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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-NH$_2$ and 2-furoyl-LIGRLO-NH$_2$) 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-β_1 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.[1] Women with preeclampsia are at an increased risk of developing cardiovascular disease.[2] 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.[3] Elevated sVEGFR-1 antagonizes the action of vascular endothelial growth factor and placenta growth factor resulting in impaired human placental angiogenesis[4] and glomerular endothelial cell damage, proteinuria, and hypertension in rodent models,[5] 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[6] and regulates inflammatory angiogenesis,[7] suppresses sVEGFR-1 release from endothelial cells.[8]

During placentation, the trophoblasts invade the maternal tissues but avoid immune rejection.[9] 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.[10] 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[11] and eclampsia.[12]

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).[13] PAR-2 plays an

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important role in inflammation and regulates vascular function.[14,15] 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.[16] Indeed, T-cell proliferation, interferon-γ, and IL-18 levels are significantly reduced in PAR-2 knockout mice,[16,17] whereas endotoxin-stimulated macrophages show significantly greater IL-10 expression[18] and enhanced IL-4 secretion[19] 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.[20] Although PAR-2 activity is known to be upregulated in the vasculature in inflammatory conditions,[21] 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.[8] 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[8] and dominant-negative PKC (dnPKC) isozymes[22] were amplified and titered and the optimal multiplicity of infection determined by Western blotting as 50 infectious units (ifu) per cell for HO-1 and 100 ifu per cell for the dnPKC isozyme adenoviruses. HUVECs were infected overnight 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,[23] HO-1,[8] and PKC$_{\beta1}$ (sense: 5'-GGGAGAAACUUGAACGCAAtt-3'; antisense: 5'-UUGCGUUCAAGUUUCUCCCtt-3') and a universal control siRNA (Dharmacon) were introduced into HUVECs using the Amaxa Nucleofector HUVEC II kit (Amaxa) and incubated overnight before treatment.

ELISAs

The sVEGFR-1 concentration in cell supernatants was determined as described.[4] 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.[4]

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 *Bgl*II and *Hind*III sites of pGL2 (Promega). HEK293 cells, which express functional PAR-2,[24] 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$\pm$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.[20] 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 S2A, available in the online Data Supplement). To confirm that the sVEGFR-1 release was generated through PAR-2 activation, HUVECs were coincubated with 2f-LIGRLO-NH$_2$ and the PAR-2 antagonist, FSLLRY-NH$_2$,[25] which abolished both the sVEGFR-1 release and VEGFR-1 promoter activity (Figure 1C and 1D). Moreover, specificity of PAR-2-stimulated VEGFR-1 promoter activity was demonstrated in PAECs engineered to express PAR-2 (Figure S1) but not in PAECs transfected with empty vector (Figure 1E and 1F). Furthermore, PAR-2 activation induced robust activation of VEGFR-1 promoter and also increased sVEGFR-1 release from trophoblasts (Figure S2C), and PAR-2 activators did not significantly alter cellular activity, confirming that the effect on sVEGFR-1 expression was not because of an increase in endothelial cell proliferation or survival (Figure S2B).

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

Both PKC[26] and Src[27] are involved in PAR-2 signaling. Soluble VEGFR-1 release and VEGFR-1 promoter activity
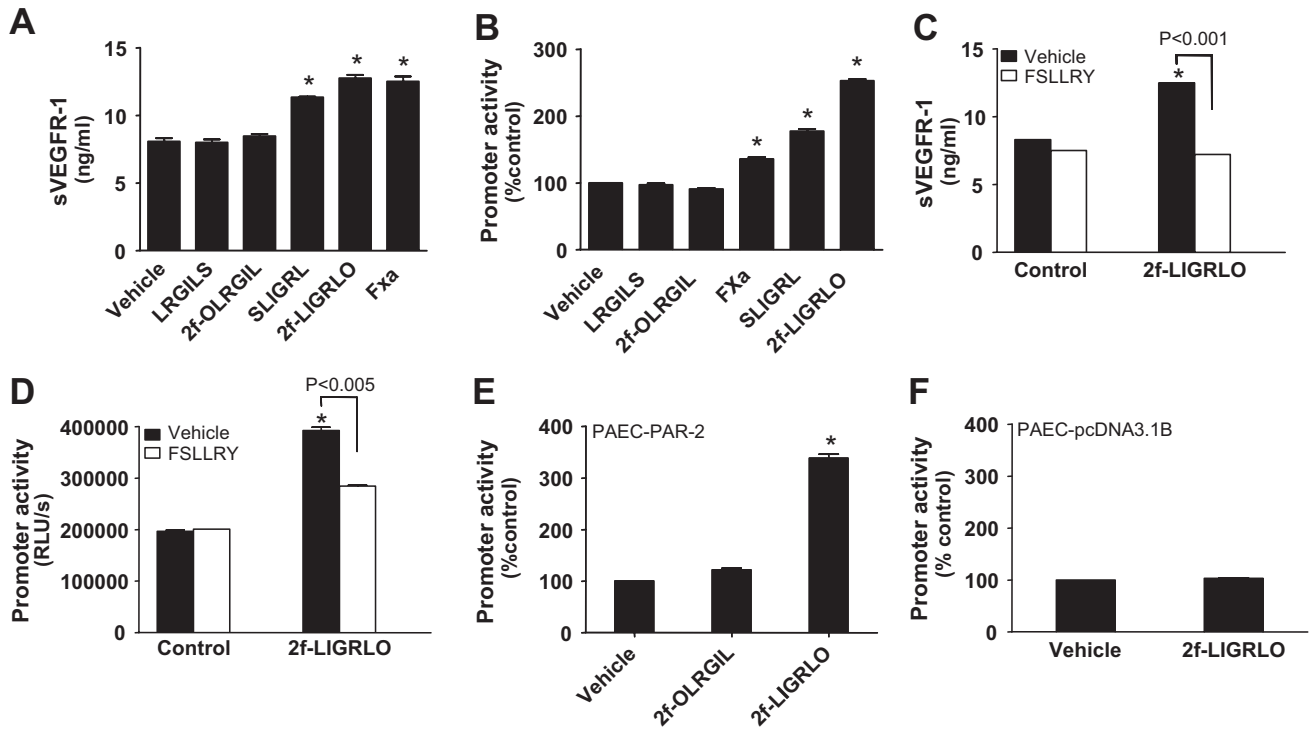
Figure 1. Selective PAR-2 activation stimulates sVEGFR-1 release in endothelial cells. A, Confluent HUVECs and (B) HEK-293 cells transfected with a ≈1.3-kb fragment of the VEGFR-1 promoter with a luciferase reporter were incubated with PAR-2 activating peptide (100 μmol/L of SLIGRL-NH$_2$ or 50 μmol/L of 2f-LIGRLO-NH$_2$) or FXa (100 nmol/L) for 24 hours, and the cell supernatants assayed for sVEGFR-1 by ELISA and VEGFR-1 promoter activity in cell lysates were determined by luciferase assay. The corresponding reverse peptides (LRGILS-NH$_2$ or 2f-OLRGIL-NH$_2$) were used as negative controls. C, HUVECs and (D) HEK-293 cells transfected with VEGFR-1 promoter were incubated for 24 hours with 2f-LIGRLO-NH$_2$ (10 μmol/L) in the presence or absence of a PAR-2 antagonist (400 μmol/L of FSLLRY-NH$_2$), and sVEGFR-1 levels in cell supernatants (C) or promoter activity (D) were determined. E, Porcine aortic endothelial cells expressing PAR-2 (PAEC-PAR-2) or (F) control cells (PAEC-pcDNA3.1B) were transfected with the VEGFR-1 promoter and stimulated for 24 hours with PAR-2 activating peptide (10 μmol/L of 2f-LIGRLO-NH$_2$) or control peptide (10 μmol/L of 2f-OLRGIL-NH$_2$), and luciferase activity in the cell lysates was determined. Results are the mean ($\pm$SEM) of 3 experiments. *$P<0.01$ (A and C), $P<0.05$ (B), and *$P<0.001$ (D and E) vs control.

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, PKCβ_1, PKCδ, and PKCϵ 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 PKCϵ, 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 MEK-1/2, 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 in HEK-293 (Figure 4B) and caused a loss of PAR-2–mediated ERK-1/2 phosphorylation (Figure 4C). Furthermore, the overexpression of dominant-negative PKCβ and PKCϵ 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 PKCϵ 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-NH$_2$–stimulated phosphorylation of Raf-1 (Figure 4G). Collectively, these data demonstrate that PAR-2 stimulates sVEGFR-1 expression and release by activat-
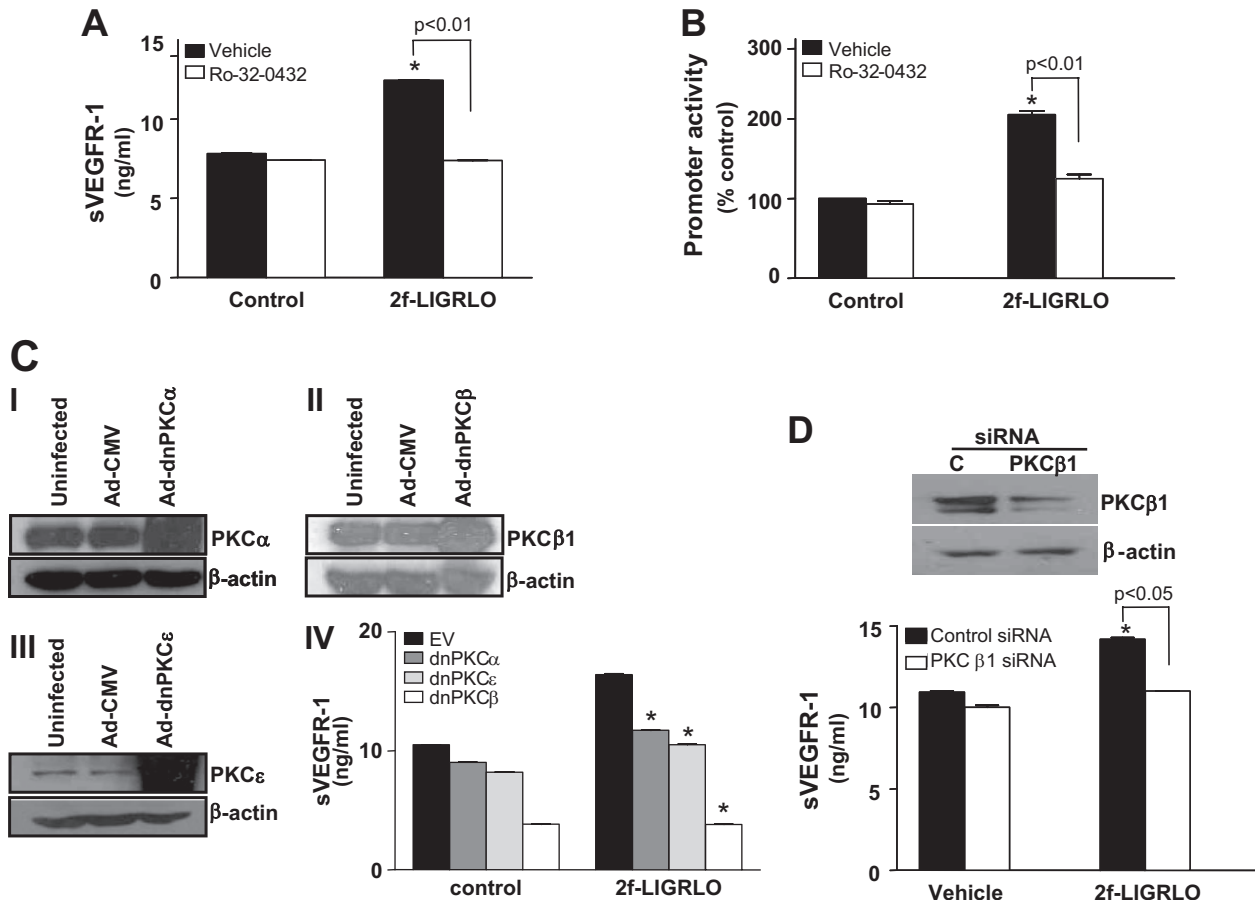
Figure 2. PAR-2–induced sVEGFR-1 release from endothelial cells depends on PKC activity. A, HUVECs and (B) HEK293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with the PKC inhibitor (Ro-32-0432; 1 μmol/L) for 45 minutes and then stimulated with PAR-2 activating peptide (10 μmol/L of 2f-LIGRLO-NH$_2$) 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 PKCα (dnPKCα), PKCβ (dnPKCβ), PKCε (dnPKCε), 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-NH$_2$) 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 ($\pm$SEM). *$P < 0.01$ (A and B vs control and C vs EV+2f-LIGRLO); *$P < 0.05$ (D vs control).

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 peptide or EGF, and EGFR phosphorylation was determined by ELISA. PAR-2 activation increased EGFR phosphorylation in HUVECs, which was inhibited by the EGFR kinase inhibitor AG1478 (3 μmol/L; Figure 5A).[34] In addition, 2f-LIGRLO-NH$_2$, FXa, and EGF induced similar levels of Raf-1^{S338} phosphorylation in endothelial cells (Figure S3C). EGFR inhibition abrogated sVEGFR-1 release and downstream ERK-1/2 phosphorylation in response to the PAR-2 agonists demonstrating the requirement of EGF transactivation for PAR-2–mediated sVEGFR-1 release (Figure 5B). Further-

more, the inhibition EGFR resulted in the loss of ERK-1/2 activity after the acute stimulation of HUVECs with either 2f-LIGRLO or FXa (Figure 5C). As anticipated, the inhibition of ERK-1/2 prevented sVEGFR-1 production in response to FXa and EGF (Figure S3D).

Src activity has been reported to act both upstream and downstream of EGFR transactivation after PAR-2 stimulation in different cell types.[27,35] PAR-2–induced EGFR phosphorylation was inhibited by the Src inhibitor PP2, indicating that Src activity is required for EGFR transactivation (Figure 5D). In addition, the PAR-2–mediated activation of Src was not inhibited by AG1478, supporting these findings (Figure 5E). Transactivation of the EGFR by PAR-2 can occur through the release of EGFR agonists, such as transforming growth factor-α or heparin-binding EGF from the cell surface through the activation of matrix metalloproteinases (MMPs).[30] To determine whether the transactivation of the EGFR by PAR-2 observed in our studies occurred via a similar extracellular route, HUVECs were preincubated with the MMP inhibitor, GM6001, or the reverse-control peptide (rGM6001; 10 μmol/L), before stimulation with
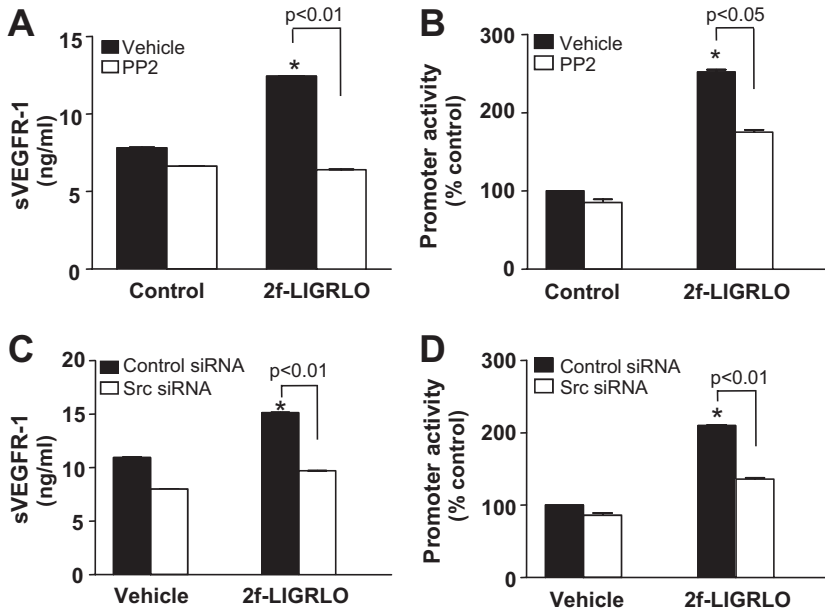
Figure 3. PAR-2–induced sVEGFR-1 production requires Src activation. A, HUVECs and (B) HEK-293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with an Src inhibitor (PP2; 10 μmol/L) for 45 minutes and then stimulated with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH$_2$) 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-NH$_2$) 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 ($\pm$SEM). *$P<0.01$ (A and D) and *$P<0.05$ (D and C) vs control.

2f-LIGRLO-NH$_2$ (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.[8] 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.[8]
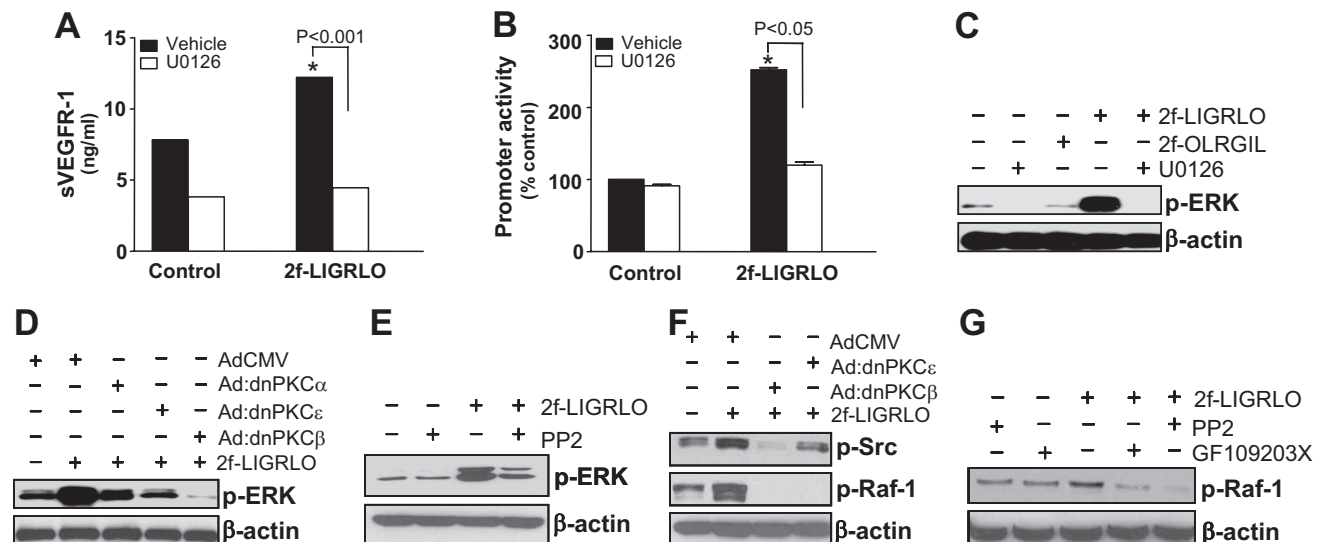
Figure 4. ERK-1/2 activation is required for PAR-2–induced sVEGFR-1 release. A, HUVECs and (B) HEK293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with an MEK-1/2 inhibitor (U0126; 10 μmol/L) for 45 minutes, stimulated with PAR-2–activating peptide (2f-LIGRLO-NH$_2$; 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 phosphor–ERK-1/2 (p-ERK), phosphor-Src (pY416Src), and phospho–Raf-1 (pS338Raf-1); HUVECs were either pre-incubated 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-NH$_2$; 10 μmol/L) for 10 minutes. *$P<0.01$ (A) and *$P<0.05$ (B) vs control.
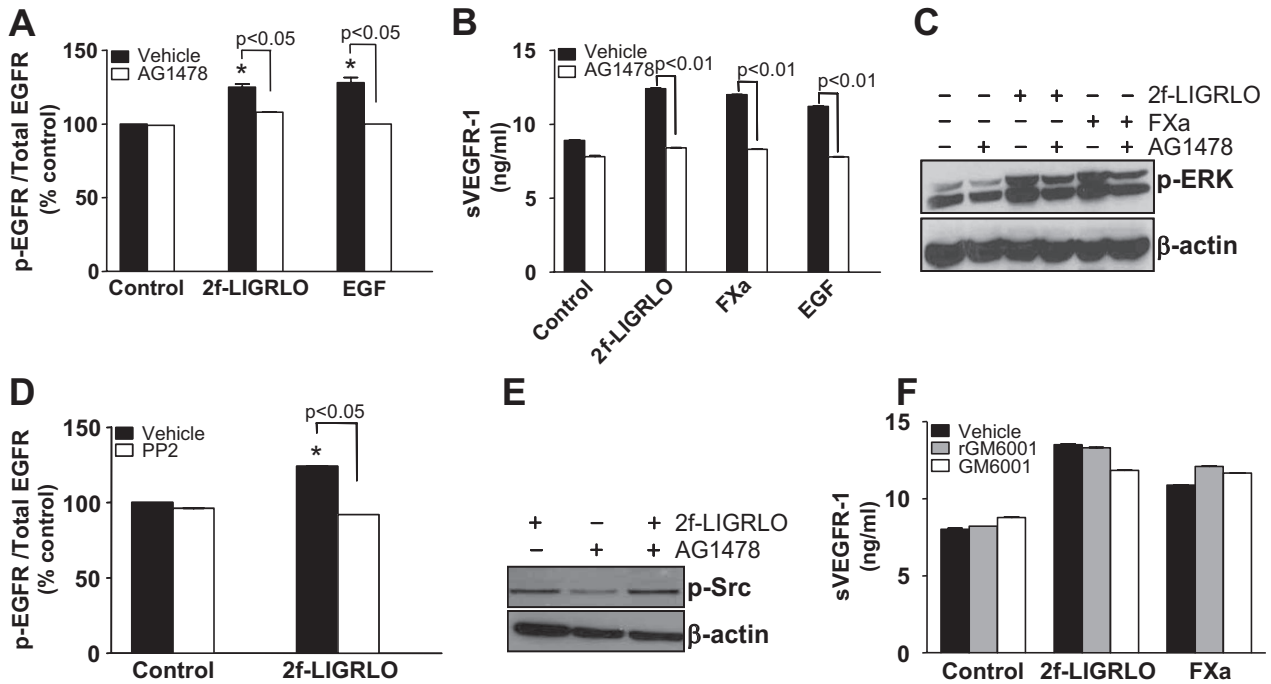
Figure 5. EGFR transactivation is required for PAR-2–induced sVEGFR-1 release. A, HUVECs were pretreated for 45 minutes with AG1478 (3 μmol/L), stimulated for 10 minutes with PAR-2–activating peptides (2f-LIGRLO-NH$_2$; 10 μmol/L) or EGF (50 ng/mL), and the level of phosphorylated and total EGFRs were determined in cell lysates (100 μg per well) by ELISA (R&D Systems). B, HUVECs were pretreated with AG1478 and stimulated for 24 hours with PAR-2–activating peptide (2f-LIGRLO-NH$_2$), FXa (200 nmol/L), or EGF (50 ng/mL), and sVEGFR-1 was assayed in the cell culture supernatants by ELISA. C, After pretreatment with the EGFR inhibitor (AG1478), HUVECs were incubated with 2f-LIGRLO-NH$_2$ and FXa (200 nmol/L) for 10 minutes, and cell lysates (30 μg per lane) were subjected to Western blotting for phosphor-ERK1/2 (p-ERK) and β-actin as a loading control. D, HUVECs were incubated with the Src inhibitor PP2 for 45 minutes and then stimulated for 10 minutes with 2f-LIGRLO-NH$_2$, and the levels of EGFR and phosphorylated EGFR were determined in cell lysates (100 μg per well) by ELISA. E, HUVECs were treated with AG1478 before incubation with 2f-LIGRLO-NH$_2$ for 10 minutes, and cell lysates were Western blotted for phosphorylated-Src (p-Src). F, HUVECs were preincubated with the metalloproteinase inhibitor (GM6001; 10 μmol/L) or control (rGM6001; 10 μmol/L) for 45 minutes before stimulation with 2f-LIGRLO-NH$_2$ or FXa (200 nmol/L), and sVEGFR-1 was assayed in cell supernatants by ELISA. Results represent the mean ($\pm$SEM); n=3. *$P<0.05$ (A and D) vs control.

Discussion

This study shows that receptor-selective PAR-2 activation induces VEGFR-1 promoter activity and sVEGFR-1 release from endothelial cells through the sequential activation of PKC, Src, Raf-1, and ERK-1/2 and depends on EGFR transactivation (Figure 7). Furthermore, it demonstrates that upregulation of HO-1 with Simvastatin or overexpression of HO-1 or CO suppresses PAR-2–mediated sVEGFR-1 release and supports our earlier study showing that the HO-1/CO pathway inhibits cytokine-induced sVEGFR-1 release.[8]

The transactivation of the EGFR by G-coupled protein receptors, including PAR-1, PAR-2, and PAR-4, is well established.[29,30,36] 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[35] and a similar mechanism in cardiomyocytes after PAR-4 stimulation.[36] The inhibition of either the Src or EGFR did not completely block PAR-2–stimulated ERK-1/2 phosphoryla-

tion, 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 EGF by MMP such as the TNF-α–converting enzyme.[29,30] PAR transactivation of EGFR was reported to occur in an MMP-independent manner, requiring Src activation in cardiac fibroblasts[36] and intestinal epithelial cells.[35] 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.[37] Angiotensin II type 1 receptor density increases in preeclamptic placentas,[38] and angiotensin II type 1 activating autoantibodies induce a preeclampsia-like condition in mice.[39] Given the ability of angiotensin II to transactivate the EGFR[31] 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.
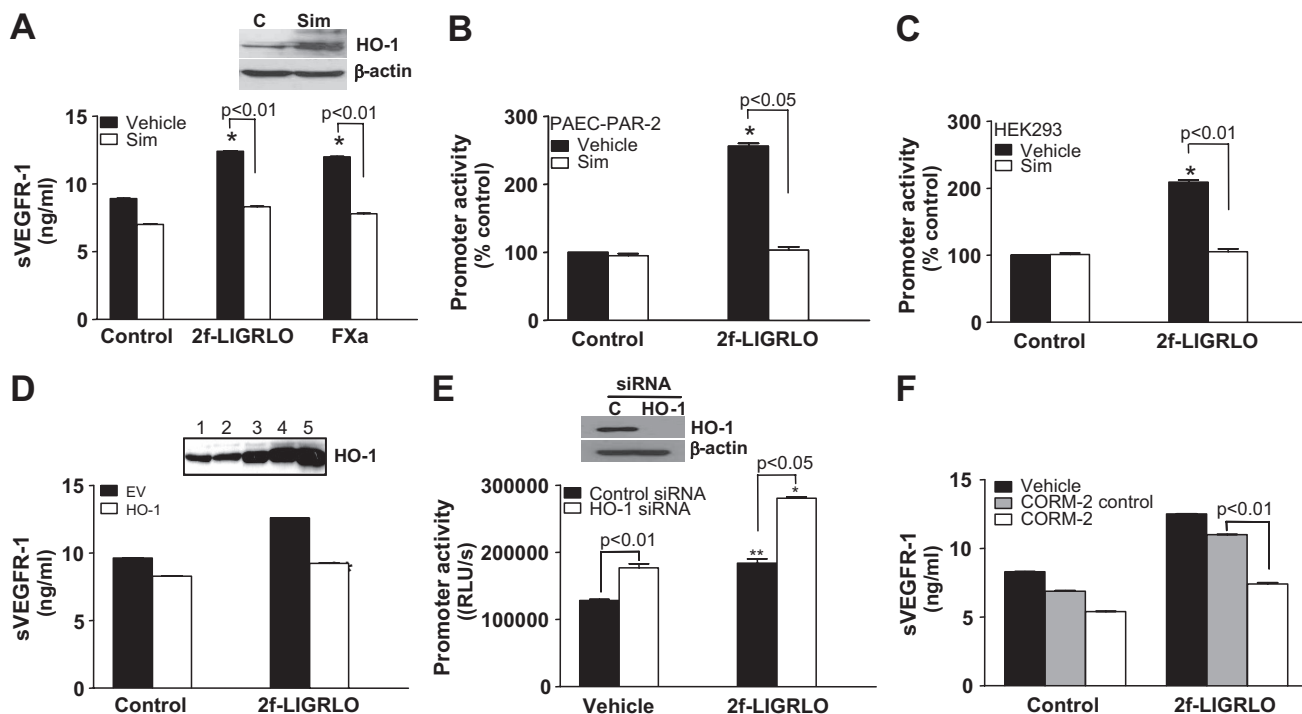
Figure 6. PAR-2-induced sVEGFR-1 release is inhibited by statins, HO-1, and CO. A, HUVECs were stimulated with PAR-2–activating peptide (2f-LIGRLO-NH$_2$; 10 μmol/L) or FXa (200 nmol/L) in the presence or absence of Simvastatin (10 μmol/L) for 24 hours, and sVEGFR-1 was quantified in cell supernatants by ELISA. HO-1 upregulation in HUVECs incubated with Simvastatin (10 μmol/L) for 24 hours was confirmed by Western blotting (inset). B, PAEC-PAR-2 and (C) HEK-293 cells were transfected with the VEGFR-1 promoter-reporter construct, stimulated with PAR-2–activating peptide (2f-LIGRLO-NH$_2$) for 24 hours in the presence or absence of Simvastatin (10 μmol/L), and luciferase activity was determined in cell lysates. D, HUVECs were then infected with 50 ifu per cell of HO-1 or control (EV) adenovirus for 48 hours before stimulation with 2f-LIGRLO-NH$_2$ for 24 hours (*$P<0.001$ vs control). HUVECs infected with low (5 ifu per cell; lanes 2 and 4) or high (50 ifu per cell; lanes 3 and 5) concentrations of HO-1 adenovirus for 24 (lanes 2 and 3) or 48 (lanes 4 and 5) hours or uninfected control cells (lane 1) were immunoblotted for HO-1 (inset). E, HEK293 cells containing the VEGFR-1 promoter-reporter construct were electroporated with HO-1 siRNA and incubated overnight. After stimulation with 2f-LIGRLO-NH$_2$ (10 μmol/L) for 24 hours, luciferase activity was determined in cell lysates. The medium was then collected and assayed for sVEGFR-1 by ELISA. F, HUVECs were treated with 50 μmol/L of CORM-2 or CORM-2 control for 45 minutes and then stimulated for 24 hours with 2f-LIGRLO-NH$_2$. Results represents mean ($\pm$SEM); n=3. A through C, *$P<0.01$ vs control; D, *$P<0.001$ vs control; **$P<0.001$ vs EV+2f-LIGRLO; E, *$P<0.05$, *$P<0.001$, and **$P<0.05$ vs control.

PAR-2 inhibition can suppress TNF-α expression in inflammatory settings[40] and improve wound healing in mice by reducing inflammation.[41] 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,[20] 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[20] and placenta[42] 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.[43] HO-1 activity is essential for the successful outcome of pregnancy,[44] 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,[45] 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 know as sFlt-1) appears to be "the final common pathway"
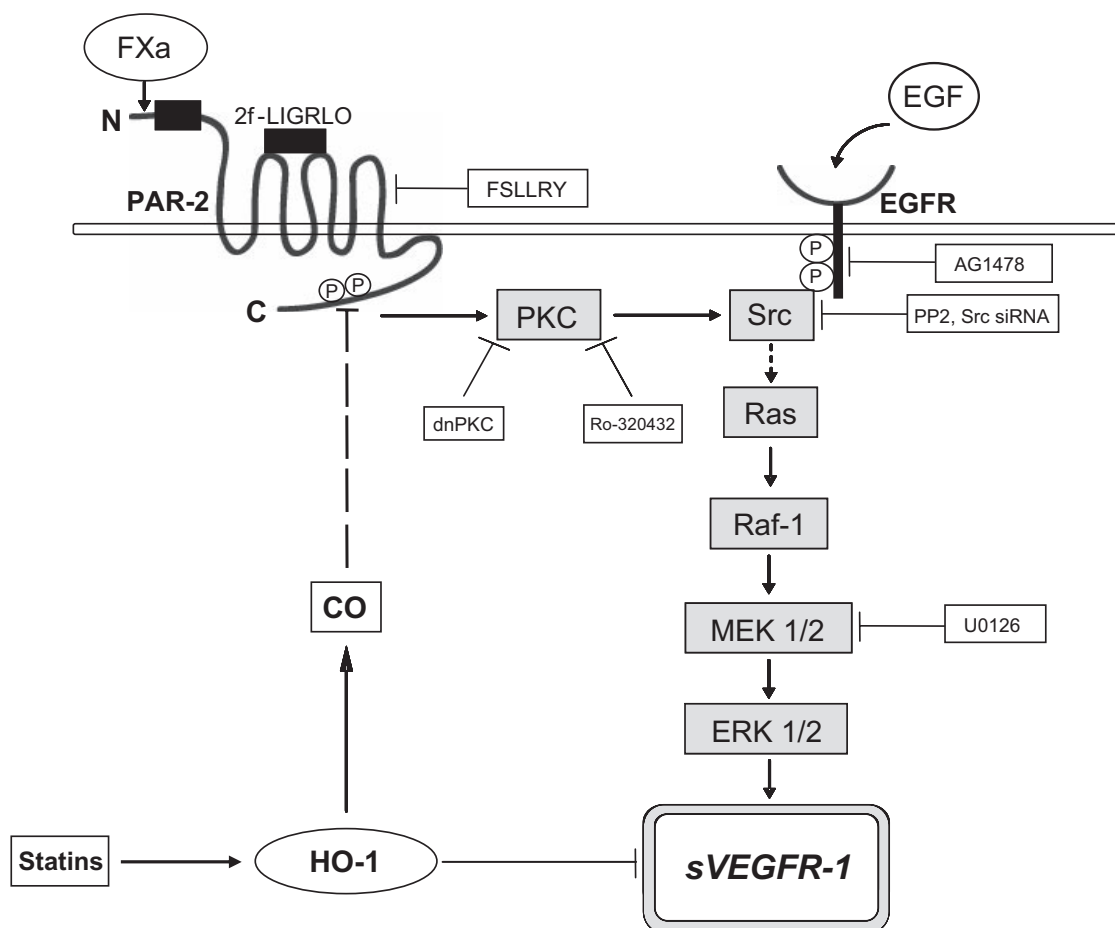
Figure 7. Proposed model for PAR-2–induced sVEGFR-1 release via transactivation of the EGFR. The proposed PAR-2 signaling pathway places the activation of PKC upstream of Src-family enzymes and the EGFR, which leads to the activation of the MAP kinase pathway, including Ras, Raf-1, and ERK-1/2 expression and release of sVEGFR-1. The PAR-2 activator FXa and synthetic PAR-2–activating peptide (2f-LIGRLO), along with the specific PAR-2 inhibitor (FSLLRY) and the various points of inhibition, are indicated. HO-1 and CO negatively regulate sVEGFR-1 production in response to PAR-2 activation, and statins upregulate HO-1.

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[43] 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.

Disclosures

None.

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ONLINE SUPPLEMENT

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* (Brauschweig, 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, dnPKCβ_1, 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-Src[3], HO-1[4] and PKC$_{\beta 1}$ (*sense* 5′-GGGAGAAACUUGAACGCAAtt-3′; *antisense* 5′-UUGCGUUCAAGUUUCUCCCtt-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 previously[5] 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.[6] *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 [6]. 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 [6] 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.[5] EGFR was measured in cell lyates 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 lyates 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-1*luc*) 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[7] 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.[8] Following stimulation for 24 hours, luciferase activity was determined in cell lysates using the Dual Luciferase Assay (Promega) as described previously.[8]

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-NH$_2$ (Figure S1B). In addition, PAR-2 desensitisation was achieved after a second challenge with SLIGRL-NH$_2$ at 10 minutes. No PAR-2 staining, or calcium mobilisation in response to SLIGRL-NH$_2$, 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-NH$_2$ 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.[9] 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. [10] More recently EGFR-1 has been detected in HUVEC and other types of endothelial cells. [11, 12] 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.

5

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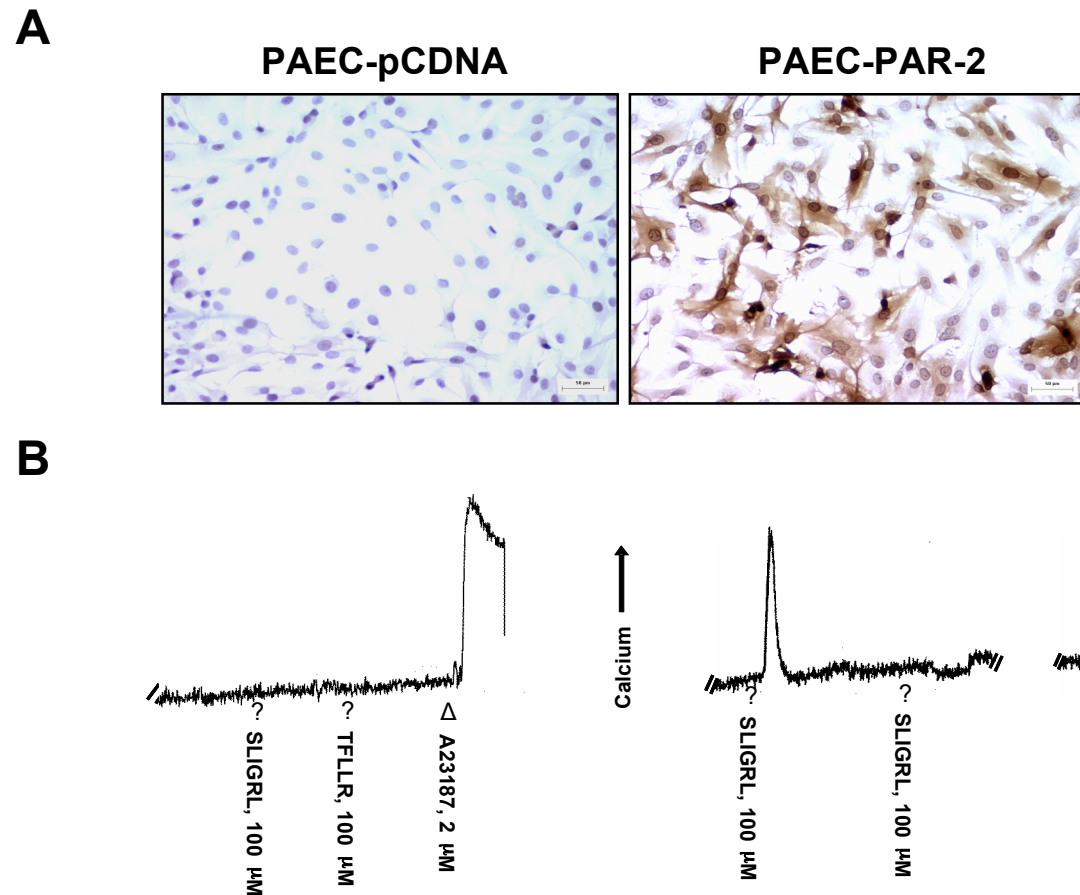
7

A

PAEC-pCDNA   **PAEC-PAR-2**

B

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-NH$_2$ (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.
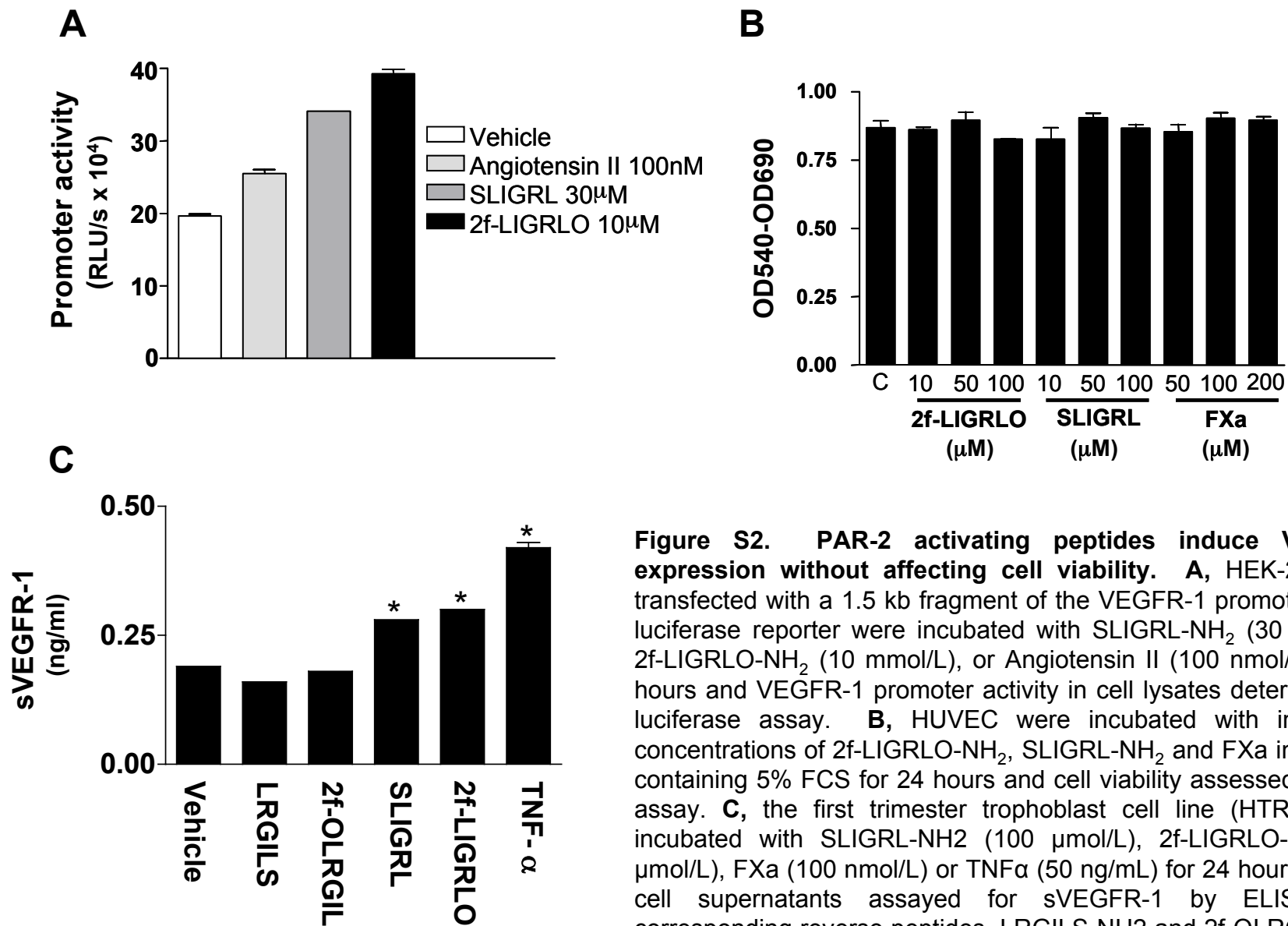
Figure S2

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 mmol/L), 2f-LIGRLO-NH$_2$ (10 mmol/L), or Angiotensin II (100 nmol/L) 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-NH2 (100 µmol/L), 2f-LIGRLO-NH2 (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-NH2 and 2f-OLRGIL-NH2, and TNFα were used as negative and positive controls respectively.
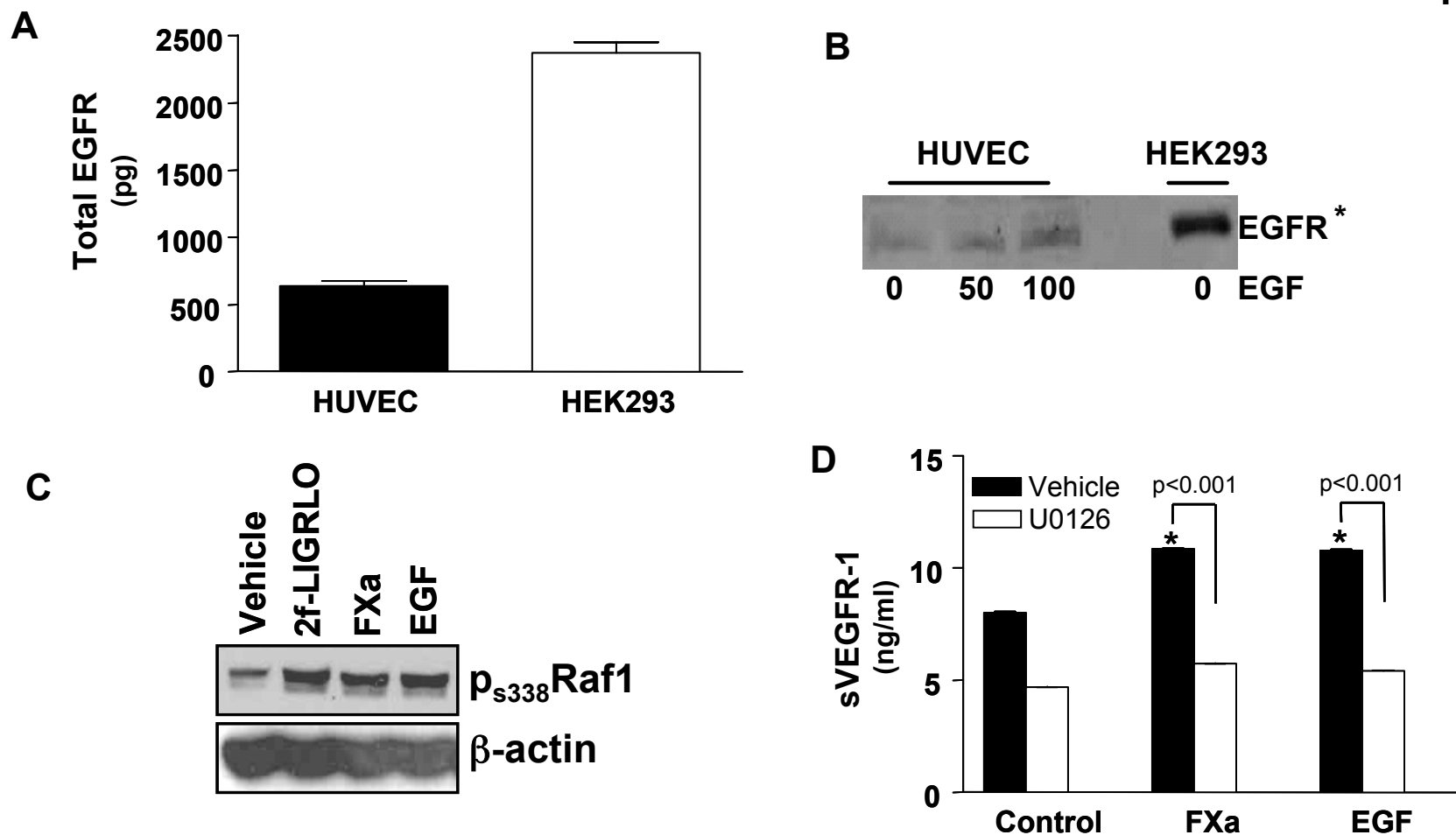
Figure S3. HUVEC express functional EGF receptors and their activation results in sVEGFR-1 release. A, The presence of EGFR was detected in both HUVEC and HEK-293 cell lysates (100 μg/well) by ELISA (R&D Systems). **B,** HUVEC were incubated with increasing concentrations of EGF for 10 minutes and cell 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-NH$_2$ (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.