Activation of proteinase-activated receptor 2 stimulates soluble vascular endothelial growth factor receptor 1 release via epidermal growth factor receptor transactivation in endothelial cells

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Activation of Proteinase-Activated Receptor 2 Stimulates Soluble Vascular Endothelial Growth Factor Receptor 1 Release via Epidermal Growth Factor Receptor Transactivation in Endothelial Cells

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Abstract—The proteinase-activated receptor 2 (PAR-2) expression is increased in endothelial cells derived from women with preeclampsia, characterized by widespread maternal endothelial damage, which occurs as a consequence of elevated soluble vascular endothelial growth factor receptor-1 (sVEGFR-1; commonly known as sFlt-1) in the maternal circulation. Because PAR-2 is upregulated by proinflammatory cytokines and activated by blood coagulation serine proteinases, we investigated whether activation of PAR-2 contributed to sVEGFR-1 release. PAR-2–activating peptides (SLIGRL-NH₂ and 2-furoyl-LIGRLO-NH₂) and factor Xa increased the expression and release of sVEGFR-1 from human umbilical vein endothelial cells. Enzyme-specific, dominant-negative mutants and small interfering RNA were used to demonstrate that PAR-2–mediated sVEGFR-1 release depended on protein kinase C-β1 and protein kinase C-ε, which required intracellular transactivation of epidermal growth factor receptor 1, leading to mitogen-activated protein kinase activation. Overexpression of heme oxygenase 1 and its gaseous product, carbon monoxide, decreased PAR-2–stimulated sVEGFR-1 release from human umbilical vein endothelial cells. Simvastatin, which upregulates heme oxygenase 1, also suppressed PAR-2–mediated sVEGFR-1 release. These results show that endothelial PAR-2 activation leading to increased sVEGFR-1 release may contribute to the maternal vascular dysfunction observed in preeclampsia and highlights the PAR-2 pathway as a potential therapeutic target for the treatment of preeclampsia. (Hypertension. 2010;55:689-697.)

Key Words: PAR-2 ■ sVEGFR-1/sFlt-1 ■ endothelium ■ factor Xa ■ HO-1 ■ preeclampsia

Preeclampsia is a pregnancy-specific multiorgan syndrome characterized by widespread maternal endothelial damage with a clinical presentation of hypertension and proteinuria after 20 weeks’ gestation. Women with preeclampsia are at an increased risk of developing cardiovascular disease. The antiangiogenic factors, soluble vascular endothelial growth factor receptor 1 (VEGFR; sVEGFR-1, also known as sFlt-1) and soluble endoglin, are increased dramatically before the clinical onset of preeclampsia. Elevated sVEGFR-1 antagonizes the action of vascular endothelial growth factor and placenta growth factor resulting in impaired human placental angiogenesis and glomerular endothelial cell damage, proteinuria, and hypertension in rodent models, indicating that it is a major contributory factor to the development of preeclampsia. Furthermore, the anti-inflammatory enzyme heme oxygenase 1 (HO-1), which is decreased in preeclamptic placentas and regulates inflammatory angiogenesis, suppresses sVEGFR-1 release from endothelial cells.

During placentation, the trophoblasts invade the maternal tissues but avoid immune rejection. Preeclampsia is associated with a failure to switch from the T helper 1 cytokine profile (eg, interferon-γ, tumor necrosis factor [TNF]-α, interleukin [IL]-8, and IL-18) to T helper 2 cytokine profile (eg, IL-4 and IL-10), indicating a lack of immune tolerance. A rise in circulating levels of proinflammatory cytokines (eg, TNF-α and IL-1β) upregulates tissue factor expression leading to activation of the coagulation system, which can result in disseminated intravascular coagulation, particularly in early onset severe preeclampsia and eclampsia.

The main physiological activators of the proteinase-activated receptors (PAR-1 and PAR-2) are serine proteinases, such as thrombin and factors VIIa and Xa (FXa). PAR-2 plays an
important role in inflammation and regulates vascular function. Proinflammatory cytokines, including interferon-γ and TNF-α, induce PAR-2 expression and, in turn, PAR-2 activation promotes the production of interferon-γ, TNF-α, IL-8, and IL-18 in various cell types, including the endothelium. Indeed, T-cell proliferation, interferon-γ, and IL-18 levels are significantly reduced in PAR-2 knockout mice. whereas endotoxin-stimulated macrophages show significantly greater IL-10 expression and enhanced IL-4 secretion in PAR-2 null mice.

PAR-2 expression is reported to be increased in human umbilical vein endothelial cells (HUVECs) derived from preeclamptic pregnancies, and the conditioned medium from preeclamptic placental villous tissue explants upregulates PAR-2 in cultured endothelial cells. Although PAR-2 activity is known to be upregulated in the vasculature in inflammatory conditions, the potential relationship between PAR-2 activation and sVEGFR-1 release is unknown. Therefore, we speculated that the activation of PAR-2 could increase endothelial sVEGFR-1 release. In this study, we report the ability of PAR-2 agonists to increase sVEGFR-1 release from endothelial cells via protein kinase C (PKC)–mediated intracellular transactivation of epidermal growth factor (EGF) receptor (EGFR) 1 and subsequent downstream mitogen-activated protein (MAP) kinase signaling. Furthermore, we show that PAR-2–stimulated sVEGFR-1 release was suppressed by HO-1 overexpression and enhanced by HO-1 knockdown, indicating that HO-1 is a central regulator of sVEGFR-1 expression.

### Materials and Methods

A full description of materials and methods used can be found in the online Data Supplement (please see http://hyper.ahajournals.org).

### Cell Culture

HUVECs were isolated and cultured as described. Human embryonic kidney cells (HEK-293) were maintained in DMEM containing 10% FCS, whereas porcine aortic endothelial cells (PAECs) expressing PAR-2 (PAEC-PAR-2) and cells containing the vector alone (PAEC-pCDNA3.1B) were propagated in G418-containing F12-HAM nutrient mix supplemented with 10% FCS.

### Adenoviruses

The recombinant, replication-deficient adenoviruses encoding rat HO-1 and dominant-negative PKC (dnPKC) isozymes were amplified and titered, and the optimal multiplicity of infection determined by Western blotting as 50 infectious units (ifu) per well. The HO-1 and 100 ifu per well for the dnPKC isozyme adenoviruses. HUVECs were infected with adenoviruses and then incubated for 24 hours in basal medium containing 5% FCS.

### Small Interfering RNA-Mediated Gene Knockdown

The small interfering RNAs (siRNAs) targeted against c-Src, were amplified and titered, and the optimal multiplicity of infection determined by Western blotting as 50 infectious units (ifu) per well. The HO-1 and 100 ifu per well for the dnPKC isozyme adenoviruses. HUVECs were infected with adenoviruses and then incubated for 24 hours in basal medium containing 5% FCS.

### ELISAs

The sVEGFR-1 concentration in cell supernatants was determined as described. EGFR was measured using the EGFR DuoSet IC ELISA (R&D Systems) and phosphorylated EGFR by a sandwich ELISA using an anti-EGFR capture antibody and phosphotyrosine detection antibody.

### Western Blotting

After stimulation, cells were lysed in radioimmunoprecipitation assay buffer and 30 μg of protein were Western blotted using rabbit antiphospho-extracellular signal-regulated kinase (ERK)1/2, anti--Src phospho-Y416, or anti–Raf-1-phospho-S338 (Cell Signaling) antiactivated EGFR (BD Biosciences) antibodies.

### VEGFR-1 Promoter Reporter Assays

A 1.3-Kb fragment of the human VEGFR-1 promoter-luciferase construct was used to determine the ability of PAR-2 to activate the VEGFR-1 gene. The reporter plasmid was constructed by cloning a PCR fragment corresponding with sequences from −1214 to +155 relative to the first exon in the VEGFR-1 gene into the BglII and HindIII sites of pGL2 (Promega). Hek293 cells, which express functional PAR-2, and porcine aortic endothelial cells were transfected with the VEGFR-1 promoter construct using Exgen 500 (Fermentas). For details see the online Supplemental Methods.

### Statistical Analysis

All of the data are expressed as the mean±SEM. Statistical analysis was performed using the 2-tailed Student t test. P<0.05 was considered statistically significant.

### Results

**PAR-2 Activation Stimulates sVEGFR-1 Release**

Endothelial cells derived from patients with preeclampsia exhibit increased PAR-2 expression. To determine the effect of PAR-2 activation on sVEGFR-1 production, HUVECs were stimulated with the PAR-2 selective activating peptides (SLIGRL-NH$_2$ and 2f-LIGRLO-NH$_2$) or FXa for 24 hours and sVEGFR-1 quantified in the culture medium by ELISA. PAR-2 activating peptides induced sVEGFR-1 release, whereas the corresponding reverse-control peptides (LRGILS-NH$_2$ and 2f-OLRGIL-NH$_2$) failed to induce sVEGFR-1 release (Figure 1A). Similarly, FXa (100 nmol/L) induced sVEGFR-1 release. A 1.3-Kb human VEGFR-1 promoter luciferase reporter was used to assess the ability of PAR-2 to activate the VEGFR-1 gene in PAECs and HEK293 cells. Activation of PAR-2 significantly increased VEGFR-1 promoter activity, indicating that PAR-2 regulates the production of sVEGFR-1 transcription (Figure 1B and Figure 2A).

**PAR-2–Induced sVEGFR-1 Release Depends on PKC and Src Activity**

Both PKC and Src are involved in PAR-2 signaling. Soluble VEGFR-1 release and VEGFR-1 promoter activity...
were blocked by a PKC inhibitor (Ro-32-0432), indicating its involvement in PAR-2–mediated sVEGFR-1 production (Figure 2A and 2B). HUVECs express PKCα, PKCβ1, PKCδ, and PKCe isozymes.28 To evaluate the PKC subtype involved in PAR-2–mediated sVEGFR-1 release, HUVECs were infected with adenoviruses encoding dominant-negative isozymes of PKC. PAR-2–induced release of sVEGFR-1 was inhibited by both PKCα and PKCe, and the basal level of sVEGFR-1 was suppressed by the overexpression of PKCβ in endothelial cells (Figure 2C). PKCβ1 knockdown in HUVECs abrogated PAR-2–mediated sVEGFR-1 release (Figure 2D). Western blot analysis confirmed endogenous expression of PKC isozymes and the modulation by adenovirus overexpression or knockdown (Figure 2C). Similarly, Src family kinase inhibitor PP2 inhibited sVEGFR-1 release and VEGFR-1 promoter activity, implicating its involvement in PAR-2–stimulated sVEGFR-1 expression (Figure 3A and 3B). These results were confirmed using siRNA-mediated knockdown of Src (Figure 3C and 3D).

MAP Kinase Activation and EGFR Transactivation Are Required for PAR-2–Induced sVEGFR-1 Expression

The activation of G protein–coupled receptors including PAR-1, PAR-2, and angiotensin II receptors, is widely reported to phosphorylate MAP kinase via PKC-mediated transactivation of EGFR.29–31 To investigate whether MAP kinase activation is required for PAR-2–induced sVEGFR-1 release, HUVECs were preincubated with MAP kinase kinase (MEK)1/2 inhibitor (U0126) and stimulated with PAR-2 ligand. Inhibition of MEK1/2, which is immediately upstream of ERK-1/2 in the MAP kinase pathway, resulted in a complete loss of PAR-2–mediated sVEGFR-1 release (Figure 4A) and VEGFR-1 promoter activity (Figure 4B) and caused a loss of PAR-2–mediated ERK-1/2 phosphorylation (Figure 4C). Furthermore, the overexpression of dominant-negative PKCβ and PKCe or Src-kinase inhibition suppressed PAR-2–mediated ERK-1/2 phosphorylation (Figure 4D and 4E).

To determine the sequence of events leading to PAR-2–mediated sVEGFR-1 release, we examined the phosphorylation of Src at Y416 and Raf-1 at S338 in relation to downstream activation of ERK-1/2 and whether PKC activation is upstream of Src and Raf-1 in PAR-2–stimulated VEGFR-1 release. Overexpression of dominant-negative PKCβ or PKCe attenuated PAR-2–stimulated Src phosphorylation and completely inhibited the phosphorylation of Raf-1 (Figure 4F). In addition, the PKC (GF109203X) and Src (PP2) inhibitors completely abrogated 2f-LIGRLO-NH2–stimulated phosphorylation of Raf-1 (Figure 4G). Collectively, these data demonstrate that PAR-2 stimulates sVEGFR-1 expression and release by activat-
ing PKC, leading to sequential Src, Ras, Raf-1, and ERK-1/2 activation.

Subsequently, we investigated whether EGFR transactivation is required for PAR-2–induced ERK1/2 activation and sVEGFR-1 expression. However, early studies reported the absence of EGFR in endothelial cells,32 and EGFR-1 has only recently been detected in HUVECs.33 ELISA and Western blot analysis confirmed the presence of functional EGFR in HUVEC lysates (Figure S3). To investigate whether PAR-2 activation can lead to EGFR transactivation, HUVECs were stimulated with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH2) for 24 hours, and the conditioned medium was assayed for sVEGFR-1 by ELISA. D, PKC-β1 siRNA was introduced into HUVECs using an Amaxa nucleofector and the knockdown of PKCβ1 confirmed by Western blotting. PAR-2–mediated sVEGFR-1 release was inhibited in cells treated with PKC-β1 siRNA. Results represent the mean (±SEM). *p<0.01 (A and B vs control and C vs EV silencing). **p<0.05 (D vs control).

Figure 2. PAR-2–induced sVEGFR-1 release from endothelial cells depends on PKC activity. A, HUVECs and (B) HEK293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with the PKC inhibitor (Ro-32-0432; 1 μmol/L) for 45 minutes and then stimulated with PAR-2 activating peptide (10 μmol/L of 2f-LIGRLO-NH2) for 24 hours, and the cell culture supernatants were assayed for sVEGFR-1 by ELISA (A) and cell lysates assayed for luciferase activity (B), respectively. C, HUVECs were infected overnight with 100 ifu per cell of adenoviruses expressing dominant-negative PKC α/β/ε (dnPKCα/β/ε) or empty vector (EV), incubated for 24 hours, and the expression of PKC isoforms were examined in cell lysates by Western blotting. These cells were stimulated with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH2) for 24 hours, and the conditioned medium was assayed for sVEGFR-1 by ELISA. D, PKC-β1 siRNA was introduced into HUVECs using an Amaxa nucleofector and the knockdown of PKCβ1 confirmed by Western blotting. PAR-2–mediated sVEGFR-1 release was inhibited in cells treated with PKC-β1 siRNA. Results represent the mean (±SEM). *p<0.01 (A and B vs control and C vs EV silencing). **p<0.05 (D vs control).
2f-LIGRLO-NH₂ (10 μmol/L) or FXa (200 nmoL/L; Figure 5F). The MMP inhibitor did not significantly suppress sVEGFR-1 release, indicating that MMPs are not involved in PAR-2–stimulated sVEGFR-1 release. On the basis of these results, we conclude that the PAR-2–mediated transactivation of EGFR occurs through an intracellular route via a PKC- and Src-dependent pathway.

Statins and HO-1 Activity Downregulate PAR-2s-Induced sVEGFR-1 Release

Statins (which upregulate HO-1), HO-1, and its gaseous product CO act as negative regulators of sVEGFR-1 release in endothelial cells. Consistent with this concept, simvastatin inhibited PAR-2–mediated sVEGFR-1 release (Figure 6A) and VEGFR-1 promoter activity (Figure 6B and 6C). The overexpression of HO-1 also significantly inhibited the release of sVEGFR-1 (Figure 6D), whereas loss of HO-1 enhanced VEGFR-1 promoter activity (Figure 6E). The lipid soluble CO-releasing molecule (CORM-2) reduced PAR-2–induced sVEGFR-1 release, whereas the inactive CORM-2 had no significant effect (Figure 6F). These results further support a potentially beneficial role for HO-1 and its product CO in preeclampsia, as reported previously.

Figure 3. PAR-2–induced sVEGFR-1 production requires Src activation. A, HUVECs and (B) HEK-293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with an Src inhibitor (PP2; 10 μmol/L) for 45 minutes and then stimulated with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH₂) for 24 hours. The cell culture supernatants were assayed for sVEGFR-1 by ELISA (A) and cell lysates were assayed for luciferase activity (B), respectively. C, HUVECs and (D) HEK293 cells containing the VEGFR-1 luciferase reporter were electroporated with Src siRNAs using an Amaxa Nucleofector and incubated overnight. After stimulation with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH₂) for 24 hours, sVEGFR-1 was measured in the cell culture supernatants by ELISA (C) and cell lysates were assayed for luciferase activity (D), respectively. Results represent the mean (±SEM). * P < 0.01 (A and D) and * P < 0.05 (B) vs control.

Figure 4. ERK-1/2 activation is required for PAR-2–induced sVEGFR-1 release. A, HUVECs and (B) HEK293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with an MEK-1/2 inhibitor (U0126; 10 μmol/L) for 45 minutes, stimulated with PAR-2–activating peptide (2f-LIGRLO-NH₂; 10 μmol/L) for 24 hours, and the conditioned medium was assayed for sVEGFR-1 by ELISA (A) and luciferase activity determined in cell lysates (B), respectively. C through G, Western blot analysis: cell lysates were immunoblotted for phospho-ERK-1/2 (p-ERK), phospho-Src (pY416Src), and phospho–Raf-1 (pS338Raf-1); HUVECs were either preincubated for 45 minutes with (C) MEK-1/2 (U0126; 10 μmol/L), (E) Src (PP2; 10 μmol/L), and (G) PKC (GF109203X; 5 μmol/L) inhibitors or (D and F) infected overnight with adenoviruses expressing dominant-negative PKCα (dnPKCα), PKCβ (dnPKCβ), or PKCε (dnPKCε) and stimulated with PAR-2–activating peptide (2f-LIGRLO-NH₂; 10 μmol/L) for 10 minutes. * P < 0.01 (A) and * P < 0.05 (B) vs control.
intestinal epithelial cells depended on Src activation and a recent report showing that EGFR transactivation and PAR-2–mediated EGFR transactivation. This is consistent with PAR-2. In this study, Src activity was required for PKC, Src, Raf-1, and ERK-1/2 and depends on EGFR from endothelial cells through the sequential activation of receptors, including PAR-1, PAR-2, and PAR-4, is well similar mechanism in cardiomyocytes after PAR-4 stimulation. The transactivation of the EGFR by G-coupled protein receptors, including PAR-1, PAR-2, and PAR-4, is well established. Inhibition of Src or EGFR completely abrogated PAR-2–mediated sVEGFR-1 expression, indicating that both Src and EGFR activation is required in concert with PKC for the efficient release of sVEGFR-1 in response to PAR-2. In this study, Src activity was required for PAR-2–mediated EGFR transactivation. This is consistent with a recent report showing that EGFR transactivation and MAP kinase activity in PAR-2–induced chloride secretion in intestinal epithelial cells depended on Src activation and a similar mechanism in cardiomyocytes after PAR-4 stimulation. The inhibition of either the Src or EGFR did not completely block PAR-2–stimulated ERK-1/2 phosphorylation, suggesting that ERK-1/2 may also be activated directly by PKC via Raf-1. However, activation of ERK-1/2 appears to be the final pathway for PAR-2–mediated release of sVEGFR-1. In many cell systems, EGFR transactivation is mediated by the proteolytic cleavage of cell membrane–bound EGFR ligands, including transforming growth factor-α and heparin-binding EGFR by MMP such as the TNF-α–converting enzyme. PAR transactivation of EGFR was reported to occur in an MMP-independent manner, requiring Src activation in cardiac fibroblasts and intestinal epithelial cells. In this study, MMP inhibition did not prevent PAR-2–mediated sVEGFR-1 release supporting EGFR transactivation occurring via an intracellular route. The observation that EGFR transactivation leads to increased sVEGFR-1 release may have broader significance in preeclampsia. The infusion of angiotensin II selectively upregulates the production of sVEGFR-1 in pregnant mice. Angiotensin II type 1 receptor density increases in preeclamptic placentas, and angiotensin II type 1 activating autoantibodies induce a preeclampsia-like condition in mice. Given the ability of angiotensin II to transactivate the EGFR and the signal transduction pathway identified for PAR-2 in this study, we suggest that this represents a common mechanism by which G protein–coupled receptors could induce sVEGFR-1 production.
PAR-2 inhibition can suppress TNF-α expression in inflammatory settings\(^4^0\) and improve wound healing in mice by reducing inflammation.\(^4^1\) Given the increased procoagulant activity observed in preeclampsia and the ability of the coagulation proteases factors VIIa and FXa to activate PAR-2, coupled with the reported increased PAR-2 expression on endothelium derived from preeclamptic women,\(^2^0\) we suggest that PAR-2 activation may be a contributing factor to the increases in circulating sVEGFR-1 in this syndrome. Furthermore, the reported increased expression of PAR-1 in the endothelium\(^2^0\) and placenta\(^1^2\) of preeclamptic women, combined with the observed excessive generation thrombin, the ligand for PAR-1, in preeclampsia, indicates that other PAR receptors may also be involved in sVEGFR-1 production in this setting. Although the trophoblast is the main source of sVEGFR-1, and PAR-2 activation increases sVEGFR-1 release from trophoblasts, this study confirms that the endothelium may be a significant source of sVEGFR-1. PAR-2 activation leading to sVEGFR-1 release from the endothelium is relevant not only in the placental-based perturbation in preeclampsia, but inflammatory conditions, such as cardiovascular diseases and sepsis, may contribute directly to the endothelial dysfunction.

A recent report showed that, in mice lacking PAR-2, exposure to antiphospholipid antibodies did not induce fetal injury or miscarriage. Furthermore, statin treatment reduced the adverse effects of PAR-2 activation induced by antiphospholipid antibodies and prevented pregnancy loss.\(^4^3\) HO-1 activity is essential for the successful outcome of pregnancy,\(^4^4\) and HO protein expression is reduced in preeclamptic placentas.\(^6\) The concentration of CO in the exhaled breath of women with preeclampsia is significantly less than in normal pregnancy,\(^4^5\) indicating lower HO activity in these patients. HO-1 and CO inhibit VEGF-stimulated sVEGFR-1.\(^8\) In this study, we demonstrate that upregulation of HO-1 with Simvastatin or activation of the HO-1 pathway suppresses PAR-2-mediated sVEGFR-1 release. More importantly, these studies highlight the potential efficacy of statins in controlling complications of pregnancy, which are being investigated in a randomized, placebo-controlled trial (Statins to Ameliorate early onset Pre-eclampsia [StAmP]) for use of statins to ameliorate early onset preeclampsia.

**Perspectives**

The antiangiogenic soluble factor sVEGFR-1 (commonly known as sFlt-1) appears to be “the final common pathway”
inducing the maternal clinical signs of preeclampsia. This study demonstrates that activation of the proinflammatory receptor PAR-2 caused the endothelium to release sVEGFR-1, and the lipid-lowering statin, simvastatin was found to completely block sVEGFR-1 expression. Recently, Redecha et al. showed that PAR-2 activation caused trophoblast injury and fetal death, which was also blocked by simvastatin. Collectively, the findings indicate that PAR-2 activation leading to increased sVEGFR-1 release may contribute to vascular dysfunction in pregnancy and identifies the PAR-2 pathway as a potential therapeutic target.

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Disclosures
None.

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Activation of PAR-2 stimulates sVEGFR-1 release via EGF receptor transactivation in endothelial cells

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Short title: HO-1 suppresses PAR-2-induced sVEGFR-1

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Methods

Reagents
All peptides were synthesized at the peptide synthesis facility, University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada. Factor Xa was obtained from Cambridge BioScience (Cambridge, UK). EGF was purchased from RELIA Tech (Braunschweig, Germany). Rabbit antibodies against phospho-ERK1/2, phospho-c-Raf and phospho-Src were purchased from Cell Signaling (Beverly, MA), and rabbit anti-HO-1 from StressGen Biotechnologies Corporation, Canada. Ro-32-0432, GF109203X, PP2 and U0126 were purchased from the Calbiochem (Nottingham, UK). Eurogentec (Southampton, UK) synthesized the siRNAs. Tricarbonyldichlororuthenium (II) dimer (CORM-2), ruthenium (III) chloride hydrate (CORM-2 control), Simvastatin, tumor necrosis factor-α (TNFα), mouse anti-β-actin monoclonal antibody and all other cell culture reagents and chemicals were obtained from Sigma Aldrich (Poole, UK).

Cell Culture
HUVEC were isolated and cultured as described previously.1 Experiments were performed on second or third passage HUVEC. HEK 293 human embryonic kidney cells were maintained in DMEM containing 10% FCS. The porcine aortic endothelial cell line expressing PAR-2 (PAEC-PAR-2) and cells containing the vector alone (PAEC-pCDNA3.1B) were routinely propagated in G418 containing F12-HAM nutrient mix supplemented with 10% (v/v) FCS. Human first trimester placental tissues derived cell-line (HTR-8) was a kind gift from Professor Charles H. Graham.

Adenoviruses
The recombinant, replication-deficient adenoviruses encoding rat HO-1 (a kind gift from Professor Augustine Choi, Pennsylvania, USA) and dominant-negative PKC (dnPKC) isozymes were prepared as described previously.2 All adenoviruses were amplified, titred and the optimal multiplicity of infection determined by Western blotting was found to be 50 ifu/cell for HO-1 and 100 ifu/cell for the dnPKC isozyme adenoviruses. HUVEC were infected overnight at 100 ifu/cell with adenoviruses expressing dnPKCα, dnPKCβ1, dnPKCε, and empty-vector (AdCMV) as a control then incubated for 24 hours in basal medium containing 5% FCS.

SiRNA-mediated gene knock-down
The siRNAs targeted against c-Src3, HO-14 and PKCβ1 (sense 5′-GGGAGAAACUUGAACGCAAtt-3′; antisense 5′-UUGCGUUCAAGUUCUCCTt-3′) and a universal control siRNA (Dharmacon) were introduced into HUVEC using the Amaxa Nucleofector HUVEC II kit according to the manufacturer’s instructions (Amaxa, Germany). Following Nucleofection the cells were incubated overnight prior to treatment.

Western Blotting
Following stimulation, cells were lysed in RIPA buffer and 30 µg protein Western blotted as described previously5 using rabbit anti-phospho-ERK1/2, anti-Src phospho-Y416 or anti-Raf-1-phospho-S338 (Cell Signaling) anti-activated EGFR (BD Biosciences) antibodies at the manufacturer’s recommended concentrations.
Generation of porcine aortic endothelial cells (PAEC) expressing PAR-2

A permanent endothelial cell line expressing PAR-2 was generated as described previously. Briefly, a rat PAR-2 cDNA in pcDNA3.1B was transfected into a PAEC which lack functional PAR-1 and PAR-2 expression (see Figure S1B), using ExGen 500 (Fermentas, UK). Cells were then subcloned and selected in geneticin-containing medium and PAR-2 receptor-bearing cells (PAEC-PAR-2) were isolated using the anti-PAR-2 B5 antibody and fluorescence-activated cell sorting to yield a permanent cell line. PAEC-PAR-2 were routinely propagated in geneticin-containing HAM F12 nutrient mix supplemented with 10% (v/v) FCS. PAEC stably transfected with the pCDNA3.1B vector alone were used as a control.

Calcium signalling assay

PAEC-PAR-2 and PAEC-pCDNA31.B cells were loaded with the intracellular calcium indicator Fluo-3 (Molecular Probes inc., Eugene, USA) at a final concentration of 22 µmol/L, as described previously. Fluorescence measurements, reflecting elevations of intracellular calcium, were conducted at 24°C using an AMINCO-Bowman series 2 luminescence spectrometer (Spectronic Unicam, Rochester, USA), with excitation at 480 nM and emission at 530 nM. The fluorescence signals caused by the addition of test agonists were expressed as described previously relative to the fluorescence peak height yielded by replicate cell suspensions treated with 2 µmol/L concentrations of the ionophore A23187 (Sigma Chemical). This concentration of A23187 was at the plateau of its concentration-response curve for fluorescence responses.

MTT Assay

HUVEC were seeded at a density of 1x 10^4 cells/well in a 96-well plate and incubated overnight at 37°C in growth medium. Cells were incubated in triplicate with PAR-2 activators in medium containing 5% FCS for 24 hours. The medium was removed, 80 µl of 0.2% BSA-M199 medium and 20 µl of 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thizolyl blue were added to each well and the plate incubated in the dark at 37°C for 4 hours. The MTT solution was removed and DMSO (150 µl/well) added and the plate agitated for 5 minutes. The optical density was measured at 540 nm and 690 nm and DMSO alone was used as a blank.

Enzyme-Linked Immunosorbent Assays

The sVEGFR-1 concentration in cell supernatants was determined as described previously. EGFR was measured in cell lysates using the EGFR DuoSet IC ELISA according to the manufacturer’s instructions (R&D Systems, UK). Phosphorylated EGFR was detected using a sandwich ELISA employing an anti-EGFR capture antibody and phosphotyrosine detection antibody. Following stimulation cell monolayers growing on 6-well dishes were placed on ice and lysed in RIPA buffer (UpState) containing protease and phosphatase inhibitors (Sigma, Poole, Dorset UK). The protein concentration of cell lysates was determined using the DC Protein Assay (BioRad). Levels of total and phosphorylated EGFR were measured in cell lysates using commercial (DuoSet® IC; R&D Systems) and in-house ELISAs respectively. Briefly, phospho-EGFR was detected on Immunosorb (Nunc, USA) 96-well plates coated with 0.8 µg/ml monoclonal anti-EGFR capture antibody (R&D Systems) in PBS overnight and blocked with 1% BSA in PBS. Cell lysates (100 µg/well), were added to the plate and incubated for 2 hours. The presence of phospho-EGFR was then detected by adding 100 µl/well biotin conjugated anti-phospho-
tyrosine MoAb (PY99; 1 µg/ml) for 2 hours and binding visualised using the diluted streptavidin-HRP. Total EGFR was measured in the same cell lysates using the EGFR DuoSet ELISA according to the manufacturer’s instructions (R&D Systems, UK). Results were normalised for protein concentration and expressed as ratio of the level of phosphorylated to total EGFR.

**VEGFR-1 promoter reporter assays**
A ~1.3 Kb fragment of the human VEGFR-1 promoter starting from -1500 bp relative to the start codon was cloned into the pGL2 luciferase reporter plasmid (pVEGFR-1lac) was used to determine the ability of PAR-2 to activate the VEGFR-1 gene in PAEC and HEK293 cells which express high levels of PAR-2 and EGFR (Figure S3). Luciferase reporter constructs were introduced into HEK293 cells using the Amaxa Nucleofector Kit V and manufacturer’s recommended conditions, and into PAEC using ExGen 500 (EuroMedex, France) as described previously. Following stimulation for 24 hours, luciferase activity was determined in cell lysates using the Dual Luciferase Assay (Promega) as described previously.

**Results**

**Generation PAEC line expressing functional PAR-2 receptors**
PAEC which do not express PAR-2 were transfected with a plasmid containing rat PAR-2 and positive clones selected in G418 containing medium to establish a stable PAR-2 expressing cell line (PAEC-PAR-2). The presence of PAR-2 receptors in these cells was demonstrated by immunostaining with an anti-PAR-2 antibody (Figure S1A). Functional activity of PAR-2 in this cell line was confirmed by monitoring calcium mobilisation in Fluo-3 loaded cells following activation with the PAR-2-selective peptide SLIGRL-NH₂ (Figure S1B). In addition, PAR-2 desensitisation was achieved after a second challenge with SLIGRL-NH₂ at 10 minutes. No PAR-2 staining, or calcium mobilisation in response to SLIGRL-NH₂, was observed in the empty vector containing PAEC-pCDNA3.1B control cell line (Figure S1).

**PAR-2 activating peptides induce robust VEGFR-1 promoter activation without affecting cell viability**
The PAR-2 peptides SLIGRL-NH₂ and 2f-LIGRLO induced VEGFR-1 promoter activity in HEK-293 cells in a similar manner to Angiotensin II (Figure S2A) which we had reported previously to stimulate sVEGFR-1 release in trophoblasts. The incubation of primary endothelial cells with the PAR-2 activators did not significantly alter their mitochondrial activity as assessed by MTT assay (Figure S2B) indicating that the effect of PAR-2 activation on sVEGFR-1 expression was specific and not due to a more general effect on endothelial cell proliferation or survival.

**HUVEC express functional EGF receptors and their direct activation results in sVEGFR-1 release**
Several earlier studies have reported the absence of EGFR in endothelial cells. More recently EGFR-1 has been detected in HUVEC and other types of endothelial cells. To confirm that HUVEC were expressing EGFR under the culture conditions employed for our studies, HUVEC lysates were analysed by both ELISA and Western blotting for the presence of EGFR. HUVEC were found to express approximately 550 pg of EGFR per
100 µg of cell lysate by ELISA (Figure S3A). This was ~ 4-fold less than in HEK-293 cells which were used as a positive control. To demonstrate activation of EGFR in endothelial cells HUVEC were incubated with EGF for 10 minutes and cell lysates Western blotted using a monoclonal antibody specific for the activated conformation of EGFR. The level of activated EGFR detected in HUVEC was found to be greater following stimulation of the cells with increasing concentrations of EGF (Figure S3B). Moreover, stimulation HUVEC with EGF resulted in an increase in Raf-1 S338 phosphorylation and increase in sVEGFR-1 release which was blocked by the p42/p44 Erk inhibitor, U0126. Collectively, these results demonstrate the presence of functional EGFR in HUVEC and that its direct activation can induce sVEGFR-1 production through the p42/p44 MAP Kinase pathway.
References


Figure S1. Generation of a porcine aortic endothelial cell (PAEC) line expressing functional PAR-2. A, PAEC were transfected with either a plasmid containing rat PAR-2 cDNA (PAEC-PAR-2) or empty vector (PAEC-pCDNA) and selected with G418. Cells growing on microculture slides were stained with anti-human PAR-2 antibody (clone B5). B, To demonstrate the presence of functional PAR-2 in PAEC-PAR-2 cells were loaded with Fluo-3 and stimulated with the PAR-2-selective peptide SLIGRL-NH₂ (100 μmol/L) and calcium mobilisation investigated. No calcium response was observed in PAEC-pCDNA following stimulation with PAR-2 (SLIGRL) or PAR-1 (TFLLR) specific peptides. The ionophore A23187 (2 μmol/L) was used as a positive control.
Figure S2. PAR-2 activating peptides induce VEGFR-1 expression without affecting cell viability. **A**, HEK-293 cells transfected with a 1.5 kb fragment of the VEGFR-1 promoter with a luciferase reporter were incubated with SLIGRL-NH$_2$ (30 µmol/L), 2f-LIGRLO-NH$_2$ (10 µmol/L), or Angiotensin II (100 nM) for 24 hours and VEGFR-1 promoter activity in cell lysates determined by luciferase assay. **B**, HUVEC were incubated with increasing concentrations of 2f-LIGRLO-NH$_2$, SLIGRL-NH$_2$ and FXa in medium containing 5% FCS for 24 hours and cell viability assessed by MTT assay. **C**, the first trimester trophoblast cell line (HTR-8) were incubated with SLIGRL-NH$_2$ (100 µmol/L), 2f-LIGRLO-NH$_2$ (50 µmol/L), FXa (100 nmol/L) or TNFα (50 ng/mL) for 24 hours and the cell supernatants assayed for sVEGFR-1 by ELISA. The corresponding reverse peptides, LRGILS-NH$_2$ and 2f-OLRGIL-NH$_2$, and TNFα were used as negative and positive controls respectively.
Figure S3. HUVEC express functional EGF receptors and their activation results in sVEGFR-1 release.  

A, The presence of EGFR was detected in both HUVEC and HEK-293 cell lysates (100 µg/well) by ELISA (R&D Systems).  

B, HUVEC were incubated with increasing concentrations of EGF for 10 minutes and cell lysates (50 µg/lane) subjected to Western blotting with an antibody specific for activated EGFR.  HEK-293 cells were used as a positive control.  

C, HUVEC were serum-starved, stimulated for 10 minutes with 2f-LIGRLO-NH₂ (10 µmol/L), FXa (200 nmol/L) or EGF (50 ng/mL) and the cells lysates Western blotted for phospho S338 Raf-1 and β-actin as a loading control.  

D, HUVEC were pre-treated for 45 minutes with U0126 (10 µmol/L) and stimulated with FXa (200 nmol/L) or EGF (50 ng/mL) for 24 hours and sVEGFR-1 assayed in the cell culture supernatants by ELISA.  *p<0.01 versus control.