Plasmin Induces Endothelium-Dependent Nitric Oxide–Mediated Relaxation in the Porcine Coronary Artery

Tetsuhiro Fujiyoshi, Katsuya Hirano, Mayumi Hirano, Junji Nishimura, Shosuke Takahashi, Hideo Kanaide

Objective—Plasmin is a key enzyme in fibrinolysis. We attempted to determine the possible role of plasmin in the regulation of vascular tone, while also investigating the mechanism of plasmin-induced vasorelaxation.

Methods and Results—In porcine coronary artery, plasmin induced an endothelium-dependent relaxation. This relaxing effect was mostly abolished by a proteinase inhibitor, a plasin inhibitor, or a nitric oxide (NO) synthase inhibitor. The preceding stimulation with plasmin significantly inhibited the subsequent relaxation induced by thrombin but not that induced by proteinase-activated receptor-1–activating peptide. The relaxation induced by trypsin and substance P remained unaffected by the preceding plasmin stimulation. The pretreatment with plasmin, thrombin, or trypsin significantly attenuated the plasmin-induced relaxation. In porcine coronary artery endothelial cells (PCAECs) and human umbilical vein endothelial cells (HUVECs), plasmin induced a transient elevation in the cytosolic Ca²⁺ concentrations ([Ca²⁺]). The preceding stimulation with plasmin inhibited the subsequent [Ca²⁺], elevation induced by thrombin but not that induced by trypsin. In PCAECs, plasmin concentration-dependently induced NO production.

Conclusions—The present study demonstrated, for the first time, that plasmin induced an endothelium-dependent NO-mediated relaxation in the porcine coronary artery, while also showing plasmin to specifically inactivate the thrombin receptor. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: plasmin ■ proteinase-activated receptor ■ vasorelaxation ■ nitric oxide ■ endothelium

Proteinases involved in the blood coagulation cascade not only play an important role in hemostasis but also exert various vascular effects such as the regulation of the vascular tone, tissue remodeling, and angiogenesis. 1–4 Proteinase-activated receptors (PARs) play a major role in mediating such cellular effects of proteinases. 3–6 PARs belong to a unique family of G protein–coupled receptor.3 Four members of PARs have been cloned. PAR1, PAR3, and PAR4 serve as receptors for thrombin, whereas PAR1, PAR2, and PAR4 serve as receptors for trypsin.5 The activation of PAR is initiated by the proteolytic cleavage at the specific site of the extracellular domain.3 Under physiological conditions, thrombin and trypsin mainly have been reported to induce an endothelium-dependent vasorelaxation in various type of blood vessel.5–7 We have previously reported that thrombin and trypsin induced a transient elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]) in vascular and aortic valve endothelial cells8–10 and an endothelium-dependent relaxation in the porcine coronary artery.9,10,11

Plasmin, as a key enzyme in fibrinolysis, has been reported to induce the cell migration in Chinese hamster ovary cells,12 the cell proliferation in the murine thoracic aorta smooth muscle cells,13 and an increase in the expression of Cy661, a growth factor–like gene, in fibroblast.14 These reports suggested PAR1 to mediate the cellular effects of plasmin. However, the roles of plasmin in the regulation of vascular tone still remain to be determined. Plasmin has been reported to cleave PAR1 at the C-terminal side of the tethered ligand region, thus inactivating the responsiveness of PAR1.15,16 Plasmin has been shown to cleave the extracellular region of PAR2 at the N-terminal side of the trypsin cleavage site as well as the trypsin site,16 although it was also shown to attenuate the subsequent PAR2-mediated [Ca²⁺], elevation in the rat brain capillary endothelial cell.17 Therefore, the receptor(s) that mediate the cellular effect of plasmin still remain controversial. The NO-dependent relaxant effect of plasmin has been reported in rat arteries.18 However, the precise mechanism for the plasmin-induced relaxation and the receptors involved still remain to be elucidated.

In the present study, we aimed to determine the role of plasmin in the regulation of vascular tone and investigated the mechanism of plasmin-induced vasorelaxation in the porcine coronary artery. The effects of plasmin on [Ca²⁺], and the production of NO were also determined in the cultured
endothelial cells. The present study demonstrated, for the first time, that plasmin induced an endothelium-dependent NO-mediated relaxation in the porcine coronary artery, whereas it inhibited the endothelium-dependent relaxation induced by thrombin.

### Materials and Methods

The contractile responses were evaluated using the isolated strips of the porcine right coronary artery, as previously described. The changes in [Ca$^{2+}$], were monitored in the fura-2–loaded porcine coronary artery endothelial cells (PCAECs) and human umbilical vein endothelial cells (HUVECs), as previously described. The NO production was monitored in PCAECs using diaminorhosamine-4M fluorometry, as previously described.

An expanded Materials and Methods section can be found in the online data supplement at http://atvb.ahajournals.org.

### Results

#### Plasmin-Induced Endothelium-Dependent Relaxation in the Porcine Coronary Artery

In the porcine coronary arterial strips, both with and without an endothelium on exposure to 30 nmol/L U46619 (a thromboxane A$_2$ analog), the tension rapidly increased as it reached steady state, and it remained at this level for more than 15 minutes. The application of 300 nmol/L plasmin induced a rapid and transient relaxation during U46619-induced contraction (Figure 1a). After reaching its lowest level, the tension returned to a level similar to that seen before the application of plasmin. The evaluation of the concentration-dependent effects of plasmin indicated that plasmin induced a significant relaxation at 100 nmol/L and higher concentrations. The extent of the relaxation obtained with 300 nmol/L plasmin was 52.1±4.4% relaxation (n=4) (Figure 1b). The concentration of the stock solution of plasmin (in 50% glycerol) was 150 μmol/L. It was thus impractical to use final concentrations higher than 300 nmol/L because the final concentrations of glycerol became higher than 0.1%. In the absence of an endothelium, plasmin has no significant relaxation (Figure 1b). The requirement of the proteinase activity for the relaxing effect of plasmin was examined (Figure 1c). The 10-minute preincubation of plasmin with a serine proteinase inhibitor, 4-aminophenyl methane-sulfonfluoride (p-APMSF) or a plasmin inhibitor, tranexamic acid, substantially abrogated the relaxing effect of plasmin. In the presence of an NO synthase inhibitor, N$^\omega$-nitro-l-arginine methyl ester (L-NAME), the plasmin-induced relaxation was almost completely abolished (Figure 1c). p-APMSF and tranexamic acid had no effect on the U46619-induced contraction and the relaxation induced by 30 μmol/L sodium nitroprusside (data not shown). The similar relaxant effect of plasmin was also observed during the precontraction induced by 118 mmol/L K$^+$ or 30 μmol/L prostaglandin (PG) F$_2$α (data not shown). Thrombin and trypsin also concentration-dependently induced a transient relaxation with an intact endothelium (Figure 1 in the online data supplement). These relaxations were abolished by p-APMSF, but they were resistant to tranexamic acid. These observations are consistent with the findings of our previous reports.

#### Inhibition of Thrombin-Induced Relaxation by the Plasmin Pretreatment in Porcine Coronary Artery

To determine the possible involvement of PARs in the plasmin-induced relaxation, we examined the cross-desensitization between plasmin and thrombin or trypsin. After the 20-minute treatment with plasmin at the indicated concentrations, the strips were precontracted with U46619 in the absence of plasmin, and then consecutively stimulated with 3 U/mL thrombin, 100 nmol/L trypsin, and 100 nmol/L substance P (Figure 2a). Substance P was used as a control stimulation to induce an endothelium-dependent relaxation in the porcine coronary artery. The level of the 30 nmol/L U46619-induced contraction after the pretreatment with plasmin (137.6±9.2%, n=5) did not differ from that obtained without the pretreatment with plasmin (130.0±12.1%, n=5). The preceding stimulation with thrombin had no significant effect on the subsequent relaxation induced by trypsin (supplemental Figure Ic versus Figure 2a). The preceding stimulation with thrombin and trypsin had no significant influence on the substance P–induced relaxation (data not shown).

Pretreatment with 300 nmol/L plasmin significantly attenuated the thrombin-induced relaxation in comparison to that seen without the plasmin treatment, whereas the relaxations induced by trypsin and substance P remained unaffected (Figure 2a and 2b). A significant attenuation of the thrombin-induced relaxation was observed with 100 nmol/L and 300 nmol/L plasmin (Figure 2c). The longer pretreatment with plasmin did not cause greater inhibition in comparison to that seen with 20-minute pretreatment (data not shown). The
inhibitory effect of plasmin on the thrombin-induced relaxation was abolished by the pretreatment of plasmin with p-APMSF (Figure 2d). However, tranexamic acid had no significant effect on the inhibitory effect of plasmin. A PAR1 activating peptide TFLLR-NH₂ (10 μmol/L) induced a transient endothelium-dependent relaxation, to the similar extent to that obtained with 3 U/mL thrombin (Figure 3e and 3f). The pretreatment with 300 nmol/L plasmin had no significant effect on the relaxation induced by TFLLR-NH₂, whereas the pretreatment with TFLLR-NH₂ slightly but significantly attenuated the relaxation induced by the second application of TFLLR-NH₂ (Figure 3e and 3f).

**Effects of Pretreatment With Thrombin and Trypsin on the Plasmin-Induced Relaxation in the Porcine Coronary Artery**

We next examined the effects of the pretreatment with thrombin and trypsin on the plasmin-induced relaxation according to a similar protocol to that used in Figure 2. The pretreatment with thrombin or trypsin had no significant effect on the level of the U46619-induced contraction (data not shown). When the strips were pretreated with 3 U/mL thrombin or 100 nmol/L trypsin for 20 minutes, and then precontracted by U46619 in the absence of thrombin and trypsin, the subsequent application of the same stimulation failed to induce any relaxations (Figure 3a). The pretreatment with 300 nmol/L plasmin, 3 U/mL thrombin, and 100 nmol/L trypsin significantly but, only partly, attenuated the subsequent relaxation induced by plasmin (Figure 3b). The major part of the plasmin-induced relaxation was thus resistant to these pretreatments (Figure 3b).

**Negligible Involvement of Any Production of Vasoactive Peptides and Insulin-Like Growth Factor 1 Receptor in the Plasmin-Induced Relaxation**

The observations in Figure 3 suggested that PAR1 to -4 play a negligible role, if any, in the plasmin-induced relaxation.
Alternative possibility is that plasmin proteolytically generated vasoactive peptides or proteolytically activated the insulin receptor or insulin-like growth factor 1 receptor, thereby inducing the relaxation.23,24 Insulin and insulin-like growth factor 1 were reported to induce NO production and an endothelium-dependent relaxation.25 To investigate the involvement of the proteolytic generation of vasoactive peptides in the plasmin-induced relaxation, we conducted a bath-transfer experiment (Figure 4) as previously reported.26 After 300 nmol/L plasmin was applied during the U46619-induced contraction, p-APMSF was added to the bath at a final concentration of 10 μmol/L to terminate the proteolytic activity of plasmin (Figure 4a). After a 10-minute incubation, the organ bath solution was transferred to the reporter tissue (c and d).

Effect of Plasmin on the [Ca2+]i Elevation Induced by Thrombin and Trypsin in Endothelial Cells

The inhibitory effect of plasmin on the thrombin-induced relaxation suggested the endothelial cells to be a site of crosstalk between plasmin and thrombin. We thus directly examined the effect of plasmin on the [Ca2+]i elevations induced by thrombin and trypsin in endothelial cells (Figure 5). Plasmin induced a concentration-dependent, transient elevation of [Ca2+]i, at 100 nmol/L and higher concentrations in both PCAECs and HUVECs (Figure 5b and 5d through 5f). The plasmin-induced [Ca2+]i elevation was also abolished by the pretreatment of plasmin with p-APMSF (data not shown). Thrombin (3 U/mL) induced a transient [Ca2+]i elevation both in PCAECs and HUVECs, whereas trypsin (100 nmol/L) induced a significant [Ca2+]i elevation in only HUVECs (Figure 5a and 5c). The preceding stimulation with plasmin concentration-dependently inhibited the subsequent [Ca2+]i elevation induced by thrombin in both PCAECs and HUVECs (Figure 5a and 5c). However, it had no significant effect on the response to 100 nmol/L trypsin in HUVECs (Figure 5f). The addition of 30 nmol/L U46619, as in the protocol of Figure 2, did not affect the inhibitory effect of plasmin on the thrombin-induced [Ca2+]i, elevation (data not shown).

NO Production in Response to Plasmin in PCAECs
diaminorhosamine-4M fluorometry revealed plasmin to induce NO production in the concentrations similar to those required to
induce vasorelaxation (supplemental Figure II). The plasmin-induced NO production was partly inhibited in the 1,2-bis(2-aminophenoxy)ethane-N,N\textprime,N\prime,N\prime\textprime-tetraacetic acid (BAPTA)-loaded cells (data not shown), thus suggesting a link between plasmin-induced [Ca^{2+}]\textsubscript{i} elevation and NO production.

**Discussion**

The present study demonstrated, for the first time, that plasmin induces an endothelium-dependent vasorelaxation in the porcine coronary artery and [Ca^{2+}]\textsubscript{i} elevation and NO production in the cultured endothelial cells. The plasmin-induced vasorelaxation was completely abolished in the absence of endothelium or in the presence of L-NAME. Plasmin induced NO production in the cultured endothelial cells in a similar concentration range to that required to induce vasorelaxation. These observations thus suggested that the endothelium-derived NO played a major role in mediating the plasmin-induced relaxation. In the porcine coronary artery, we have previously reported the endothelium-derived NO and hyperpolarization to be the major mechanisms mediating the relaxation induced by thrombin and trypsin. Others have also indicated that endothelium-derived NO and hyperpolarization are responsible for the thrombin and trypsin-induced relaxations observed in the human, porcine, and dog coronary artery and rat aorta. In this respect, the plasmin-induced relaxation is distinct from that induced by thrombin and trypsin.

The identity of the receptor that mediates the plasmin-induced relaxation still remains to be determined. Our observations that the relaxing effect of plasmin was abolished by p-APMSF and tranexamic acid indicated that the proteolytic activity of plasmin was required for its relaxing effect, thus suggesting that a mechanism similar to that of PARs may thus be involved in the plasmin-induced relaxation. Plasmin is listed as an activating protease for PAR1, although it is also listed as an inactivating protease for PAR1. Plasmin has been shown to cleave the recombinant proteins corresponding to the extracellular domain of PAR1 mainly at the C-terminal side of the ligand region, although plasmin also cleaved it at the same site as that cleaved by thrombin. In the present study, the greater part of the plasmin-induced relaxation was resistant to the preceding stimulation with thrombin or trypsin, whereas the relaxation induced by thrombin and trypsin was almost completely abolished. These observations thus suggested that the receptors for thrombin and trypsin played only a minor role in the plasmin-induced relaxation. Some other receptors are thus suggested to be involved in the plasmin-induced relaxation. On the contrary, the preceding stimulation with plasmin significantly attenuated the thrombin-induced vasorelaxation and the [Ca^{2+}]\textsubscript{i} elevation in the cultured endothelial cells, although it had no significant effect on the trypsin-induced vasorelaxation and the [Ca^{2+}]\textsubscript{i} elevation. These observations thus suggested that plasmin inactivated the thrombin receptor, while having no significant effect on the trypsin receptor. Such an inactivating effect of plasmin on thrombin receptor is consistent with the effect of plasmin on the cleavage of PAR1. It should be noted that the inactivating effect of plasmin on the thrombin receptor was resistant, whereas the relaxing effect of plasmin was sensitive to tranexamic acid. Both effects of plasmin, however, were inhibited by p-APMSF, an irreversible proteinase inhibitor. The inhibitory effect of tranexamic acid on plasmin is considered to be attributable to the interference of the interaction between plasmin and its substrates but not caused by the direct inhibition of the proteolytic activity. Plasmin is considered to be attributable to the interference of the interaction between plasmin and its substrates but not caused by the direct inhibition of the proteolytic activity.

The currently known PARs cannot account for the plasmin-induced relaxation. An atypical PAR may be involved in the plasmin-induced relaxation. Indeed, such an atypical PAR has been proposed for trypsin. We have also proposed the possible involvement of a novel member of PAR in the trypsin-induced rat myometrial contraction. However, proposing such PAR-like mechanism for the effect of plasmin is apparently inconsistent with the fact that the plasmin-induced relaxation was mostly resistant to the preceding stimulation with plasmin. In contrast, the relaxation induced by thrombin and trypsin was almost completely abolished by the preceding same stimulation, which is consistent with the mechanism of PAR. However, such proteolytic inactivation is also a concentration-dependent phenomenon, as we have reported for the inactivating effect of trypsin on the thrombin receptor. It is thus possible that the 300 nmol/L plasmin was insufficient to cause the complete inhibition of the subsequent response to plasmin. In this case, the PAR-like mechanism may thus be involved in the plasmin-induced relaxation, and the partial attenuation of the relaxant effect of plasmin by the preceding stimulation with plasmin is considered to be attributable to the partial proteolytic inactivation of the putative plasmin receptor. The possibility of the proteolytic production of some vasoactive peptides by plasmin was not supported by the bath transfer experiments. The possible involvement of the proteolytic activation of insulin receptor was also ruled out. As a result, a novel member of PARs is thus suggested to mediate the plasmin-induced relaxation.

It has been reported that the plasma concentration of plasmin could increase from 5 to 10 nmol/L under basal conditions, up to 300 nmol/L under such pathophysiological conditions as endotoxin shock, vascular remodeling, or wound healing. Therefore, the concentrations of plasmin required to induce NO production and vasorelaxation could be achievable in situ. The observed plasmin-induced NO-mediated vasorelaxation is thus operable under either physiological or pathophysiological conditions. It is well known that the activation of PARs induce an endothelium-dependent relaxation in the isolated arteries. A limited number of studies demonstrate that the activation of PAR1 or PAR2 increases blood flow in vivo and also suggest a possible link between the in vitro observation of the endothelium-dependent relaxation and the in vivo flow regulation. It is thus conceivable that the relaxant effect of plasmin may be linked to the flow regulation. However, the in vivo role of the relaxant effect of plasmin remains to be investigated. An indication of our discovery is that plasmin can induce cellular effects in vascular endothelial cells. Thrombin is known to induce not only a transient endothelium-dependent relaxation but also the phenotypic conversion to the proinflammatory phenotype in vascular endothelial cells, thus contributing to the pathogenesis of vascular diseases.
endothelial action of plasmin may thus imply such a role for plasmin. However, this possibility remains to be elucidated. In conclusion, the present study demonstrated, for the first time, that plasmin induced an endothelium-dependent NO-mediated relaxation in the porcine coronary artery, while also showing plasmin to inactivate the receptor for thrombin. The identity of a putative plasmin receptor mediating the NO production and the relaxing effect remains to be elucidated. Plasmin is a key enzyme in fibrinolysis, thus antagonizing the thrombotic effect of thrombin. The present study thus proposes a novel function for plasmin as a regulator of vascular tone, while also proposing a new aspect of the interaction between plasmin and thrombin. Plasmin is therefore suggested to antagonize not only the thrombotic effect of thrombin but also the cellular effect of thrombin at the receptor level.

Acknowledgments
We thank Brian Quinn for linguistic comments and help with the manuscript.

Sources of Funding
This study was supported, in part, by grants from the 21st Century Centers of Excellence Program, Grants-in-Aid for Scientific Research (nos. 17590222, 17590744, 17790493), Ministry of Education, Culture, Sports, Science and Technology, Japan; and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

Disclosures
None.

References
Plasmin Induces Endothelium-Dependent Nitric Oxide-Mediated Relaxation in the Porcine Coronary Artery
Tetsuhiro Fujiiyoshi, Katsuya Hirano, Mayumi Hirano, Junji Nishimura, Shosuke Takahashi and Hideo Kanaide

Arterioscler Thromb Vasc Biol. published online February 1, 2007;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2007/02/01/01.ATV.0000259360.33203.00.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/02/05/01.ATV.0000259360.33203.00.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Data Supplements

Plasmin induces endothelium-dependent nitric oxide-mediated relaxation in the porcine coronary artery

Tetsuhiro Fujiyoshi, Katsuya Hirano, Mayumi Hirano, Junji Nishimura, Shosuke Takahashi and Hideo Kanaide

Expanded Material and Methods

Tissue preparation

The segments of the right coronary arteries (2-3 cm from the origin) were excised from the porcine hearts at a local slaughterhouse immediately after the animals had been slaughtered. The segments were brought back to the laboratory in ice-cold normal physiological salt solution (PSS). After removing the adventitia, the segments were cut into strips in a circular direction, and then were opened longitudinally (approximately 1 mm wide, 5 mm long, and 0.1 mm thick). Care was taken to avoid damaging the endothelial cells. The strips without endothelium were prepared by rubbing off the inner surface with a cotton swab.

Measurement of tension in the coronary arterial strips

The measurement of tension development was performed at 37 °C, as previously described. The strips were mounted vertically to a force transducer, TB-612T (Nihon Koden, Japan), in a quartz organ bath filled with normal PSS. The strips were stimulated with 118 mmol/L K⁺ for 10 min every 15 min during the 2-3 hours equilibration period. Every time after stimulation with 118 mmol/L K⁺, the resting tension increased, and then it was finally adjusted to 300 mg in normal PSS (5.9 mmol/L K⁺) before stating the experimental protocol. Such a level of resting tension was the minimum level to obtain the maximal tension development with 118 mmol/L K⁺. Without this repetition of stretch and high K⁺ stimulation during the equilibration period, the optimal resting tension did not stabilize, and therefore it was difficult to perform the optimal measurement of tension development. We have previously shown that U46619, a thromboxane A₂ analogue, induced a sustained contraction in the porcine coronary artery. In the present study, we thus used 30 nmol/L U46619 to induce a sustained precontraction, and then examined the endothelium-dependent relaxation induced by plasmin, thrombin, trypsin and substance P. The extent of relaxation was expressed as a percentage, while assigning the
level of the U46619-induced precontraction and the resting level of tension to be 0 % and 100 % relaxation, respectively.

Cross-desensitization protocol
The involvement of currently known PARs in the plasmin-induced relaxation was investigated by a cross-desensitization protocol (Figures 2, 3, 5), as we have previously described.3,4 Because PARs are activated by the proteolytic cleavage at the specific site, the response to the second application of the same proteinase is substantially abrogated. However, the response to the activating peptide either remains intact or is attenuated, depending on the degrees of the proteolysis-independent desensitization of PARs.5 The strips or cells were stimulated with the first proteinase, and then the samples were challenged to the stimulation with the second proteinase (Figures 2, 3, 5) or the activating peptides (Figure 2) in the absence of the first proteinase. When the responses to thrombin and trypsin after the plasmin pretreatment were examined, thrombin and trypsin were consecutively applied in this order, because our previous observations indicated that the preceding stimulation with thrombin had no effect on the response to the subsequent stimulation with trypsin, while the trypsin prestimulation was shown to abolish the response to thrombin.3,4,6

Cell culture of porcine coronary artery endothelial cells (PCAECs) and human umbilical vein endothelial cells (HUVECs)
PCAECs without any contamination of fibroblasts were established as previously described.7 In brief, the endothelial lining was mechanically rubbed off from the inner surface of isolated porcine coronary artery with a scalpel blade, and suspended in Dulbecco’s modified Eagle medium (DMEM) containing 10 % fetal bovine. After centrifugation at 1,000 rpm for 5 min, the cells were re-suspended in the growth media and mechanically dispersed by repeated pipetting, and then they were aliquoted into 24-well plates. After approximately a 2-week culture, the wells showing growth of endothelial cells without fibroblast contamination were selected, and the cells were then subcultured to obtain a sufficient amount of cells for the experiments. The cells were plated on 35-mm dishes for the measurement of [Ca^{2+}]_{i}, and on Cell Desk LF1 (Sumitomo Bakelite, Tokyo, Japan) coated with Type-1 collagen (Nitta Gelatin, Osaka, Japan) for the measurement of NO production. The cells were used just at confluence. HUVECs were isolated by treatment with dispase (Godo Shusei Co., Ltd., Tokyo, Japan) in the EDTA-containing PBS as previously described,8 and plated in 35-mm culture dishes and cultured to confluence in a MCDB104 medium supplemented with endothelial cell growth supplements (Nissui seiyaku, Tokyo, Japan).
**Measurement of \([\text{Ca}^{2+}]_i\) in PCAECs and HUVECs**

The cells were loaded with fura-2 by incubating them in DMEM containing 10 µmol/L fura-2 acetocymethyl ester (fura-2/AM) for 1 h at 37 °C.9 The cells were then equilibrated in Hepes-buffered saline (HBS) before starting fluorometry. The changes in \([\text{Ca}^{2+}]_i\) were monitored using a front-surface fluorometer as previously described.4 Fluorometry was performed at 25 °C to prevent any leakage of fura-2.1 The intensities of 500 nm fluorescence at 340 nm and 380 nm excitation and their ratio were continuously monitored. At the end of the fluorometry, the response to 50 µmol/L ionomycin was recorded. 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 µmol/L ionomycin to be 0 % and 100 %, respectively.

**Measurement of NO in PCAECs**

The NO production of PCAECs was monitored with diaminorhodamine-4M (DAR-4M) fluorometry as previously described.10 In brief, the fluorescence intensity of 10 µmol/L diaminorhodamine-4M (DAR-4M) in 1 ml HBS was first determined 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 recording so that the maximum intensity obtained with 10 µmol/L 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 were expressed in arbitrary units. The \(\text{Ca}^{2+}\)-dependency of the NO production was evaluated in the PCAECs loaded with BAPTA, an intracellular \(\text{Ca}^{2+}\) chelator, as we previously described.10

**Drugs and solutions**

The normal PSS was composed of (in mmol/L): NaCl 123, KCl 4.7, NaHCO3 15.5, KH2PO4 1.2, MgCl2 1.2, CaCl2 1.25 and D-glucose 11.5, aerated with a mixture of 5 % CO2 and 95 % O2, with the resulting pH determined to be 7.4. High K+-PSS was prepared by equimolar substitution of KCl for NaCl. HBS was composed of (in mmol/L): Hepes 10 (pH 7.4), NaCl 135, KCl 5, MgCl2 1, CaCl2 1 and D-glucose 5.5. Fetal bovine serum was obtained from Sanko Junyaku (Tokyo, Japan). U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dion-1-oic acid) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Plasmin (human plasma, 8.7 units/mg in 50 %
glycerol, >95 % pure by SDS-PAGE) was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, U.S.A.). The maximal concentration of plasmin investigated in the present study was 300 nmol/L because further increment of the plasmin concentration will cause the final concentrations of glycerol to become higher than 0.1 %. Glycerol at 0.1 % had no significant effect on the U46619-induced contraction (data not shown). DMEM (Dulbecco’s Modified Eagle’s Medium), thrombin (bovine plasma, 1050 units/mg), trypsin (bovine pancreas, 13600 units/mg), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_3$), 4-aminidophenyl methane-sulphonyl fluoride (p-APMSF), tranexamic acid (trans-4-(aminomethyl)cyclohexanecarboxylic acid) and sodium nitroprusside were obtained from Sigma (St. Louis, MO, U.S.A.). The stock solutions of p-AMPSF and tranexamic acid were prepared at 10 mmol/L and 100 mmol/L in distilled water. Nω-nitro-L-arginine methyl ester (L-NAME) was purchased from Wako Pure Chemical (Tokyo, Japan). Fura-2/AM and BAPTA/AM were obtained from Dojindo (Kumamoto, Japan). DAR-4M was purchased from Daiich Pure Chemicals (Tokyo, Japan). A PAR1 activating peptide, TFLLR-NH$_2$, was purchased from Bachem (Bubendorf, Switzerland). A competitive inhibitor of IGF-1 receptor kinase, AG538, was purchased from Calbiocem (La Jolla, CA U.S.A.)

Data analysis
All data were collected using a computerized data acquisition system (MacLab, Analog Digital Instruments, Australia; Macintosh, Apple computer, U.S.A.). The data are the mean ± SEM of the number of experiments as indicated. Significant differences were statistically analyzed by either the unpaired Student's t-test or the Turkey-Kramer test. Each data point for the concentration-response curves in Figures 1, 2, and 5 were obtained with a separate sample. Therefore, the statistical analysis was performed with the unpaired Student’s t-test as indicated in each figure legend. Otherwise, the Turkey-Kramer test was used to analyze the statistical significance. A $P$ value of less than 0.05 was considered to be statistically significant.
Supplemental Figure I. Endothelium-dependent relaxation induced by thrombin and trypsin in the porcine coronary artery.

Representative traces (a, c) and concentration-response curves (b, d) showing the effects of thrombin (a, b) and trypsin (c, d) on the contraction induced by 30 nmol/L U46619. The levels of tension at rest and during the sustained phase of the U46619-induced contraction just prior to the applications of thrombin or trypsin were assigned as values of 100 % and 0 % relaxation, respectively. The data are the mean ± SEM. *, P<0.05; **, P<0.01 vs. the values without endothelium (b, d).
Supplemental Figure II. NO production induced by plasmin in cultured endothelial cells. The concentration-response curve of the plasmin-induced NO production in PCAECs. The value at zero concentration indicated the change in DAR-4M fluorescence observed with the buffer change without stimulation with plasmin. The data are the mean ± SEM.
References for Data Supplements


