Online Supplementary information for the manuscript entitled
Rac1 regulation of the surface expression of PAR1 and responsiveness to thrombin in
vascular smooth muscle cells

Tatsuya Yufu, Katsuya Hirano, Dan Bi, Mayumi Hirano, Junji Nishimura, Yukihide Iwamoto, Hideo Kanaide

Expanded information for Methods

Materials
Thrombin (bovine plasma, 1880 NIH units/mg protein) was purchased from Sigma (St. Louis, MO, U.S.A.). Simvastatin, fluvastatin and pravastatin were supplied by Merck & Co. (Rahway, N.J., U.S.A.), Novartis Pharma Inc. (Tokyo, Japan) and Sankyo Co. (Tokyo, Japan), respectively. TFLLR-NH₂ (PAR1-activating peptide) and GYPGKF-NH₂ (PAR4-activating peptide) were purchased from Bachem (Bubendorf, Switzerland). Geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were purchased from Sigma (St. Louis, MO, U.S.A.). Geranylgeranyl transferase inhibitor (GGTI-298) and farnesyl transferase inhibitor (FTI-277) were purchased from Calbiochem (San Diego, U.S.A.). The antibodies and their source were as follows: phycoerythrine-conjugated anti-PAR1 antibody WEDE15 (Immunotech, Marseille, France), anti-(His)₆ antibody (Qiagen, Hilden, Germany), Anti-PAR1 antibody (sc-5605, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), horse radish peroxidase-conjugated anti-mouse IgG (Sigma) and horse radish peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.). Oligonucleotides was synthesized by Hokkaido System Sciences (Sapporo, Japan).

Cell culture
The human coronary artery smooth muscle cells (HCASMCs) were purchased from Kurabo (Osaka, Japan), and cultured in HuMedia-SG2 (Kurabo) as described in
manufacturer’s instruction. The rat aortic smooth muscle cells (RASMCs) in primary culture were enzymatically dispersed from the aortic media of male Wistar rats and cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and antibiotics, as previously described \(^1\). The growth medium was renewed every 2-3 days. The cells were used in all experiments on culture days 4-6 and before confluence.

**Measurement of \([\text{Ca}^{2+}]_{i}\) in smooth muscle cells**

The cells were loaded with fura-2 by incubating them in Dulbecco’s modified Eagle medium containing 10 \(\mu\text{mol/L}\) fura-2 acetoxyethyl ester for 1 h at 37 °C as previously described \(^2\). After loading with fura-2, the cells were washed and equilibrated in HEPES-buffered saline (HBS; 10 mmol/L Hepes, pH 7.4, 135 mmol/L NaCl, 5 mmol/L KCl, 1.0 mmol/L CaCl\(_2\), 1.0 mmol/L MgCl\(_2\) and 5.5 mmol/L D-glucose) for at least 30 min at room temperature before starting the measurements. The changes in \([\text{Ca}^{2+}]_{i}\) were monitored using a front-surface fluorometer as previously described \(^3\). Fluorometry was performed at 25 °C to prevent any leakage of fura-2. The 500 nm fluorescence intensities at 340 nm 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 \([\text{Ca}^{2+}]_{i}\), elevation induced by ionomycin (50 \(\mu\text{mol/L}\) for HCASMC and 25 \(\mu\text{mol/L}\) for RASMC) to be 0% and 100%, respectively. All data were collected using a computerized data acquisition system MacLab (Analog Digital Instruments, Australia).

**Immunofluorescent staining of PAR1**

HCASMCs were either untreated or treated with simvastatin for 24 h, and then harvested by incubating in PBS containing 1 mmol/L EDTA, 5 mmol/L EGTA at 37 °C for 15 min, and scraping them off from the culture dishes as previously described \(^4\). The cells were washed and suspended in PBS, and then were incubated with phycoerythrin-labeled anti-PAR1 antibodies, WEDE15, in PBS containing 1% bovine serum albumin at room temperature for 30 min. The cells were then washed 3 times in PBS and subjected to flow cytometric analysis with a flow cytometer FACSCalibur (Beckton Dickenson, San Jose, CA, U.S.A.) as previously described \(^4\). The peak fluorescence
The intensity of the cell population was determined on a histogram presentation of the fluorescence data. The fluorescence image was observed under a laser scanning confocal fluorescence microscope LSM GB200 (Olympus, Tokyo, Japan), using a 60x objective lens, 488 nm excitation and a 500-530 band pass emission filter. The fluorescence images were obtained at the nuclear level.

Expression and purification of inhibitor proteins of RhoA and Rac1/Cdc42
The RhoA-binding domain (RB) of Rho-kinase and the Rac1/Cdc42-binding domain (PBD) of p21-activated protein kinase-1 were used as inhibitors of RhoA and Rac1/Cdc42, respectively. RB and PBD were expressed in *E. coli* as (His)$_6$-tagged proteins with and without a cell-penetrating peptide of human immunodeficiency viral Tat protein (TAT) and a hemagglutinin tag (HA), as previously described. The recombinant proteins were expressed and purified as previously described. In brief, the bacterial pellet was homogenized in buffer A (6 mol/L guanidine-HCl, 100 mmol/L NaH$_2$PO$_4$, 10 mmol/L Tris-HCl, pH 8.0) and clarified by centrifugation at 43,000 g, 4 °C, 15 min, and then was loaded onto the Ni$^{2+}$-loaded Hi-Trap chelating column (Pharmacia Biotech, Tokyo, Japan). The column was washed with buffer A and buffer B (6 mol/L urea, 100 mmol/L NaH$_2$PO$_4$, 10 mmol/L Tris-HCl, 50 mmol/L imidazole, pH 6.3). The recombinant proteins were then eluted in buffer C (6 mol/L urea, 100 mmol/L NaH$_2$PO$_4$, 10 mmol/L Tris-HCl, 500 mmol/L imidazole, pH 6.3) and dialyzed against PBS (136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na$_2$HPO$_4$, 1.47 mmol/L KH$_2$PO$_4$). The protein concentration was determined using the Coomassie protein assay kit (Pierce, Rockford, IL, U.S.A.) and bovine serum albumin as the standard.

RhoA and Rac1 knock-down by RNA interference
The cellular expression of RhoA and Rac1 was specifically down-regulated by using RNA interference technique. The single-stranded sense and anti-sense RNAs corresponding to the coding region 143-163 of human RhoA, their inverted sequences and the sense and anti-sense RNAs corresponding to the coding region 13-33 of human Rac1 were synthesized to contain a 3’ TT overhang. The sequences of synthesized RNAs were as follows: 5’- GUG GAU GGA AAG CAG GUA GAG TT- 3’ (RhoA sense), 5’- CUC UAC CUG CUU UCC AUC AUC CAC TT -3’ (RhoA anti-sense), 5’- GAG
AUG GAC GAA AGG UAG GUG TT -3’ (RhoA inverted sense), 5’- CAC CUA CCU UUC GUC CAU CUC TT -3’ (RhoA inverted anti-sense), 5’- AAG UGU GUG GUG GUG GGA GAC TT -3’ (Rac1 sense), 5’- GUC UCC CAC CAC ACA CUU TT -3’ (Rac1 anti-sense). The sense and anti-sense RNAs (at 20 µmol/L) were annealed by incubating in annealing buffer (30 mmol/L HEPES, pH 7.4, 100 mmol/L potassium acetate, 2 mmol/L magnesium acetate) for 1 min at 90 °C, and then 1 min at 70 °C, followed by gradual cool-down to 37 °C for 1 h, as previously described 8,9. RNA were precipitated in ethanol and then dissolved in annealing buffer at the concentration of 20 µmol/L. The cells were plated at the density of 5x10^4 per 35 mm culture dish for fura-2 fluorometry. On the next day, the cells were exposed to 2 mL Opti-MEM1 (Invitrogen, Carlsbad, CA, U.S.A.) containing 25 nmol/L RNA and 15 nmol/L oligofectamine (Invitrogen) for 40-45 h.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of PAR1, Rac1 and RhoA mRNA
Total RNA was isolated and subjected to RT-PCR analysis, using 1 µg total RNA in 20 µl RT reaction, as previously described1. One µl RT product was then subjected to 10 µl PCR reaction. The primer used in the RT reaction of rat PAR1 was 5’-AGT AAG ATG GAT ACC TGC-3’ (rat PAR1 coding region 1243-1262), and the primers used in PCR amplification of rat PAR1 were 5’-TGA CAG TCA TAA GCA TTG AC-3’ (the sense primer: 605-634) and 5’-CCT TGA TTT ACT ACT A TG CC-3’ (the antisense primer: 1129-1149). The PCR reaction for rat PAR1 mRNA was performed as previously described 11. The primers and conditions for the RT-PCR analysis of rat β-actin mRNA were as previously described 1. The primer used in the RT reaction of human Rac1 was 5’-TTA CAA CAG CAG GCA TTT TC -3’ (human Rac1 coding region 748-767), and the primers used in PCR amplification of human Rac1 were 5’-CTA TCC TA T CCG CAA ACA GA -3’ (the sense primer: 396-415) and 5’-CTC TTC TTC TTC TTC ACG GG-3’ (the antisense primer: 729-748). The primer used in the RT reaction of human RhoA was 5’-TTT GGT CTT TGC TGA ACA CT -3’ (human RhoA coding region 624-643), and the primers used in PCR amplification of human RhoA were 5’-CTA TCC CAG AGG TGT ATG CEE CTTC TTC TTC ACG AAA TGC TTG ACT TC-3’ (the antisense primer: 455-477). The primers for human β-actin was purchased from Toyobo (Tokyo, Japan). The PCR reaction for RhoA and Rac1
consists of the initial 2 min denaturation at 94 °C, followed by 30-cycle amplification step consisting of 30 sec. denaturation at 94 °C, 90 sec. annealing at 55 °C and 30 sec extension at 72 °C (RhoA), and 35-cycle amplification step consisting of 30 sec. denaturation at 94 °C, 90 sec. annealing at 50 °C and 30 sec extension at 72 °C (RhoA).

The PCR products were separated on 3 % agarose gel electrophoresis containing 0.5 µg/mL ethidium bromide. The density of bands was determined with Gel Plotting Macros of the NIH image.

**Immunoblot analysis**

The cell extracts were prepared in RIPA buffer (50 mmol/L Tris-HCl, pH 7.2, 0.5 mol/L NaCl, 10 mmol/L MgCl₂, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % TritonX-100, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 10 µmol/L 4-aminophenylmethane sulfonyle fluoride), and then were separated with SDS-PAGE on 7.5-20 % gradient polyacrylamide gel, followed by transfer to polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA). Anti-(His)₆ antibody and anti-PAR1 antibody were used as the primary antibody at x100 and x250 dilution, respectively. Immune complex was detected using horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence technique (ECL plus; Amersham, Buckinghamshire, U.K.), and light emission was detected by X-OMAT AR film (Kodak, Rochester, NY, U.S.A.).
References for Online supplementary information


