Distinct Ca$^{2+}$ requirement for NO production between proteinase-activated receptor 1 and 4 (PAR$_1$ and PAR$_4$) in vascular endothelial cells

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Abbreviations: BAECs, bovine aortic endothelial cells; [Ca^{2+}]_i, cytosolic Ca^{2+} concentrations; DAR-4M, diaminorhodamine-4M; HA, a hemagglutinin tag; HBS, Hepes-buffered saline; HUVECs, human umbilical vein endothelial cells; L-NAME, N\(^\omega\)-nitro-L-arginine methyl ester; L-NOARG, N\(^\omega\)-nitro-L-arginine; PAR, proteinase-activated receptor; PAR_1-AP, PAR_1-activating peptide; PAR_4-AP, PAR_4-activating peptide; PAECs, porcine aorta endothelial cells.

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Abstract

Proteinase-activated receptor 1 and 4 (PAR₁ and PAR₄) are the major receptors mediating thrombin-induced NO production in endothelial cells. The intracellular signaling following their activation still remains to be elucidated. The present study provides the first evidence for the distinct Ca²⁺ requirement for the NO production between PAR₁ and PAR₄. The activation of PAR₁ by the activating peptide (PAR₁-AP) elevated [Ca²⁺]ᵢ and activated NO production in porcine aortic and human umbilical vein endothelial cells, while it had little effect on bovine aortic endothelial cells. PAR₄ activation by PAR₄-AP consistently induced NO production without an appreciable [Ca²⁺]ᵢ elevation in three types of endothelial cells. The PAR₁-mediated NO production was significantly inhibited by BAPTA, while the PAR₄-mediated NO production was resistant. NO production following the PAR₁ and PAR₄ activation was significantly inhibited by pertussis toxin, but it was resistant to a Gαᵣ/₁₁ inhibitor, YM254890. However, YM254890 abrogated the PAR₁-mediated Ca²⁺ signal. PAR₄-mediated NO production was substantially inhibited by the inhibitors of phosphotidylinositol-3 kinase and Akt, and also by the dominant negative mutant of Akt. The PAR₁-mediated NO production was relatively resistant to inhibitors of phosphotidylinositol-3 kinase. An immunoblot analysis revealed a transient increase in the phosphorylation of Akt and eNOS following the PAR₄ stimulation. In conclusion, PAR₁ and PAR₄ engage distinct signal transduction mechanisms to activate NO production in vascular endothelial cells. PAR₄ preferably activates Gαᵣ/₁₁ and induced NO production in a manner mostly independent of Ca²⁺ but dependent on the phosphotidylinositol-3 kinase-Akt pathway, while PAR₁ activates both the Ca²⁺-dependent and independent mechanisms.
Introduction

Proteinase-activated receptors (PARs), which belong to the G protein-coupled receptor family, play a critical role in the cross signaling between the coagulation system and vascular wall, thus contributing to the vascular physiology and pathophysiology (Coughlin, 2000; Steinberg, 2005; Hirano, 2007). PARs are mainly expressed in endothelial cells in normal arteries, and thus mediate the endothelium-dependent regulation of vascular tone (Hirano and Kanaide, 2003; Hirano, 2007). NO production and its resultant vasorelaxation are, in fact, the most widely documented endothelial effects of thrombin under physiological situations (Hirano and Kanaide, 2003; Steinberg, 2005). Thrombin exerts vascular effects by activating PAR₁, PAR₃, and PAR₄ (Macfarlane et al., 2001; Hollenberg and Compton, 2002). However, PAR₃ does not directly elicit intracellular signaling, but instead functions as a cofactor for PAR₄ activation (Coughlin, 2000). PAR₁ and PAR₄ are thus considered to be major signaling receptors for thrombin. However, little is known about the intracellular signaling following the PAR₄ activation, although the signal transduction pathways following the PAR₁ activation have been intensively studied (Coughlin, 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002). Nevertheless, PAR₁-activating peptides (PAR₁-APs) and PAR₄-APs have been reported to induce endothelium-dependent NO-mediated relaxation (Hollenberg et al., 1999; Mizuno et al., 2000a). The study with knock-out mice have demonstrated that PAR₁ and PAR₄ account for most, if not all, of the thrombin-induced vasorelaxation, while PAR₁ played a major role, especially at low concentrations of thrombin (Kataoka et al., 2003). It is thus conceivable that both PAR₁ and PAR₄ activate NO production in endothelial cells. However, the intracellular mechanisms for the NO production following the activation of PAR₁ and PAR₄ and their any possible differences still remains to be elucidated (Amadesi and Bunnett, 2004; Steinberg, 2005).
We have previously reported that thrombin induced NO production with a slight elevation of [Ca\(^{2+}\)]\(_i\) in the endothelial cells of the porcine aortic valve (Mizuno et al., 2000b). Thrombin was also found to induce the greater NO production for a given elevation of [Ca\(^{2+}\)]\(_i\) than the other stimulants we tested: ATP, bradykinin, and ionomycin (Mizuno et al., 2000b). Our observations thus suggested that thrombin activated the Ca\(^{2+}\)-independent as well as the Ca\(^{2+}\)-dependent mechanism of NO production. We also reported that PAR\(_4\) induced NO production without any appreciable elevation of [Ca\(^{2+}\)]\(_i\) in the cultured bovine aortic endothelial cells (BAECs) (Momota et al., 2006). There is a possibility that PAR\(_4\) mediates the Ca\(^{2+}\)-independent component of the thrombin-induced NO production. However, BAECs were found to be unique in that they were well responsive to PAR\(_4\)-AP but not PAR\(_1\)-AP (Momota et al., 2006). Therefore, the Ca\(^{2+}\)-independency of the PAR\(_4\)-mediated NO production still waits for the evaluation in other types of endothelial cells, which are responsive to both PAR\(_1\) and PAR\(_4\) activation. Furthermore, the mechanism for the PAR\(_4\)-mediated Ca\(^{2+}\)-independent NO production still remains to be elucidated.

In the present study, using diaminorhodamine-4M (DAR-4M) (Kojima et al., 2001; Momota et al., 2006) and fura-2 fluorometry, we aimed to elucidate the signal transduction pathways, especially in terms of Ca\(^{2+}\) signal, from the activation of PAR\(_1\) and PAR\(_4\) to the production of NO in three difference types of endothelial cells; BAECs, porcine aorta endothelial cells (PAECs), and human umbilical vein endothelial cells (HUVECs). BAECs are taken to represent the endothelial cells, which predominantly respond to PAR\(_4\) stimulation, while PAECs and HUVECs are taken to represent the cells, which respond to both PAR\(_1\) and PAR\(_4\) stimulation (see Results). We consistently found that the PAR\(_4\) activation induced the NO production in a manner independent of Ca\(^{2+}\) signal in three different cell types. The mechanism for the PAR\(_4\)-mediated Ca\(^{2+}\)-independent NO production was further elucidated using BAECs. The
present study thus provides the first evidence for the differential requirement of Ca^{2+} signal for the endothelial NO production between PAR\textsubscript{1} and PAR\textsubscript{4}. 
Methods

Materials

DAR-4M was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Fura-2-acetoxyethyl ester (AM) and BAPTA-AM were purchased from Dojin (Kumamoto, Japan). Anti-phospho-Akt (Ser473) and anti-phospho-eNOS (Ser1179) antibodies were purchased from Cell Signaling Technologies (Beverly, MA, U.S.A.); anti-Akt and anti-eNOS antibodies were from BD Bioscience (San Jose, CA, U.S.A.); anti-(His)_6 antibody was from Qiangen (Hilden, Germany); and anti-tubulin antibody was from Serotec (Oxford, U.K.). Thrombin (bovine plasma, 1880 NIH U/mg protein, 1 U/ml=10 nM), bradykinin, aminoguanidine, and ionomycin were purchased from Sigma (St. Louis, MO, U.S.A.). TFLLR-NH2 (PAR1-AP) and AYPGKF-NH2 (PAR4-AP) were purchased from Bachem (Bubendorf, Switzerland). The negative control peptides for PAR1-AP (FTLLR-NH2) and PAR4-AP (YAPGKF-NH2) were synthesized by Rapid Multiple Peptide Synthesis Service, University of Calgary (Calgary, Alberta, Canada). LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), wortmannin, SH-6 (a Akt inhibitor), n-(3-(aminomethyl) benzyl) acetamidine (1400W), AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline), AG1024 (3-bromo-5-t-butyl-4-hydroxy-benzylidenemalonitrile), AG538 (α-cyano-(3,4-dihydroxy)cinnamoyl-(3’,4’-dihydroxyphenyl)ketone), KN-93 (2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), GF109203X (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), and H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) were purchased from Calbiochem (San Diego, CA, U.S.A.).
(Z)-1-(N-Methyl-N-[6-(N-methylammoniohexyl)amino])diazen-1-ium-1,2-diolate (MAHMA-NONOate) was purchased from Alexis Biochemicals (Lausen, Switzerland). Nω-nitro-L-arginine methyl ester (L-NAME) and Nω-nitro-L-arginine (L-NOARG) were purchased from Wako Pure Chemicals (Tokyo, Japan). PD98059 (2′-amino-3′-methoxyflavone) was purchased from Biomol (Plymouth Meeting, PA, U.S.A). Pertussis toxin was purchased from Seikagaku Co. (Tokyo, Japan). YM254890 was kindly donated by Astellas Pharma Inc. (Takasaki et al., 2004).

Cell culture

BAECs, PAECs, and A7r5 cells were cultured in Dulbecco’s modified Eagle medium containing 10 % fetal bovine serum as previously described (Hirano et al., 2001; Eto et al., 2003; Hirano et al., 2004b). HUVECs were cultured in MCDB104 supplemented with 5 % fetal bovine serum and endothelial cell growth supplements (Nissui Pharmaceutical, Tokyo, Japan), as previously described (Nakayama et al., 2004). For the measurement of NO production, the cells were plated on Cell Desk LF1 (Sumitomo Bakelite, Tokyo, Japan) coated with Type1-P collagen (Nitta Gelatin, Osaka, Japan). Unless otherwise stated, the cells were plated on culture dishes. The cells were used at confluence (days 3-4).

DAR-4M fluorometry

The fluorescence intensity of 10 µM DAR-4M in 1 ml Hepes-buffered saline (HBS; 10 mM Hepes, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 5.5 mM D-glucose) was first measured in a quartz cuvette at 25 °C with a fluorescence spectrophotometer 650-40 (Hitachi, Tokyo, Japan), using a pre-scan mode, which automatically set the full-scale amplitude for the recoding (17.1±0.15 fluorescence units, n=31) so that the maximum intensity obtained
with 10 μM DAR-4M was 70 % of the full scale. Cell Desk LF1 containing the cells was then inserted into the cuvette, and the changes in the fluorescence intensity (excitation at 540±5 nm; emission at 580±10 nm) were recorded under such a full-scale setting. The fluorescence data obtained with the cells were normalized by multiplying with the full-scale amplitude pre-determined for each measurement, and thus expressed in arbitrary units. The pre-stimulation level was assigned to be zero fluorescence. When the effects of various inhibitors on NO production were studied, the DAR-4M fluorescence data were expressed as % of the control value. The calibration curve for DAR-4M fluorescence was obtained using MAHMA-NONOate as a standard in the absence of the cells (Fig. 1a). The indicated concentrations of MAHMA-NONOate were added to the HBS containing 10 μM DAR-4M, and then the excitation spectrum of the DAR-4M fluorescence was recorded after the incubation period sufficient for the half life of NO release of MAHMA-NONOate (~3 min at room temperature). The calibration curve was constructed with the values obtained at the peak of fluorescence intensity of the excitation spectrum.

The following observations validates DAR-4M fluorometry as a method to estimate the NO production: (1) The linear relationship between the DAR-4M fluorescence intensity and the concentration of MAHMA-NONOate (Fig. 1), (2) The concentration-dependency in the agonist-induced increases in DAR-4M fluorescence (Fig. 1), (3) The inhibition of the agonist-induced increases in the DAR-4M fluorescence by NOS inhibitors (Fig. 1), (4) No increase in the DAR-4M fluorescence by thrombin in the smooth muscle cell line A7r5 (Thrombin did induce [Ca^{2+}]_i elevation in fura-2 fluorometry) (data not shown).

**Fura-2 fluorometry**

The cells on 35-mm dishes were loaded with fura-2 in an acetoxyethyl ester form as previously
described (Eto et al., 2003). When the fura-2 fluorometry was conducted in the BAPTA-loaded cells, BAPTA-AM was added during the last 30 min of the fura-2 loading period. After loading with fura-2 and/or BAPTA, the cells were equilibrated in HBS at room temperature for 30 min and then fluorometry was started. The changes in fura-2 fluorescence (excitation at 340±10 and 380±10 nm; emission at 500±10 nm) were monitored in HBS at 25 °C using a front-surface fluorometer as previously described (Eto et al., 2003; Kanaide, 2006). The response to 50 µM ionomycin was recorded as a reference response at the end of each recording. 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 50 µM ionomycin to be 0 % and 100 %, respectively.

The effect of BAPTA loading on the resting level of \[ \text{[Ca}^{2+}]_i \] was evaluated in a separate experiment, according to the protocol that we have previously described (Kanaide, 2006). In brief, the resting level of \[ \text{[Ca}^{2+}]_i \] was first recorded, and then the maximal and minimal fluorescence ratio was sequentially recorded in HBS containing 50 µM ionomycin, and then in HBS containing 2 mM EGTA but no \text{Ca}^{2+}.

Treatment with pertussis toxin

The cells were treated with 100 ng/ml pertussis toxin in the growth media for 24 h as previously reported (Momota et al., 2006). When the cells were loaded with fura-2, fura-2-AM was added during the last 1 h of the pertussis toxin treatment. The cells were then washed and equilibrated in HBS at room temperature for at least 30 min, and then subjected to fura-2 or DAR-4M fluorometry.

Recombinant proteins

A dominant negative mutant of Akt with (TATHA-Akt) and without ((His)6-Akt) a
cell-penetrating peptide, and a RhoA-inhibitory protein with a cell-penetrating peptide (TATHA-RB) were expressed in bacteria and prepared as previously described (Hirano et al., 2004b; Koga et al., 2004; Shiga et al., 2005; Yufu et al., 2005). TATHA-Akt consisted of a hexahistidine tag, a cell-penetrating peptide of Tat, a hemagglutinin (HA) epitope and the N-terminal 147 amino acids of Akt1 (Accession No. M63167), while (His)_6-Akt lacks a cell-penetrating peptide and a HA epitope (Koga et al., 2004). TATHA-RB consisted of a hexahistidine tag, a cell-penetrating peptide, a HA epitope and the RhoA-binding region of Rho-kinase (Hirano et al., 2004b; Shiga et al., 2005). The recombinant proteins were affinity-purified through Ni^{2+}-loaded Hi-Trap chelating column on Akta Prime (Pharmacia Biotech, Tokyo, Japan). The protein concentration of the recombinant proteins was estimated with Coomassie protein assay kit with bovine serum albumin as a standard (Pierce, Rockford, IL, U.S.A.).

**Immunoblot analysis of protein transduction**

The transduction of TATHA-Akt and TATHA-RB into BAECs was confirmed by an immunoblot analysis as previously described (Hirano et al., 2004b; Koga et al., 2004; Yufu et al., 2005). In brief, the cells on 100-mm dishes were harvested and re-suspended in PBS at room temperature. TATHA-RB, TATHA-Akt and (His)_6-Akt were added to the cell suspensions at a concentration of 0.3 µM. The cells were exposed to proteins for 30 min, and then were rapidly and thoroughly washed three times in ice-cold PBS. The cells were once frozen at -80 °C, and then lysed in the buffer (50 mM Tris-HCl, pH 7.2, 0.5 M NaCl, 10 mM MgCl₂, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % TritonX-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µM 4-aminidophenylmethane sulfonfonyl fluoride), as previously described (Hirano et al., 2004b). The protein concentration of the lysates was estimated with Coomassie protein assay kit. The lysates (25 µg proteins) were then
separated with SDS-PAGE on 7.5-20 % gradient polyacrylamide gel, followed by transfer to PVDF membrane (BioRad, Hercules, CA, USA). The membranes were blocked overnight with 5 % non-fat dry milk in PBS containing 0.1 % Tween-20. The recombinant proteins were detected with anti-(His)$_6$ antibody (x500 dilution), horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence technique (Amersham, Buckinghamshire, U.K.). Tubulin was detected to validate the equal loading of the cell extract. The luminescence signal was detected and analyzed with a ChemiDoc XRS-J image analysis system (Bio-Rad, Tokyo, Japan).

Immunoblot analysis of phosphorylation of Akt and eNOS

The cells on a 60-mm culture dish were stimulated with 30 µM PAR$_4$-AP in the growth media, and then the cells were immediately washed twice in ice-cold PBS. PBS was thoroughly aspirated, and then the cells were lysed by scraping in 150 µl lysis buffer (50 mM Tris-HCl, pH 7.2, 0.5 M NaCl, 10 mM MgCl$_2$, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % TritonX-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µM 4-aminophenylmethane sulfonyle fluoride, 5 µM microcystin-LR, 20 µM NaF, 2 mM Na$_3$VO$_4$, 5 mM sodium pyrophosphate). The cell lysates were snap frozen in liquid N$_2$ and kept at -80 °C. When the effects of various inhibitors were examined, the cells were treated with inhibitors 30 min prior to and during the stimulation with PAR$_4$-AP. The cell lysates (50 µg proteins) were then subjected to an immunoblot analysis as described above. The PVDF membranes were blocked overnight with 5 % non-fat dry milk in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.05 % Tween-20. The antibodies were diluted (x250 for anti-phospho-Akt and anti-phospho-eNOS antibodies; x1000 for anti-Akt and anti-eNOS antibodies; x1000 for horseradish peroxidase-conjugated secondary antibodies) in an immunoreaction enhancer solution named Can-Get-Signal$^\text{®}$ (Toyobo, Osaka, Japan). The luminescence signal was detected and analyzed with a ChemiDoc XRS-J image
analysis system (Bio-Rad, Tokyo, Japan). The level of phosphorylation of Akt and eNOS was normalized by the total amount of Akt and eNOS, respectively, and then the value obtained with the unstimulated cells was assigned a value of 1.

Statistical analysis

The data are the means±SEM. The experimental number (n) indicates the number of independent experiments using different cell preparations. The analysis of variance (ANOVA) evaluated any statistical significance. A value of $p<0.05$ was considered to be significantly different.
Results

*NO production and [Ca^{2+}]_i elevation in response to thrombin, PAR\textsubscript{1}-AP, PAR\textsubscript{4}-AP, and bradykinin in BAECs*

The standard curve for the relationship between the DAR-4M fluorescence intensity and the concentrations of MAHMA-NONOate was first obtained (Fig. 1a). The linear relationship was observed within the range of 0-100 nM MAHMA-NONOate. The change in 10 arbitrary units of DAR-4M fluorescence was approximately equivalent to the changes in 10 nM MAHMA-NONOate. In the presence of BAECs, the buffer change from HBS to HBS induced a negligible change, if any, in the DAR-4M fluorescence (Fig. 1b). The subsequent change to HBS containing 30 µM PAR\textsubscript{4}-AP increased the DAR-4M fluorescence intensity (Fig. 1b). The fluorescence level thereafter remained sustained with no further increase (Fig. 1b), while washing out PAR\textsubscript{4}-AP in HBS containing 10 µM DAR-4M reverted the fluorescence level to that seen before stimulation (data not shown). Since DAR-4M is converted to a fluorescence triazole by reaction with NO (Kojima et al., 2001), this observation indicated a transient nature of the NO production induced by PAR\textsubscript{4}-AP, which lasted for a few minutes.

Thrombin, PAR\textsubscript{1}-AP, PAR\textsubscript{4}-AP and bradykinin all induced a concentration-dependent production of NO in BAECs (Fig. 1c-f). The maximal NO production obtained with thrombin was comparable to that obtained with PAR\textsubscript{4}-AP, while PAR\textsubscript{1}-AP induced only a small production of NO in BAECs. These findings thus suggested that PAR\textsubscript{4} may thus play a major role of in the thrombin-induced NO production in BAECs. The NO production seen with thrombin and PAR\textsubscript{4}-AP in BAECs were estimated to be approximately 80-120 nM (Fig. 1), which was consistent with those obtained *in vivo* (Vallance et al., 1995). The NOS inhibitors almost completely abolished the NO production induced by thrombin (0.3 U/ml), PAR\textsubscript{4}-AP (30 µM), and
bradykinin (10 nM) (Fig. 1g). However, iNOS inhibitors had no significant effect on the PAR₄-AP-induced NO production (Fig. 1h).

Bradykinin induced a transient elevation of [Ca²⁺]ᵢ in a concentration-dependent manner in BAECs (Fig. 2). On the other hand, thrombin, PAR₁-AP, and PAR₄-AP induced no increase in [Ca²⁺]ᵢ in BAECs (Fig. 2).

**Effect of BAPTA loading on the NO production in BAECs**

We investigated the requirement of Ca²⁺ for the PAR₄-mediated NO production using an intracellular Ca²⁺ chelator BAPTA in BAECs. The cells were treated with BAPTA-AM 30 min prior to the fura-2 or DAR-4M fluorometry. First, the concentration-dependent inhibition of the bradykinin-induced [Ca²⁺]ᵢ elevation by treatment with BAPTA-AM was examined, and 50 µM BAPTA-AM was found to be sufficient to almost completely abolish the bradykinin-induced [Ca²⁺]ᵢ elevation (Fig. 3a). This concentration of BAPTA-AM was thus used in the following evaluations (Figs. 3b, 4). In BAECs treated with 50 µM BAPTA-AM, the bradykinin-induced NO production was significantly, but partially, attenuated (Fig. 3b). However, the NO production induced by thrombin and PAR₄-AP was resistant to BAPTA loading in BAECs (Fig. 3b).

**Effect of thrombin, PAR₁-AP, and PAR₄-AP on [Ca²⁺]ᵢ and NO production in PAECs and HUVECs**

In PAECs, both thrombin and PAR₁-AP induced a concentration-dependent elevation of [Ca²⁺]ᵢ and the production of NO (Fig. 4a). However, in PAECs, PAR₄-AP also induced NO production (Fig. 4a) without any appreciable elevation of [Ca²⁺]ᵢ (data not shown). Similar results were also observed in HUVECs (Fig. 5). In HUVECs, the NO production induced by PAR₄-AP was not associated with any [Ca²⁺]ᵢ elevation, while that seen with thrombin and PAR₁-AP was associated with a significant elevation of [Ca²⁺]ᵢ. The negative control peptides for PAR₁-AP and PAR₄-AP
had no effects on either BAECs or PAECs, as evaluated by the Ca$^{2+}$ response. In PAECs, BAPTA-AM, at 50 µM, almost completely abolished the [Ca$^{2+}$]$_i$ elevation induced by PAR$_1$-AP and thrombin (data not shown). The NO production induced by PAR$_1$-AP was partially, but significantly, inhibited by BAPTA loading (Fig. 4b). However, the NO production induced by PAR$_4$-AP was resistant to BAPTA (Fig. 4b). BAPTA did not significantly inhibit the thrombin-induced NO production in PAECs (Fig. 4b).

The effect of BAPTA on the resting level of [Ca$^{2+}$]$_i$ was examined in PAECs. The resting level of the control cells seen in HBS containing 1 mM CaCl$_2$ was significantly higher than the level obtained in 2 mM EGTA-containing HBS after the treatment with 50 µM ionomycin (data not shown). However, the resting level of the BAPTA-loaded cells was similar to the level obtained in 2 mM EGTA-containing HBS (data not shown). These findings indicated that BAPTA loading significantly decreased the resting level of [Ca$^{2+}$]$_i$.

Involvement of G$\alpha$$_{i/o}$ in the NO production following the PAR$_1$ and PAR$_4$ activation

We examined the effect of pertussis toxin on the NO production as previously reported (Momota et al., 2006), and thereby evaluated the involvement of G$\alpha$$_{i/o}$ in the NO production. In BAECs, the NO production induced by 0.1 U/ml thrombin and 30 µM PAR$_4$-AP was significantly inhibited by the treatment with pertussis toxin, while that seen with 1 µM ionomycin was resistant to pertussis toxin (Fig. 6a). These observations are thus consistent with those of our previous report (Momota et al., 2006). In PAECs, the NO production induced by not only thrombin and PAR$_4$-AP but also PAR$_1$-AP was significantly inhibited by pertussis toxin (Fig. 6b). The NO production induced by ionomycin in PAECs was resistant to pertussis toxin (Fig. 6b). The observed inhibitory effect of pertussis toxin on the NO production induced by thrombin and PAR$_1$-AP was not associated with the inhibition of the Ca$^{2+}$ signal in PAECs (Fig. 5c).
Involvement of $G \alpha_{q/11}$ in the $[Ca^{2+}]_i$ elevation following the PAR1 activation

The involvement of $G \alpha_{q/11}$ in the PAR1 and PAR4 signaling was investigated using YM254890 (Takasaki et al., 2004). The cells were treated with YM254890 30 min prior to and during the agonist stimulations. We first determined the concentration-dependent effect of YM254890 on the bradykinin-induced $[Ca^{2+}]_i$ elevation in BAECs, and we found that 30 nM YM254890 completely abolished the bradykinin-induced $[Ca^{2+}]_i$ elevation (Fig. 7a). This concentration was thus used in the following evaluations. YM254890, at 30 nM, also abolished the $[Ca^{2+}]_i$ elevations induced by 3 U/ml thrombin and 30 µM PAR1-AP in PAECs (Fig. 7b). However, YM254890 had no significant effect on the NO production induced by PAR1-AP, PAR4-AP, or ionomycin in BAECs and PAECs (Figs. 7c, d). The thrombin-induced NO production was significantly inhibited by YM254890 in both BAECs and PAECs (Figs. 7c, d).

Thrombin, PAR1-AP, and PAR4-AP in BAECs (Fig. 2) and PAR4-AP in PAECs (Fig. 3) induced no appreciable elevation of $[Ca^{2+}]_i$. The effects of pertussis toxin or YM254890 on the $[Ca^{2+}]_i$ elevation were not examined under these situations.

Involvement of the phosphotidylinositol-3 kinase-Akt pathway in the PAR4-mediated Ca$^{2+}$-independent NO production

We investigated the involvement of the phosphotidylinositol-3 kinase (PI3K)-Akt pathway in the PAR4-mediated Ca$^{2+}$-independent NO production in BAECs (Fig. 8). The PAR4-AP -induced NO production was almost completely inhibited by 30 µM LY294002, 10 µM wortmannin, and 10 µM SH6, a Akt inhibitor, (Fig. 8a). In contrast, the PAR1-AP-induced NO production in PAECs was resistant to wortmannin. However, it was significantly inhibited by LY294002, but to a lesser extent than that seen with PAR4-AP (Fig. 8b). All inhibitors had no significant effect on the
bradykinin-induced $[\text{Ca}^{2+}]_i$ elevation and NO production (Fig. 8c) in BAECs.

The involvement of Akt in the PAR₄-mediated NO production in BAECs was then further investigated using a dominant negative mutant of Akt (TATHA-Akt) (Figs. 8d, e). The RhoA inhibitory protein (TATHA-RB) was used to investigate any possible involvement of RhoA in NO production. TATHA-Akt and TATHA-RB were introduced to the cells by using a cell-penetrating peptide-mediated protein transduction technique (Hirano et al., 2004a; Hirano et al., 2004b; Koga et al., 2004). TATHA-Akt, but not TATHA-RB, significantly inhibited the PAR₄-AP-induced NO production in BAECs. The dominant negative mutant of Akt without a cell-penetrating peptide ((His)₆-Akt) had no significant effect (Fig. 8d). The intracellular transduction of TATHA-Akt and TATHA-RB, but not (His)₆-Akt, was detected by an immunoblot analysis (Fig. 8e).

Insulin has been reported to activate the PI3K-Akt pathway (Hemmings, 1997; Saltiel and Pessin, 2002; Wymann et al., 2003) and induce NO production with no increase in $[\text{Ca}^{2+}]_i$ (Montagnani et al., 2001). In BAECs, insulin did induce a concentration-dependent NO production, with the maximal production at 100 nM (Fig. 9a). However, insulin induced no $[\text{Ca}^{2+}]_i$ elevation (data not shown). An inhibitor of EGF receptor tyrosine kinase (AG1478) and inhibitors of IGF-1 and insulin receptor kinase (AG1024 and AG538) significantly inhibited the insulin-induced NO production (Fig. 9b). However, none of these inhibitors of receptor tyrosine kinases had any significant effect on the PAR₄-AP-induced NO production in BAECs (Fig. 9b).

*Increases in the phosphorylation of Akt and eNOS by PAR₄-AP*

After the application of 30 µM PAR₄-AP, the phosphorylation level of both Akt (Ser473) and eNOS (Ser1179) transiently increased, while reaching the peak within 1 min and then returning close to the pre-stimulation level within 15 min in BAECs (Fig. 10a). Pre-incubation with
LY294002 (10 μM) or wortmannin (10 μM) inhibited the phosphorylation of Akt and eNOS seen at 1 min (Figs. 10b, c). The phosphorylation of Akt and eNOS thereafter remained suppressed in the presence of LY294002 (data not shown). SH-6 (10 μM) also inhibited the PAR4-AP-induced Akt phosphorylation (Fig. 10b). The inhibitors of Ca2+-calmodulin-dependent kinase II, protein kinase C, protein kinase A or MEK had no significant effect on the PAR4-AP-induced phosphorylation of Akt and eNOS (Fig. 10c).
Discussion

The intracellular signal transduction following the PAR₁ activation has been comprehensively studied (Coughlin, 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002), while the PAR₄-initiated cell signaling still remains largely unknown (Steinberg, 2005). Nevertheless, the mechanisms underlying the NO production mediated by PAR₁ and PAR₄ remain to be investigated. The present study provides the first evidence for the distinct Ca²⁺ requirement for the NO production following the activation of PAR₁ and PAR₄ in vascular endothelial cells. The PAR₄ activation induced NO production without an appreciable elevation of [Ca²⁺]ᵢ and also in a manner resistant to BAPTA. Importantly, the PAR₄-mediated Ca²⁺-independent NO production was consistently observed in three different types of endothelial cells. The present study thus advanced our previous study in BAECs (Momota et al., 2006), and could draw a conclusion that PAR₄ preferably elicits some type of signal transduction other than the Ca²⁺ signal in vascular endothelial cells, thereby activating the NO production in a manner independent of Ca²⁺. On the other hand, PAR₁ activation induced NO production with a concomitant elevation of [Ca²⁺]ᵢ, and such NO production was significantly but partly inhibited by BAPTA. These observations thus suggest PAR₁ to induce the NO production via both Ca²⁺-dependent and independent mechanisms.

In PAECs, which respond to both the activation of PAR₁ and PAR₄, the thrombin-induced NO production was similar to that seen with PAR₁-AP and PAR₄-AP, and no apparent additive effect of PAR₁-AP and PAR₄-AP was observed. Although BAPTA inhibited the PAR₁-mediated NO production, thrombin-induced NO production was relatively resistant to BAPTA. These observations suggest that thrombin-induced NO production was mediated mainly by PAR₄, when the PAR₁ signaling was blocked. There may be some redundancy between PAR₁
and PAR$_4$ in NO production.

Our observations, which suggest the existence of the distinct signal transduction mechanisms between PAR$_1$ and PAR$_4$ in vascular endothelial cells, are consistent with those of the previous studies in human platelets (Ma et al., 2005; Holinstat et al., 2006). However, our observations contrast with those of a recent report which showed the PAR$_4$-mediated human platelet aggregation to be abolished by BAPTA, while the PAR$_1$-mediated aggregation was resistant (Holinstat et al., 2006). Instead, our findings of the poor coupling of PAR$_4$ to the Ca$^{2+}$ signal are consistent with those in mouse cardiomyocytes (Sabri et al., 2003). In cardiomyocytes, PAR$_4$ was demonstrated to be a weak activator of phospholipase C, which can be linked to a Ca$^{2+}$ signal by a generation of inositol trisphosphate (Sabri et al., 2003). As a result, the specificity of receptor coupling to the downstream signaling pathways seems to vary depending on the cell type and/or species. However, the mechanism underlying the cell-type- or species-specific coupling still remains to be elucidated.

iNOS is a Ca$^{2+}$-independent NO synthase (Alderton et al., 2001). However, its contribution to the PAR$_4$-induced NO production has been ruled out. PAR$_4$ is thus suggested to activate eNOS in a Ca$^{2+}$-independent manner. The Ca$^{2+}$-independent activation of eNOS and NO production has been reported to be accompanied by the Akt-catalyzed phosphorylation of eNOS at Ser1179 (Ser1177 in human) (Sessa, 2004). Our observations suggested the major contribution of the PI3K-Akt pathways to the PAR$_4$-induced Ca$^{2+}$-independent NO production. The observation of an increase in the phosphorylation level of Akt and eNOS, and the inhibition of these phosphorylation by the PI3K inhibitor further support the involvement of PI3K-Akt in the PAR$_4$-induced NO production. In contrast, the PI3K-Akt pathway was not suggested to play a major role in the PAR$_1$-induced NO production. The present study thus also provides the first evidence that PAR$_4$-mediated signaling is distinct from that of PAR$_1$ regarding PI3K-Akt as well.
as the Ca$^{2+}$ signal.

Insulin induced NO production in a Ca$^{2+}$-independent manner in BAECs, as previously reported (Montagnani et al., 2001), and this NO production was significantly inhibited by the receptor tyrosine kinase inhibitors. However, the PAR$_4$-induced NO production was resistant to these inhibitors, thus ruling out the involvement of insulin receptor activation or the transactivation of the receptor tyrosine kinases (Zwick et al., 1999) in the PAR$_4$-induced NO production. On the other hand, G protein-coupled receptors can directly activate the PI3K-Akt pathway through interactions between the $\beta\gamma$ subunit of G proteins and a $\gamma$ type of PI3K (Wymann et al., 2003). This mechanism is consistent with our findings that the pertussis toxin significantly inhibited the PAR$_4$-induced NO production. Collectively, our results suggest that PAR$_4$ is preferentially coupled to Go$_{i/o}$, and then activates the PI3K-Akt pathway, thereby inducing the NO production mostly in a Ca$^{2+}$-independent manner.

Our results further demonstrated the differential coupling of G proteins to the [Ca$^{2+}$]$_i$ elevation and the NO production. Our observations suggest that Go$_{q/11}$ plays a major role in the PAR$_1$-mediated generation of a Ca$^{2+}$ signal, while Go$_{i/o}$ is suggested to be involved in the NO production following the activation of not only PAR$_4$ but also PAR$_1$. It should be noted that the inhibition of Go$_{q/11}$ signaling by YM254890 had no significant effect on the PAR$_1$-mediated NO production, while it abolished a transient [Ca$^{2+}$]$_i$ elevation. On the other hand, the inhibition of Ca$^{2+}$ signaling by BAPTA significantly inhibited the PAR$_1$-mediated NO production. These results suggest that the transient elevation of [Ca$^{2+}$]$_i$ is not necessary for the PAR$_1$-mediated NO production, although a part of the PAR$_1$-mediated NO production is Ca$^{2+}$-dependent. It is conceivable that YM254890 inhibited an agonist-induced elevation of [Ca$^{2+}$]$_i$ without affecting the resting level of [Ca$^{2+}$]$_i$. In contrast, BAPTA not only inhibited the [Ca$^{2+}$]$_i$ elevation but also decreased the resting [Ca$^{2+}$]$_i$ level. The basal level of [Ca$^{2+}$]$_i$ is thus considered to be sufficient to
support the PAR1-mediated Ca\textsuperscript{2+}-dependent NO production.

It should also be noted that YM254890 had no significant effect on the NO production induced by PAR\textsubscript{1}-AP and PAR\textsubscript{4}-AP, while it significantly inhibited the thrombin-induced NO production in both BAECs and PAECs. These observations simply suggest that a part of the thrombin-induced NO production was mediated by some new receptor other than PAR\textsubscript{1} and PAR\textsubscript{4} (Hamilton et al., 1998). However, it is also possible that the coupling of PARs to the downstream signaling pathways may differ depending on the mode of receptor activation, either proteolytic or non-proteolytic (Blackhart et al., 2000; Al-Ani et al., 2004; Kim et al., 2004), the state of receptor oligomerization (Leger et al., 2006), or the involvement of the transactivation of PAR\textsubscript{2} following the proteolytic activation of PAR\textsubscript{1} (O'Brien et al., 2000). These possibilities still remain to be elucidated.

In conclusion, the present study demonstrated the distinct mechanisms underlying the NO production induced by the activation of PAR\textsubscript{1} and PAR\textsubscript{4} in vascular endothelial cells; PAR\textsubscript{4} preferentially activates the PI3K-Akt pathway, and induced the NO production in a manner mostly independent of Ca\textsuperscript{2+}. In contrast, PAR\textsubscript{1} activates both Ca\textsuperscript{2+}-dependent and -independent NO production. G\textsubscript{i/o} plays an important role in mediating the NO production, while G\textsubscript{q/11} is coupled to the Ca\textsuperscript{2+} signal. The intracellular signal transduction elicited by PAR\textsubscript{1} has been intensively investigated, while the PAR\textsubscript{4}-induced signal transduction still remains largely unknown. Our study is thus considered to shed some light on how PAR\textsubscript{4} contributes to the NO production in vascular endothelial cells. PAR\textsubscript{4} may therefore play an important role in mediating the Ca\textsuperscript{2+}-independent component of the thrombin-induced NO production.
Acknowledgments

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Footnotes

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Legends for figures

**Figure 1.** NO production induced by thrombin, PAR₁-AP, PAR₄-AP, and bradykinin in BAECs.

**a.** A standard curve for the relationship between MAHMA-NONOate concentrations and DAR-4M fluorescence. The data are the mean±SEM (n=7).

**b.** Representative recording of the change in the DAR-4M fluorescence intensity induced by 30 µM PAR₄-AP.

**c-f.** The concentration-dependent NO production by thrombin (c), PAR₄-AP (d), PAR₁-AP (e), and bradykinin (f). The zero concentration indicates the value obtained by the buffer change without any stimulation.

**g, h.** The effects of NOS inhibitors (100 µM N⁰⁻nitro-L-arginine methyl ester for thrombin; 1 mM N⁰⁻nitro-L-arginine for bradykinin and PAR₄-AP) and iNOS inhibitors (50 and 100 µM 1400W and 1 mM aminoguanidine) on the NO production induced by 0.3 U/ml thrombin, 30 µM PAR₄-AP, and 10 nM bradykinin. Since L-NOARG interfered with the DAR-4M fluorometry on thrombin, we used L-NAME to examine whether the thrombin-induced changes in the DAR-4M fluorescence depended on the activity of NOS. The data are the mean±SEM (n=4). *, p<0.05; n.s., not significantly different vs. NO production in the absence of NOS or iNOS inhibitors.

**Figure 2.** Effect of thrombin, PAR₁-AP, PAR₄-AP, and bradykinin on [Ca²⁺]ᵢ in BAECs.

Representative traces (**a**) and the concentration-response curves (**b**) showing the changes in the fura-2 fluorescence ratio induced by thrombin, PAR₁-AP, PAR₄-AP, and bradykinin. The unit concentration of thrombin was converted to the molar concentration, while assigning 1 U/ml to be 10 nM. The data are the mean±SEM (n=4).

**Figure 3.** Effect of BAPTA loading on the [Ca²⁺]ᵢ increase and NO production in BAECs.

**a.** The concentration-dependent inhibition of the 10 nM bradykinin-induced [Ca²⁺]ᵢ elevation by
BAPTA loading. The data are the mean±SEM (n=4). b, The NO production induced by 10 nM bradykinin, 0.3 U/ml thrombin, and 30 µM PAR4-AP in BAECs untreated (-) and treated (+) with 50 µM BAPTA-AM 30 min prior to the measurement. The data are the mean±SEM (n=4). *, p<0.05; n.s., not significantly different.

**Figure 4.** NO production induced by thrombin, PAR1-AP, and PAR4-AP in PAECs.

a, [Ca²⁺]i elevation and NO production induced by thrombin, PAR1-AP, and PAR4-AP in PAECs. The data are the mean±SEM (n=4). b, The effect of BAPTA loading on the NO production induced by 3 U/ml thrombin, 30 µM PAR1-AP, and 30 µM PAR4-AP in PAECs. The NO production obtained without BAPTA loading was assigned to be 100%. The values obtained with the buffer change alone are also shown (buffer). The data are the mean±SEM (n=7 for thrombin, n=6 for PAR1, n=4 for PAR4). *, p<0.05 vs. BAPTA-AM (-).

**Figure 5.** NO production induced by thrombin, PAR1-AP, and PAR4-AP in HUVECs.

[Ca²⁺]i elevation and NO production induced by thrombin, PAR1-AP, and PAR4-AP in HUVECs. The data are the mean±SEM (n=4). *, p<0.05 vs. the basal level.

**Figure 6.** Effects of pertussis toxin on the NO production and [Ca²⁺]i elevation induced by thrombin, PAR1-AP, and PAR4-AP in BAECs and PAECs.

a, b, NO production induced by 0.1 U/ml thrombin, 30 µM PAR1-AP, 30 µM PAR4-AP, and 1 µM ionomycin in BAECs (a) and PAECs (b), with (+) and without (-) pertussis toxin treatment. c, [Ca²⁺]i elevation induced by 3 U/ml thrombin and 30 µM PAR1-AP in PAECs, with (+) and without (-) pertussis toxin treatment. The cells were treated with 100 ng/ml pertussis toxin in the cultured media for 24 h, and then they were subjected to the measurement of [Ca²⁺]i and NO
production in the absence of pertussis toxin. The data are the mean±SEM (n=10-14 for a, n=4-7 for b, n=3-4 for c). *, p<0.05; n.s., not significantly different.

**Figure 7.** Effects of YM254890 on the NO production and [Ca\(^{2+}\)]\(_i\) elevation induced by thrombin, PAR\(_1\)-AP, and PAR\(_4\)-AP in BAECs and PAECs.

a, The concentration-dependent effects of YM254890 on the [Ca\(^{2+}\)]\(_i\) elevation induced by 100 nM bradykinin in BAECs. b, The effects of YM254890 on the [Ca\(^{2+}\)]\(_i\) elevation induced by 3 U/ml thrombin and 30 µM PAR\(_1\)-AP in PAECs. The cells were treated with YM254890 30 min prior to and during the stimulations. c, d, NO production induced by 0.1 U/ml thrombin, 30 µM PAR\(_1\)-AP, 30 µM PAR\(_4\)-AP, and 1 µM ionomycin in BAECs (c) and PAECs (d), with (+) and without (-) the treatment with 30 nM YM254890. The data are the mean±SEM (n=3 for a, b; n=3-5 for c; n=3-4 for d,). *, p<0.05; n.s., not significantly different.

**Figure 8.** The effect of inhibitors of the PI3K-Akt pathway on the NO production induced by PAR\(_4\)-AP, PAR\(_1\)-AP, and bradykinin.

a-c, NO production induced by 30 µM PAR\(_4\)-AP in BAECs (a), 30 µM PAR\(_1\)-AP in PAECs (b), and 10 nM bradykinin in BAECs (c), in the presence and absence of LY294002 (LY), wortmannin (WM) or SH-6. In c, the effect of the PI3K-Akt inhibitors on the bradykinin-induced [Ca\(^{2+}\)]\(_i\) elevation is also shown. The inhibitors were added at the indicated concentrations 30 min prior to and during the stimulations. d, NO production induced by 30 µM PAR\(_4\)-AP in BAECs untreated and treated with 0.3 µM TATHA-Akt, 0.3 µM TATHA-RB or 0.3 µM (His)\(_6\)-Akt. The recombinant proteins were added to the cells 30 min prior to and during the stimulation with PAR\(_4\)-AP. e, Immunoblot detection (IB) of the recombinant proteins using anti-(His)\(_6\) antibody in the extract of BAECs, untreated and treated with 0.3 µM TATHA-Akt, 0.3 µM TATHA-RB or 0.3
μM (His)₆-Akt for 30 min. The purified recombinant proteins (50 ng) were loaded as a positive control for immunoblot detection. Tubulin was detected to validate the equal loading of the cell extract (25 μg proteins). The data are the mean±SEM (n=4). *, p<0.05; n.s., not significantly different vs. the NO production seen without inhibitors or in untreated cells.

**Figure 9.** Effects of the receptor tyrosine kinase inhibitors on the NO production induced by insulin and PAR₄-AP in BAECs.

*a*, The concentration-dependent NO production by insulin in BAECs. *b*, NO production induced by 100 nM insulin and 30 μM PAR₄-AP in BAECs, in the presence or absence of AG1478, AG1024 or AG538 at the indicated concentrations. The NO production obtained without the receptor tyrosine kinase inhibitors was assigned a value of 100 %. The data are the mean±SEM (n=3-4). *, p<0.05, vs. the NO production seen without inhibitors.

**Figure 10.** Increases in the phosphorylation of Akt and eNOS by PAR₄-AP in BAECs.

*a*, Time course of the phosphorylation of Akt at Ser473 and eNOS at Ser1179 induced by 30 μM PAR₄-AP in BAECs. The data are the mean±SEM (n=7). *b*, Representative immunoblot findings and the summary of the effects of wortmannin (10 μM), LY294002 (30 μM) or SH-6 (10 μM) on the phosphorylation of Akt and eNOS at 1 min after the stimulation with PAR₄-AP in BAECs. The data are the mean±SEM (n=4 for Akt; n=11 for eNOS). *c*, Effects of KN-93 (1 μM), GF109203X (1 μM), H-89 (10 μM), PD98059 (10 μM), and LY294002 (30 μM) on the PAR₄-AP-induced phosphorylation of Akt and eNOS in BAECs. The data are the mean±SEM (n=7). The cells were either untreated or treated with inhibitors for 30 min, and then were stimulated with 30 μM PAR₄-AP in the continuous presence of the inhibitors. The level of phosphorylation seen in the unstimulated cells was assigned a value of 1. †, p<0.05 vs.
unstimulated control; *, $p<0.05$ vs. PAR$_{4}$-AP.
Figure 2

(a) BAECs

- 0.3 μM Thrombin and 50 μM ionomycin
- 30 μM PAR<sub>2</sub>-AP and 50 μM ionomycin
- 30 μM PAR<sub>1</sub>-AP and 50 μM ionomycin
- 100 nM Bradykinin and 50 μM ionomycin

(b) BAECs

Graph showing percentage of fura-2 fluorescence against agonist concentration (M) with data points for Thrombin, PAR<sub>2</sub>-AP, PAR<sub>4</sub>-AP, and Bradykinin.
Figure 3
Figure 4

PAECs

(a) % Fura-2 fluorescence

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(b) DAR-4M fluorescence (arbitrary units)

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(b) DAR-4M fluorescence (% of BAPTA-AM)

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<th>PAR4-AP (30 μM)</th>
<th>PAR4-AP (50 μM)</th>
<th>BAPTA-AM (50 μM)</th>
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Figure 5

HUVECs

% fura-2 fluorescence

DAR-4M fluorescence (arbitrary units)

Thrombin (1 U/ml)  PAR-A (30 nM)  PAR-A (30 nM)

* *
Figure 6

(a) BAECs

(b) PAECs

(c) PAECs
Figure 7

(a) BAECs

(b) PAECs

(c) BAECs

(d) PAECs
Figure 8

(a) BAECs

(b) PAECs

(c) BAECs

(d) BAECs

(e) BAECs
Figure 9

**a** BAECs

![Graph showing DAR-4M fluorescence as a function of insulin concentration for BAECs.](image)

**b** BAECs

![Graph showing DAR-4M fluorescence (% of control) for various treatments in BAECs.](image)
Figure 10