measurements. The changes in $[Ca^{2+}]_i$ in HUVECs were monitored using a front-surface fluorometer as previously described [17,18,23]. Fluorometry was performed at 25 °C to prevent leakage of fura-2 [24]. The 500 nm fluorescence intensities at 340 and 380 nm excitations and their ratio were continuously monitored. The fluorescence ratio data were expressed as a percentage, while assigning the values at rest and at the peak $[Ca^{2+}]_i$ elevation induced by 10 μM ATP to be 0 and 100%, respectively. All data were collected using a computarized data acquisition system MacLab (Analog Digital Instruments).

2.3. Immunofluorescent staining of PAR1

The HUVECs were untreated or treated with thrombin and trypsin for the indicated period of time, and the reaction was terminated by adding 4-aminido-phenylmethane–sulphonyl fluoride to the final concentration of 10 μM. The cells were then harvested by incubating them in PBS containing 1 mM EDTA, 5 mM EGTA at 37 °C for 15%, and scraping off from the culture dishes as described [25]. The cells were washed and suspended in PBS at approximately 1×10^5 to 10×10^5 cells ml^{-1}, and then were incubated with phycoerythrin-labeled anti-PAR1 antibodies, SPAN12 and WEDE15, at a final concentration of 5 μg ml^{-1} in PBS containing 1% bovine serum albumin at room temperature (25 °C) for 30 min. The antibodies SPAN12 and WEDE15 were raised against the synthetic peptides corresponding to the residues 35–46 and 51–64, respectively [9,26]. The cells were washed three times in

PBS after staining and subjected to flow cytometric analysis and microscopic observation. In one experiment (Fig. 3), the cells were fixed with 2% paraformaldehyde in PBS at room temperature for 20 min, permeabilized with PBS containing 0.1% Triton X-100 at room temperature for 5 min, and then were subjected to immunofluorescence staining.

The fluorescence profile of the cells stained with anti-PAR1 antibodies were analysed on a flow cytometer FACSCalibur (BD Bioscience). The fluorescence at 488 nm laser excitation was detected by a FL2 detector through a 585 nm band pass filter. A program Cell Quest ver.3.2.1 (BD Bioscience) was used to collect and analyse the fluorescent data. The fluorescence intensity at the peak of the cell population was determined on a histogram presentation of the fluorescence data (Fig. 3). The fluorescence image was observed under a laser scanning confocal fluorescence microscope LSM GB200 (Olympus), using a 60× objective lens, 488 nm excitation and a 500–530 band pass emission filter. The fluorescence images were obtained at the nuclear level and saved as TIFF files for representative photos.

2.4. Construction of expression plasmids for human PAR1 and its mutants

A cDNA for human PAR1 was obtained by reverse transcription-polymerase chain reaction (RT-PCR). The total RNA was isolated from white blood cells of four healthy Japanese volunteers, and then subjected to RT reaction with random hexamer as primer. The RT product was amplified by PCR with the following upper and lower primers containing EcoRI and BamHI, respectively, as cloning sites: 5′-gCg CAg AAT TCg ggA CAA Tgg ggC CgC ggC g-3′ for upper primer and 5′-AAA Cgg ATC CTT TTA ACC TCC CAg CAg TCC-3′ for lower primer. The PCR products were digested with EcoRI and BamHI, and ligated to pcDNA3.1(−) (Invitrogen) at EcoRI and BamHI sites to obtain pcDNA-PAR1^{wt}. The determination of the nucleotide sequences indicated the following two conversions in amino acid residues from those reported in the database (Accession number M62424): V238 to L and S364 to C. These two differences were common to all clones obtained from four volunteers, and the V238 to L conversion was also reported in the genomic clone [27]. Three putative sites (41–42, 70–71 and 82–83) for trypsin cleavage of PAR1 [16] were mutated separately or in combinations. For each site, the two residues flanking the cleavage point were mutated to a combination of A and P [6], by using the overlap extension PCR technique [28]. The mutants used in the present study were PAR1^{70} (containing mutation of R70+L71 to A+P), PAR1^{82} (containing mutation of K82+Q83 to A+P), PAR1^{70+82} (combined mutant of PAR1^{70} and PAR1^{82}), and PAR1$^{41+70+82}$ (PAR1^{70+82} containing additional mutation of R41+S42 to A+P). The nucleotide sequences were determined to contain no unintended mutations. The plasmid DNA was isolated with a Qiagen plasmid kit (Qiagen) for transfection.

2.5. Transfection of HeLa cells

HeLa cells were plated in 35 mm culture dish on a day before transfection. After rinsing with DMEM, the cells were transfected by incubation in 1 ml DMEM containing 2 μg Lipofectin (Life Technologies) and 2 μg plasmid DNA at 37 °C for 3 h. Lipofectin and plasmid DNA had been incubated in 200 μl DMEM at room temperature for 15 min before transfection. After 3 h of incubation, the transfection mixture was replaced with 10% serum-containing growth media, and the cell culture was resumed. The cells on 1 or 2 days after transfection were subjected to fura-2 fluorometry as described above for HUVECs. The preliminary experiment demonstrated that both thrombin and PAR1-APs failed to induce an elevation of $[Ca^{2+}]_i$ in non-transfected HeLa cells, while ATP and trypsin induced a significant $[Ca^{2+}]_i$ elevation (data not shown).

2.6. Drugs and solutions

Thrombin (bovine plasma, 1880 NIH units per mg protein; the molar concentration of thrombin was estimated from the activity, assigning 1 U ml^{-1} to be 10 nM), trypsin (bovine pancreas, 10,900 U per mg protein) and 4-aminidophenylmethane–sulphonyl fluoride were purchased from Sigma. Thrombin and trypsin were dissolved in distilled water and kept in small aliquots at −30 °C until use. The enzyme stock was applied to the bathing buffer by 100-fold and higher dilution without changing pH of the buffer. Human TRAP7 (SFLLRNP) was purchased from Bachem. A high affinity PAR1-AP, haPAR1-AP (Ala-Phe(pF)-Arg-Cha-homoArg-Tyr-NH_2) [19] was purchased from Neosystem. The phycoerythrin-labeled anti-PAR1 antibodies, SPAN12 and WEDE15, were purchased from Immunotech. The epitope peptides of SPAN12 (NATLDPRSFLLR) and WEDE15 (KYEPFWEDEEKNES) were synthesized by Gene Net.

2.7. Statistical analysis

The values are expressed as the mean ± S.E.M. Student's *t*-test was used to determine statistical significance. *P* values of less than 0.05 were considered to have statistical significance.

3. Results

3.1. PAR agonists-induced elevations of $[Ca^{2+}]_i$ in HUVECs

In the previous report, the proteolytic inactivation of PAR1 by trypsin was studied mainly in in situ endothelial

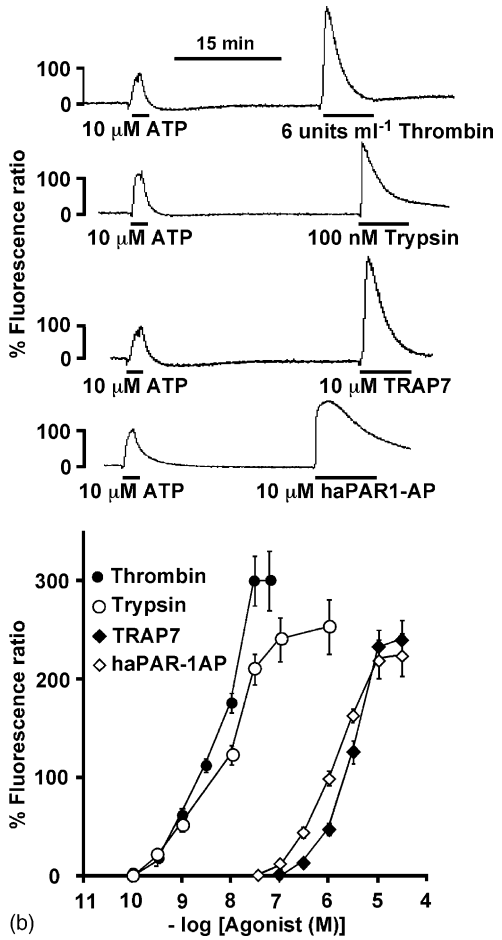

Fig. 1. $[Ca^{2+}]_i$ elevations induced by thrombin, trypsin, TRAP7 and haPAR1-AP in HUVECs. Representative recordings (a) and the concentration-response curves (b) for the elevation of $[Ca^{2+}]_i$ induced by thrombin, trypsin, TRAP7 and haPAP1-AP in HUVEC. The levels of $[Ca^{2+}]_i$ at rest and at the peak response to 10 μM ATP were assigned to be 0 and 100%, respectively. The data are the mean ± S.E.M. ($n = 5$). The concentration of thrombin was estimated from the proteolytic activity, while assigning 1 U ml^{-1} to be 10 nM.

cells of the porcine aortic valve [17]. However, the amino acid sequences of the porcine PAR1 corresponding to the epitopes of SPAN12 and WEDE15 were found to differ from those of the human PAR1 [17]. We therefore selected HUVECs to study the mechanism of proteolytic inactivation of PAR1 in the present study. We thus first validated the usage of HUVECs by examining the responsiveness toward PARs agonists (Fig. 1) and the proteolytic inactivation of PAR1 in HUVECs (Fig. 2). Thrombin, trypsin, TRAP7 and haPAR1-AP induced a rapid and transient increase in $[Ca^{2+}]_i$ in HUVECs in a concentration-dependent manner (Fig. 1). The $[Ca^{2+}]_i$ elevations induced by thrombin and trypsin, but not those induced by the activating peptides, were completely abolished by pretreatment of enzymes with a serine protease inhibitor, 4-aminido-phenyl-methane–sulphonyl fluoride (data not shown). The concentrations required to induce the maximal response (6 U ml^{-1} thrombin, 100 nM trypsin, 10 μM TRAP7 and 10 μM haPAR1-1AP) was used in the following experiments.

3.2. Cross-desensitization of thrombin and trypsin and the responsiveness to PAR1-APs after receptor cleavage in HUVECs

HUVECs were sequentially stimulated in various combinations of thrombin and trypsin for 10 min at 15 min intervals (Fig. 2a and b). Once the cells were stimulated with thrombin or trypsin, the subsequent application of thrombin induced no $[Ca^{2+}]_i$ elevation (Fig. 2a and c). On the other hand, trypsin induced no $[Ca^{2+}]_i$ elevation after the preceding stimulation with trypsin (Fig. 2b and c). However, the preceding stimulation with thrombin demonstrated no effect on the subsequent response to trypsin (Fig. 2b and c), suggesting that the trypsin-induced $[Ca^{2+}]_i$ elevation was mediated by PARs other than PAR1, i.e., mainly by PAR2.

Next, HUVECs were stimulated first with thrombin or trypsin, and then with TRAP7 or haPAR1-AP at 15 min intervals. Once HUVECs were stimulated with thrombin, the subsequent application of TRAP7 induced a $[Ca^{2+}]_i$ elevation significantly ($P < 0.05$) smaller than that obtained without the preceding stimulation with thrombin (Fig. 2d and f). On the other hand, the $[Ca^{2+}]_i$ elevation obtained with TRAP7 after the preceding stimulation with trypsin did not significantly ($P > 0.05$) differ from that seen without the preceding stimulation (Fig. 2d and f). The similar results were obtained with haPAR1-AP (Fig. 2e and f). The longer incubation with thrombin (60 min) did not cause further attenuation of the haPAR1-AP-induced $[Ca^{2+}]_i$ elevation (Fig. 2e and f). The most critical observation was that the responsiveness to TRAP7 and haPAR-AP remained intact after the preceding stimulation with trypsin, in contrast to the observation that the responsiveness to thrombin was abolished in the same situation. The observations shown in Figs. 1 and 2 were thus consistent with those seen in the porcine aortic valvular endothelial cells [17] and validated the use of HUVECs.

3.3. Analysis of cleavage of PAR1 by thrombin and trypsin by immunofluorescence staining of PAR1

To determine the broad boundaries of the cleavage site in PAR1, we performed immunofluorescence staining of PAR1 with two antibodies, SPAN12 and WEDE15 (Fig. 3). The antibody SPAN12 was raised against the region 35–46 flanking the thrombin cleavage site. Therefore, this antibody recognizes only intact PAR1 but not PAR1 cleaved at the thrombin site [9,26,29]. The antibody WEDE15 was raised against residues 51–64, the hirudin-like region located closer to the C-terminus than the thrombin site. Therefore, this antibody recognizes both intact PAR1 and PAR1 cleaved at the thrombin site.

In the untreated control cells, both antibodies specifically stained the cell surface when the cells were not permeabilized (Fig. 3a, intact). On the other hand, when

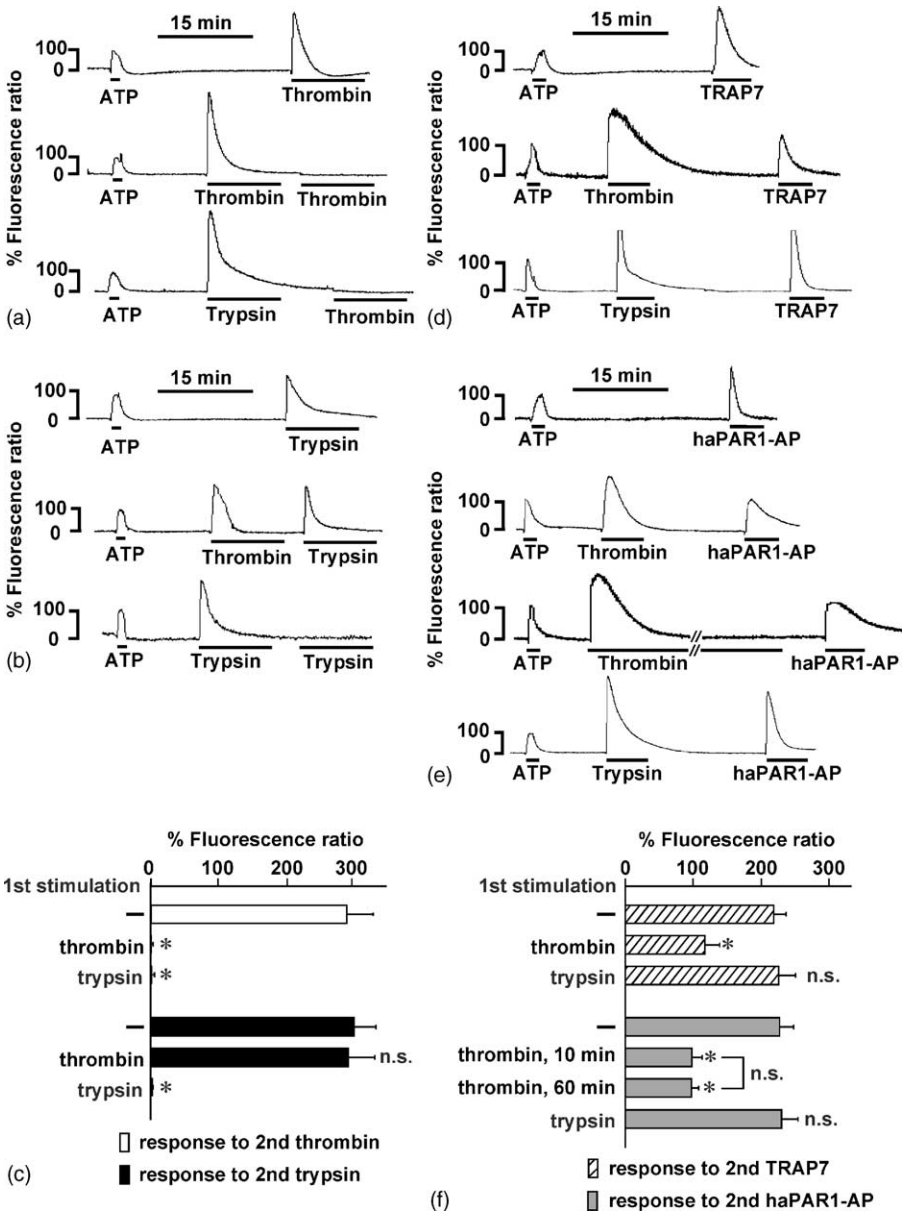

Fig. 2. Desensitization of responses to thrombin, trypsin, TRAP7 and haPAR1-AP by thrombin and trypsin in HUVECs. Representative recordings (a, b, d, e) and the summary (c and f) for $[Ca^{2+}]_i$ elevations induced by 6 U ml^{-1} thrombin, 100 nM trypsin, 10 μM TRAP7 and haPAR1-AP, without and with the preceding stimulation with 6 U ml^{-1} thrombin and 100 nM trypsin. The HUVECs were sequentially stimulated at 15 min intervals in the combinations as indicated. The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned to be 0 and 100%, respectively. The data are the mean ± S.E.M. ($n = 5$); ∗, significantly different ($P < 0.05$) and n.s., not significantly different ($P > 0.05$) from the control values obtained without the preceding stimulation.

the cells were fixed in paraformaldehyde and permeabilized with 0.1% Triton X-100 (Fig. 3a, permeabilized), the antibodies detected the antigen inside the cells as well as on the cell surface. These observations validated the method for analyzing the cleavage of the cell surface receptor in the cells with intact plasma membrane. Without permeabilization, the treatment of HUVECs with 6 U ml^{-1} thrombin or 100 nM trypsin for 3 min completely abolished the surface staining seen with SPAN12. The treatment with trypsin also completely abolished the cell surface staining of WEDE15. However, the treatment with thrombin only slightly attenuated the staining with WEDE15 (Fig. 3a) On the other hand, the internal staining with SPAN12 and WEDE15 in the permeabilized cells remained unaffected after the treatment with thrombin and trypsin (Fig. 3a, permeabilized).

The specificity of the immunofluorescence staining was examined as shown in Fig. 3b. The staining with SPAN12 was abolished by its epitope peptide at 10-fold and higher molar excess, while it was resistant to 1000-fold molar excess of the epitope peptide of WEDE15. Similarly, the staining with WEDE15 was abolished by its epitope peptide at 10-fold and higher molar excess, while it was resistant to 1000-fold molar excess of the epitope peptide

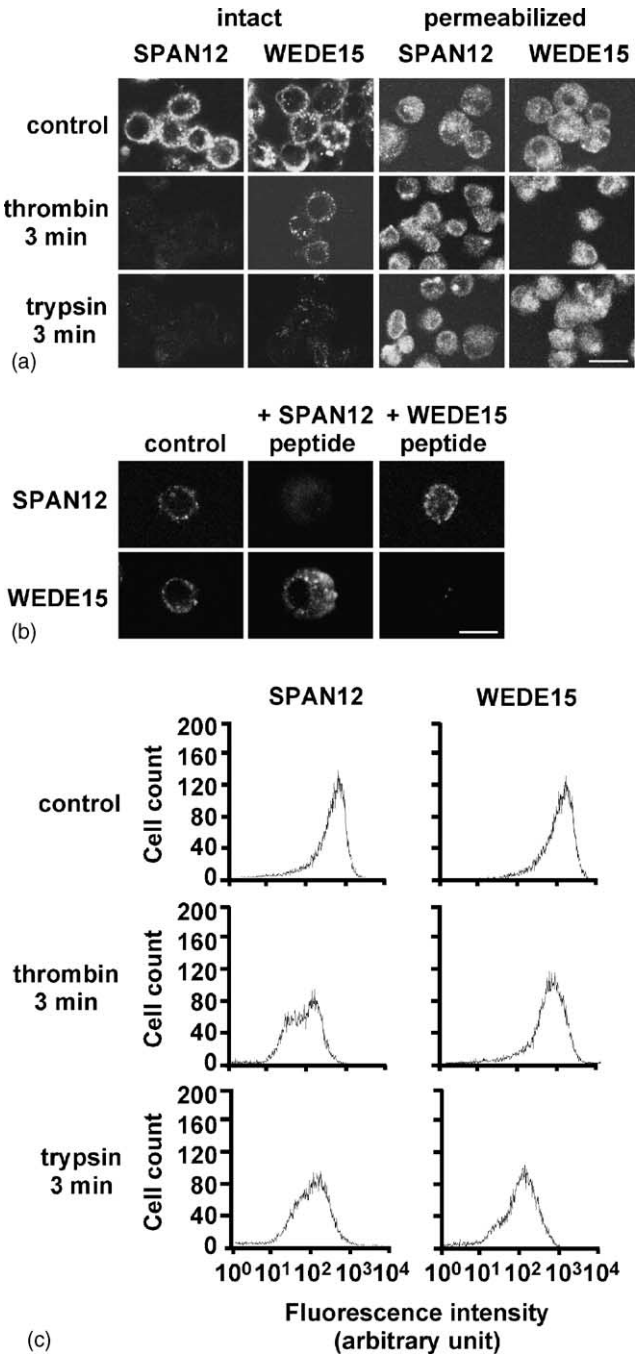

Fig. 3. Immunofluorescence detection of PAR1 after the treatment with thrombin and trypsin in HUVECs. (a) Representative photos of the immunofluorescence staining with SPAN12 and WEDE15 in the intact and permeabilized HUVECs, before (control) and after 3 min treatment with 6 U ml^{-1} thrombin and 100 nM trypsin. Scale bar, 20 μm. (b) Representative photos of the immunofluorescence staining with SPAN12 and WEDE15 in the presence and absence of 1000-fold molar excess of epitope peptides of SPAN12 and WEDE15. Scale bar, 20 μm. (c) Representative histograms of the flow cytometric data obtained with the SPAN12- and WEDE15-stained intact HUVECs untreated (control) or treated with 6 U ml^{-1} thrombin and 100 nM trypsin for 3 min. Shown are representative results of four independent experiments.

of SPAN12. These results thus validate the determination of the boundary of cleavage sites by immunofluorescence staining.

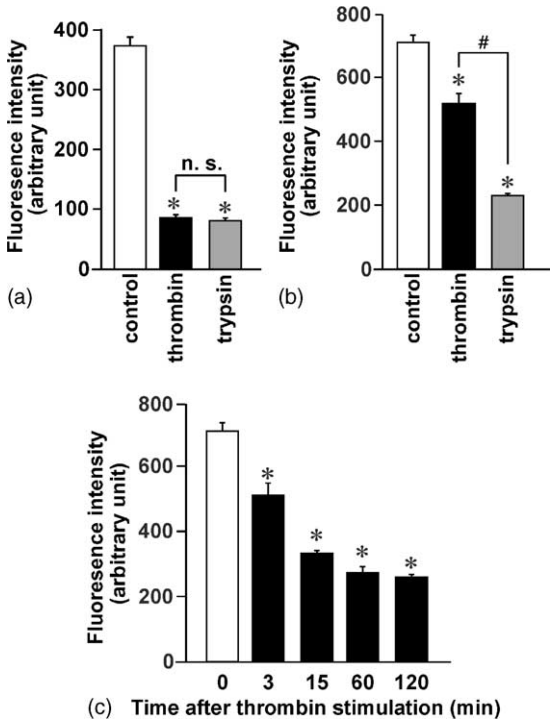

Fig. 4. The quantitative analysis of immunofluorescence staining of PAR1 with flow cytometry. (a and b) The fluorescence peak obtained with flow cytometry of the HUVECs stained with SPAN12 (a) and WEDE15 (b), before (control) and 3 min after treatment with 6 U ml^{-1} thrombin and 100 nM trypsin. (c) The time-dependent decrease in the fluorescence peak obtained with WEDE15, after treatment with 6 U ml^{-1} thrombin. The fluorescence peak was determined on a histogram of flow cytometric data (Fig. 3) and expressed in arbitrary units. The data are the mean ± S.E.M. ($n = 4$); *, significantly ($P < 0.05$) different from the control values (a and b) and the value obtained at time 0 (c); #, $P < 0.05$; n.s., not significant ($P > 0.05$).

The immunofluorescence staining was quantitatively evaluated using flow cytometry (Figs. 3c and 4). Both thrombin and trypsin similarly induced approximately a four-fold reduction of the fluorescence peak obtained with SPAN12 (Figs. 3c and 4a). The longer incubation (>120 min) with thrombin and trypsin caused no further decrease in fluorescence peak (data not shown). This fluorescence level is thus considered to be a background level, which was consistent with the observation with a confocal microscopy (Fig. 3a). On the other hand, the fluorescence peak obtained with WEDE15 was substantially decreased by 3 min treatment with trypsin, while it was only slightly but significantly ($P < 0.05$) decreased by 3 min treatment with thrombin (Figs. 3c and 4b). There was a significant ($P < 0.05$) difference in the fluorescence peak between the treatment with thrombin and trypsin (Fig. 4b). The longer incubation with trypsin caused no further decrease in the fluorescence peak (data not shown), and this level was thus considered to be the background level. However, the longer incubation with thrombin caused a time-dependent further decrease in the fluorescence peak (Fig. 4c). Eventually, the fluorescence peak obtained with 60 and 120 min treatment was similar ($P > 0.05$) to the level seen with trypsin, i.e., the background level (Fig. 4c).

3.4. The internalization of PAR1 induced by thrombin

Since WEDE15 should recognize PAR1 cleaved at thrombin site, the disappearance of cell surface staining of WEDE after thrombin treatment suggested that thrombin caused the receptor internalization. We thus examined internalization of PAR1 with confocal microscopy as shown in Fig. 5. HUVECs were first stained with WEDE15, and then treated with thrombin (Fig. 5; pre-stained). The reaction was terminated by the addition of 10 μM 4-aminido-phenylmethane-sulphonyl fluoride at 3, 15 and 60 min after thrombin treatment. The WEDE15 stained the cell surface before treatment with thrombin (Fig. 5, control). The fluorescence remained mostly on the cell surface after the 3 min treatment. However, the fluorescence was observed inside the cells after the 15 min treatment, and the internal fluorescence further increased by the 60 min treatment (Fig. 5, pre-stained). As a control, HUVECs were stained with WEDE15 after the treatments with thrombin (Fig. 5, post-stained). The surface staining with WEDE15 was still observed after the 3 min treatment. Thereafter, the surface fluorescence decreased, and mostly disappeared after the 60 min treatment. The observations of the post-stained cells were thus consistent with the results obtained with flow cytometry (Fig. 4c).

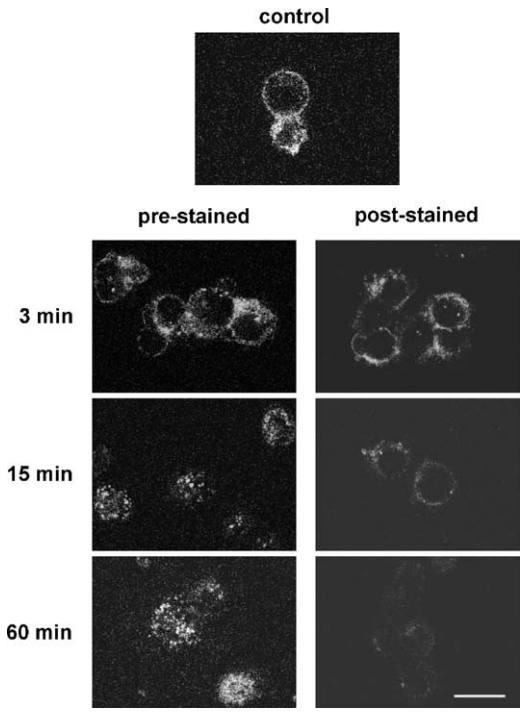

Fig. 5. The microscopic observation of the internalization of PAR1 in HUVEC. Representative photos of immunofluorescence staining with WEDE15. The HUVECs were first stained with WEDE15 and then treated with 6 U ml^{-1} thrombin for 3, 15 and 60 min (pre-stained), or the cells were first treated with 6 U ml^{-1} thrombin for 3, 15 and 60 min and then stained with WEDE15 (post-stained). The treatment with thrombin was terminated by addition of 10 μM 4-aminido-phenylmethane–sulphonyl fluoride. Control, fluorescence image obtained with HUVECs untreated with thrombin. Representative photos of two independent experiments are shown.

3.5. Determination of trypsin cleavage sites in PAR1-tansfected HeLa cells

Thrombin and haPAR1-AP did not induce any significant elevation of $[Ca^{2+}]_i$ in non-transfected HeLa cells, while ATP and trypsin induced a significant elevation of $[Ca^{2+}]_i$ (data not shown), indicating that HeLa cells lack thrombin receptor including PAR1 but express trypsin receptor PAR2. The response to 10 μM ATP was thus used as a reference response in HeLa cells as in HUVECs. On the other hand, the HeLa cells transfected with $PAR1^{wt}$ responded to thrombin and haPAR1-AP (Fig. 6a). In these cells, the preceding stimulation with thrombin significantly inhibited the subsequent response to haPAR1, while trypsin had no significant effect (Fig. 6d). This observation was consistent with that seen in HUVECs (Fig. 2). In the cells transfected with $PAR1^{70+82}$, thrombin and haPAR1-AP induced a transient elevation of $[Ca^{2+}]_i$ (Fig. 6b) and the preceding stimulation with thrombin significantly inhibited the subsequent response to haPAR1-AP (Fig. 6d). Importantly, the preceding stimulation with trypsin also inhibited the subsequent response to haPAR1-AP (Fig. 6b and d). This observation was in contrast to that seen in the HeLa cells transfected with $PAR1^{wt}$ or HUVECs. It is conceivable that $PAR1^{70+82}$ was cleaved at 41–42 and activated by trypsin as in the case with thrombin, and that trypsin thereby demonstrated a similar effect to that seen with thrombin. On the other hand, trypsin had no effect on the subsequent response to haPAR1-AP in the cells transfected with $PAR1^{70}$ and $PAR1^{82}$ (data not shown). We thus further examined the effect of trypsin in the cells transfected with $PAR1^{41+70+82}$ (Fig. 6c). In these cells, haPAR1-AP induced a transient elevation of $[Ca^{2+}]_i$, while thrombin induced no $[Ca^{2+}]_i$ elevation. These findings are consistent with the fact that $PAR1^{41+70+82}$ lacks a thrombin cleavage site. The preceding stimulation with thrombin had no effect on the subsequent response to haPAR1-AP (Fig. 6d). Trypsin also had no effect on the subsequent response to haPAR1-AP (Figs. 6c and d).

4. Discussion

Phosphorylation and proteolysis of the receptor are known to play an important role in the regulation of the responsiveness of thrombin receptor [1–3,30]. The phosphorylation causes association with arrestin-like molecules and receptor internalization [1,3]. The proteolysis makes the receptor unresponsible to a second stimulation with the same proteolytic activation, as shown in the Fig. 2. We herein determined that trypsin cleaves PAR1 at three sites; the residues 41–42 (thrombin cleavage site), 70–71 and 82–83 in the cells. These sites are consistent with those suggested by the in vitro experiment using recombinant extracellular fragment of PAR1 [16]. Therefore, the present study provides the first physiological evidence for the

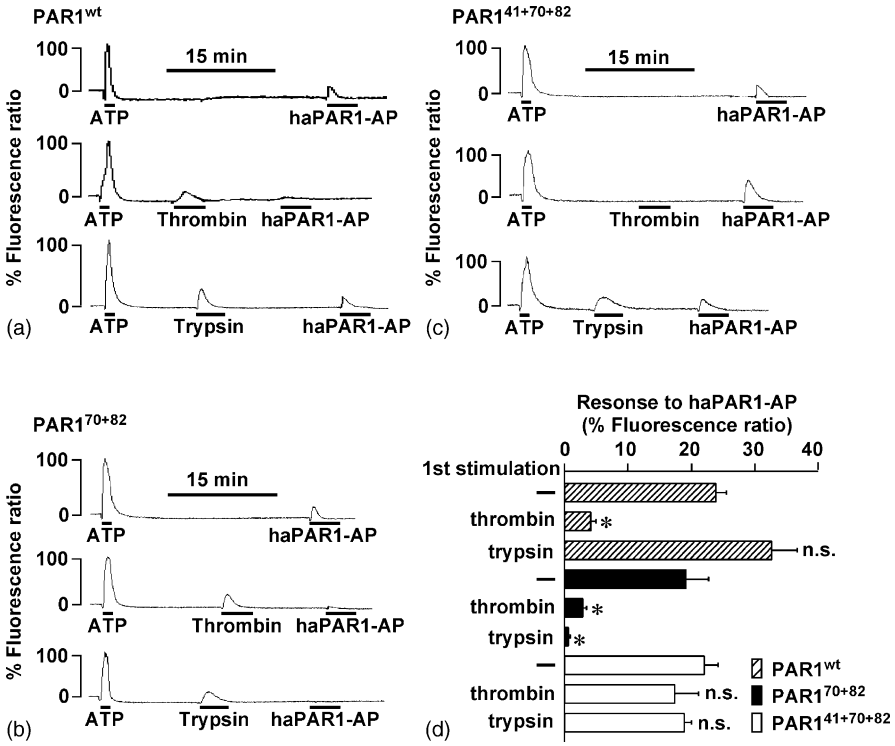

Fig. 6. Response to haPAR1-AP after treatment with thrombin and trypsin in HeLa cells transfected with proteolysis-resistant mutants of PAR1. Representative traces (a–c) and summary (d) for the responses to 10 μM haPAR1-AP without and with the preceding stimulations with 6 U ml^{-1} thrombin or 100 nM trypsin in the cells transfected with $PAR1^{wt}$ (a), $PAR1^{70+82}$ (b) and $PAR1^{41+70+82}$ (c). The cells were sequentially stimulated at 15% intervals. The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned to be 0 and 100 nM, respectively. The data are the mean ± S.E.M. ($n = 3$); *, significantly different ($P < 0.05$) and n.s., not significantly different ($P > 0.05$) from the control values obtained without the preceding stimulation.

trypsin cleavage sites of PAR1. We suggest that the cleavage at the latter two sites dominates the cleavage at the site 41–42, thereby inactivating responsiveness of PAR1 to thrombin under physiological situations. The inactivation of PAR1 by trypsin was not due to the internalization or proteolytic disintegration of the receptor, because the responsiveness to PAR1-APs remained intact after trypsin treatment. However, it is suggested to be due to the proteolytic removal of the tethered ligand region, as evidenced by immunofluorescence staining.

Trypsin has been shown to cleave the recombinant extracellular domain of PAR1 and cellular PAR1 at the thrombin site (the residues 41–42) [1–3,16]. However, we herein demonstrated that trypsin did not activate PAR1 (unproductive) as previously reported [2]. The conclusion of unproductive cleavage of PAR1 by trypsin is based on the following two observations. First, the response to trypsin after the preceding stimulation with thrombin was similar to that observed without the preceding stimulation. If PAR1 was involved in the response to trypsin, the response to trypsin after the preceding stimulation with thrombin should then be smaller than the control response. Second, the responsiveness to the agonist peptides was fully retained after the preceding stimulation with trypsin, while it was desensitized after the preceding stimulation with thrombin. If trypsin activated PAR1 by the same mechanism as thrombin, the responses to the agonist peptides should also be desensitized after the treatment with trypsin. Accordingly, the $[Ca^{2+}]_i$ response to trypsin was suggested to be mediated by PARs other than PAR1, mainly by PAR2 [13].

Although trypsin causes unproductive cleavage of PAR1 under physiological conditions, it could activate PAR1 under special conditions, where the both sites 70–71 and 82–83 were made cleavage-resistant ($PAR1^{70+82}$), thus leaving the thrombin site cleavable. In the cells transfected with $PAR1^{70+82}$, trypsin desensitized the subsequent response to haPAR1-AP as thrombin did, thus suggesting that trypsin activated PAR1. Since mutations of both sites 70–71 and 82–83 converted the responsiveness to PAR1-APs to be sensitive to trypsin treatment, but mutations of one of these sites ($PAR1^{70}$ and $PAR1^{82}$) failed to do so, these two sites could account for all of the unproductive cleavage and inactivation of PAR1 by trypsin. Collectively, our findings suggest that trypsin cleaves PAR1 at three sites 41–42, 70–71 and 82–83 in the cells. The cleavage at the latter two sites dominates the cleavage at the sites 41–42 in the wild type PAR1, resulting in unproductive cleavage and inactivation of PAR1.

The immunofluorescence staining of PAR1 clearly demonstrated that trypsin indeed cleaved PAR1 and removed the ligand region in the HUVECs. The two antibodies used in the present study were raised against the different epitopes in the N-terminal extracellular domain of PAR1 [9,26], and the specificity of the antibodies was

validated in the present study. Since trypsin removed both epitopes from the cell surface by 3 min treatment, it is thus concluded that trypsin cleaves PAR1 at the sites within the epitope of WEDE15 or closer to the C-terminus in the N-terminal extracellular domain. These observations are thus consistent with the major cleavage sites determined by the study with mutant PAR1.

It is noteworthy that the longer treatment with thrombin removed the epitope of WEDE15 from the cell surface, to the extent similar to that seen with trypsin treatment. The removal of the epitope of WEDE15 from the cell surface by thrombin was considered to be partly due to the receptor internalization, as clearly demonstrated by immunostaining in the pre-stained protocol (Fig. 5). However, the disappearance of the staining with WEDE15 is apparently inconsistent with the observation that haPAR1-AP did induce substantial elevation of $[Ca^{2+}]_i$ even after the 60 min treatment with thrombin (Fig. 3). The receptor internalization alone cannot account for all of the disappearance of the staining with WEDE15. It is possible that PAR1-APs activated the receptors other than PAR1 after longer treatment with thrombin. TRAP7 was indeed reported to activate PAR2 as well as PAR1 [31,32]. However, haPAR1-AP which has higher specificity toward PAR1 [19] induced a similar substantial elevation of $[Ca^{2+}]_i$ after the 60 min treatment with thrombin. Furthermore, the preceding stimulation with trypsin abolished the subsequent response to PAR2-activating peptides [17], while it had no effect on the subsequent response to PAR1-APs (the present study). Therefore, the activation of PARs other than PAR1 by the agonist peptides is less likely. Alternatively, PAR1 missing the epitope of WEDE15 was suggested to be retained on the cell surface and to support the responsiveness toward PAR1-APs. Thrombin is thus suggested to induce not only receptor internalization but also additional cleavage of PAR1 similar to that seen with trypsin.

However, it is unlikely that thrombin per se catalysed this additional cleavage of PAR1. It has been recently reported in rat astrocytes that the activation of PAR1 induced activity of unidentified trypsin-like protease, which in turn cleaves PAR1 either within the tethered-ligand region or at the sites closer to the C-terminus, thereby terminating the intracellular signalling by the tethered ligand [10]. This mode of inactivation of PAR1 appeared to be similar to that observed with trypsin in the present study. It is possible that thrombin induced an additional cleavage of PAR1 in the vascular endothelial cells in a manner similar to that suggested in the rat astrocytes. Furthermore, we observed that thrombin used in the present study induced aggregation of fibrin when applied to fibrinogen, and that the longer incubation did not induce any lysis of the aggregate, thus suggesting no significant contamination of plasmin activity (data not shown). However, the contribution of any contaminated protease to the thrombin-induced additional cleavage of PAR1 could not be completely excluded at this moment.

Since trypsin is not activated under physiological conditions, trypsin-induced cleavage of PAR1 may thus not play a physiological role in the regulation of PAR1 activity. However, trypsin is activated under pathological conditions. The endothelial cells found around tumor cells and in patients with disseminated intravascular coagulation were shown to produce trypsin [33]. The circulating typsin activity increase in the acute pancreatitis [34] and some cancer cells produce proteases including trypsin [35–38]. The trypsin-induced inactivation of PAR1 thus may play some role under such conditions, and may regulate hemodynamics in the pancreatitis and cancer metastasia and angiogenesis. In addition, any proteases which remove the epitope of WEDE15 can inactivate PAR1. Loew et al. [16] reported that plasmin (residues 70–71 and 76–77), cathepsin G (residues 55–56 and 69–70), elastase (residues 72–73 and 86–87), proteinase 3 (residues 48–49, 72–73 and 92–93) and calpain I (residues 76–77) cleaved the extracellular domain of PAR1 at such sites within 5 min. It is thus possible that these proteases can also cause unproductive cleavage and inactivation of PAR1. In this context, it is an intriguing possibility that plasmin antagonises thrombin not only by promoting fibrinolysis but also by inactivating PAR1.

In conclusion, trypsin was determined to cleave PAR1 at three sites; residues 41–42, 70–71 and 82–83, in vascular endothelial cells. However, we suggest that the cleavage at the latter two sites dominated that at the residues 41–42, and that trypsin cleavage thus caused no activation of PAR1 (unproductive cleavage) but rather inactivated its responsiveness to thrombin. In the present study, trypsin was studies as a representative protease, however, other proteases can induce such mechanism of inactivation of PAR1 [1,2]. Unexpectedly, the longer incubation with thrombin induced a complete loss of the epitope of WEDE15 on the cell surface. This loss of the epitope of WEDE15 was attributed to the internalization of PAR1 and additional cleavage similar to that observed with trypsin. We thus suggest that inactivation of PAR1 by a limited proteolysis as represented by trypsin-induced inactivation contributes not only to the heterologous inactivation of PAR1 by proteases other than thrombin, but also to the termination of the intracellular signaling initiated by the thrombin-cleaved PAR1 in vascular endothelial cells.

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