Synergistic Induction of Mitogen-Activated Protein Kinase Phosphatase-1 by Thrombin and Epidermal Growth Factor Requires Vascular Endothelial Growth Factor Receptor-2

Unni M. Chandrasekharan, Matthew Waitkus, Corttrell M. Kinney, Alicia Walters-Stewart, Paul E. DiCorleto

Objective—To determine the molecular mechanism underlying the synergistic response of mitogen-activated protein kinase phosphatase-1 (MKP-1), which is induced by thrombin and epidermal growth factor (EGF).

Methods and Results—MKP-1 induction by thrombin (approximately 6-fold) was synergistically increased (approximately 18-fold) by cotreatment with EGF in cultured endothelial cells. EGF alone did not induce MKP-1 substantially (<2-fold). The synergistic induction of MKP-1 was not mediated by matrix metalloproteinases. The EGF receptor kinase inhibitor AG1478 blocked approximately 70% of MKP-1 induction by thrombin plus EGF (from 18- to 6-fold) but not the response to thrombin alone. An extracellular signal–regulated kinase (ERK)–dependent protease-activated receptor-1 (PAR-1) signal was required for the thrombin alone effect; an ERK-independent PAR-1 signal was necessary for the approximately 12-fold MKP-1 induction by thrombin plus EGF. VEGF induction of MKP-1 was also approximately 12-fold and c-Jun N-terminal kinase (JNK) dependent. Inhibitors of extracellular signal–regulated kinase and JNK activation blocked thrombin plus EGF-induced MKP-1 completely. Furthermore, VEGF receptor 2 depletion blocked the synergistic response without affecting the induction of MKP-1 by thrombin alone.

Conclusion—We have identified a novel signaling interaction between protease-activated receptor-1 and EGF receptor that is mediated by VEGF receptor 2 and results in synergistic MKP-1 induction. (Arterioscler Thromb Vasc Biol. 2010;30:1983-1989.)

Key Words: endothelium signal transduction thrombin vascular biology

It was previously shown that mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) is a key signaling mediator in thrombin- and vascular endothelial cell (EC) growth factor (VEGF)–mediated activation of ECs.1,2 Furthermore, MKP-1 plays a positive role in atherogenesis in a mouse model.3 MKP-1 inactivates MAP kinases by the dephosphorylation of both threonine and tyrosine residues,4 but other substrates also exist. Recently, it was shown that MKP-1 dephosphorylates serine 10 of histone H3.5 MKP-1 induction by thrombin (approximately 6-fold) was synergistically increased (approximately 18-fold) by cotreatment with EGF in cultured endothelial cells. EGF alone did not induce MKP-1 substantially (<2-fold). The synergistic induction of MKP-1 was not mediated by matrix metalloproteinases. The EGF receptor kinase inhibitor AG1478 blocked approximately 70% of MKP-1 induction by thrombin plus EGF (from 18- to 6-fold) but not the response to thrombin alone. An extracellular signal–regulated kinase (ERK)–dependent protease-activated receptor-1 (PAR-1) signal was required for the thrombin alone effect; an ERK-independent PAR-1 signal was necessary for the approximately 12-fold MKP-1 induction by thrombin plus EGF. VEGF induction of MKP-1 was also approximately 12-fold and c-Jun N-terminal kinase (JNK) dependent. Inhibitors of extracellular signal–regulated kinase and JNK activation blocked thrombin plus EGF-induced MKP-1 completely. Furthermore, VEGF receptor 2 depletion blocked the synergistic response without affecting the induction of MKP-1 by thrombin alone.

Conclusion—We have identified a novel signaling interaction between protease-activated receptor-1 and EGF receptor that is mediated by VEGF receptor 2 and results in synergistic MKP-1 induction. (Arterioscler Thromb Vasc Biol. 2010;30:1983-1989.)

Key Words: endothelium signal transduction thrombin vascular biology

G protein coupled receptors (GPCRs) and receptor tyrosine kinases, such as EGFRs, have been involved in the progression of a variety of diseases.14,15 EC migration is an early event in inflammatory angiogenesis and normal vasculogenesis. Recently, it was demonstrated that MKP-1 is a key signaling mediator in VEGF-induced EC migration.2 Herein, we extend the previous study and demonstrate, for the first time to our knowledge, that thrombin or lysophosphatidic acid (LPA) induction of MKP-1 is synergistically increased by EGF in ECs. We have elucidated the signaling mechanism responsible for the synergy and demonstrated that VEGF receptor (VEGFR)-2 activity is critical for the synergistic induction of MKP-1 by thrombin and EGF.

Methods

An expanded “Methods” section is available in the supplemental data (available online at http://atvb.ahajournals.org.). The methods for EC isolation and culture, Northern blot analysis, Real-Time PCR, and RNA interference have been previously published.2,16
A real-time PCR assay was performed to determine pre-mRNA of MKP-1. The MKP-1–specific primers included (1) an intron-specific forward primer (5′-AGT ACA TTT ATC TCT GGA AC-3′) and (2) an exon-specific reverse primer (5′-CGT AGA GTG GGG TAC TGC AG-3′). Metalloproteinase inhibitor BB3103 was also used in the present study.

Results

Synergistic Induction of MKP-1 by Thrombin Plus EGF

It was previously shown that thrombin induces MKP-1 in ECs via protease-activated receptor-1 (PAR-1), the predominant thrombin receptor present in human ECs. Thrombin–mediated cellular events have been reported to be due to transactivation of EGFR. We reasoned that if the induction of MKP-1 by thrombin was mediated by EGFR transactivation, then EGF alone might also induce MKP-1 in ECs. To test this hypothesis, we treated ECs with EGF in the presence or absence of thrombin and measured MKP-1 mRNA by Northern blot analysis. As shown in Figure 1A, EGF treatment, 16 ng/mL, resulted in only a small fraction of the thrombin response; however, we observed a robust MKP-1 induction when ECs were treated with thrombin and EGF in combination. We tested EGF at various concentrations, ranging from 1 to 100 ng/mL; and the MKP-1 expression reached a maximum response at 16 ng/mL (approximately 2-fold; data not shown). This result excluded the possibility that the minimal effect of EGF in MKP-1 induction in ECs was due to a suboptimal EGF concentration. As shown in Figure 1B, thrombin or EGF treatment resulted in an approximate 4- or 2-fold increase, respectively, in the MKP-1 message; thrombin plus EGF increased the MKP-1 message synergistically (approximately 12-fold). The kinetics of the response to the combined agonists were similar to those of either agonist alone (Figure 1C).

Figure 1. Synergism in MKP-1 induction by thrombin (Thro) plus EGF and kinetics of ERK activation. A, Confluent cultures of umbilical vein ECs were serum starved for 2 hours before thrombin, 5 U/mL; EGF, 16 ng/mL; or thrombin plus EGF at 37°C for 1 hour. Cells were lysed, and 10 μg of total RNA was subjected to Northern blot analysis using MKP-1 or ribosomal protein L-32 (RPL-32, loading control) probes. B, Quantitative analysis of 3 independent experiments performed as described in A. C, The kinetics of MKP-1 mRNA induction by TRAP-1, EGF, or TRAP-1 plus EGF were determined by quantitative real-time PCR. The fold induction was quantified relative to the untreated condition. D, Kinetics of ERK activation. Western blotting was performed either with phosphorylated ERK1/2 (pERK)-, ERK-, or GAPDH-specific antibodies. E, Quantification of the ERK activation from 3 separate immune blots, normalized to the GAPDH and expressed as fold induction compared with the untreated condition.

We also examined MKP-1 protein levels under these conditions. Because the commercial antibodies to MKP-1 were not sensitive enough to detect MKP-1 by either Western analysis or immunoprecipitation in human ECs, we used mouse aortic ECs. MKP-1 protein was increased by approximately 8.0-fold by thrombin plus EGF (supplementary Figure I). Similar to thrombin, agents such as LPA, lysophosphatidylcholine (LPC), and endothelin-1 are physiologically important agonists that elicit their biological effects via activation of their respective GPCRs. LPA, but not LPC or endothelin-1, increased MKP-1 gene induction (supplemental Figure II). Furthermore, LPA plus EGF showed synergy in MKP-1 induction, similar to thrombin receptor-activating peptide (TRAP)-1 plus EGF.

Then, we determined the effect of TRAP-1 alone or in combination with EGF on ERK activation (Figure 1D and E). TRAP-1 induced a rapid, but moderate, activation of ERK. EGF treatment resulted in a robust activation of ERK within 5 minutes, peaking at 20 minutes (approximately 3.7-fold) and persisting (>2-fold) for up to 180 minutes. The combination of TRAP-1 and EGF caused more rapid ERK dephos-
MKP-1 Induction by Thrombin Plus EGF Is PAR-1 Dependent, EGFR Kinase Activity Dependent, and Matrix Metalloproteinase Independent

The synergistic induction of MKP-1 by PAR-1–specific agonistic peptide (TRAP-1 or SFLLRN) and EGF is shown in Figure 2A. Peptides specific to PAR-4 (AYPGKF) or PAR-2 (SLIGKV) independently or in combination with EGF failed to induce MKP-1 induction (data not shown). AG1478, a specific EGFR kinase inhibitor, completely blocked EGF-induced MKP-1; it partially blocked the TRAP-1 plus EGF response. Furthermore, AG1478 did not inhibit TRAP-1–induced MKP-1 (Figure 2B). Quantification of multiple Northern blot analyses revealed that MKP-1 mRNA levels in the EGF plus TRAP-1–treated ECs in the presence of AG1478 were equivalent to MKP-1 mRNA in the presence of TRAP-1 alone (Figure 2C). In the presence of AG1478, the TRAP-1 plus EGF–induced MKP-1 mRNA level (approximately 12-fold) was reduced to approximately 3.5-fold; the latter was equivalent to TRAP-1 alone–mediated MKP-1 induction. These results suggest that the synergy in MKP-1 induction by thrombin plus EGF is the result of convergence of 2 independent signaling pathways. The combination of an AG1478-insensitive PAR-1 signal and an AG1478-sensitive EGFR pathway resulted in the synergistic induction of MKP-1.

GPCR-mediated matrix metalloproteinase activation releases a membrane-bound EGF family of ligands that, in turn, activate the EGFR.13 To determine whether matrix metalloproteinases are involved in the synergistic induction of MKP-1, we treated ECs with TRAP-1 plus EGF in the presence of BB3103 (British Biotech Pharmaceuticals, Oxford, UK), a specific inhibitor of matrix metalloproteinases and determined MKP-1 mRNA level by Northern blot analysis. As shown in Figure 2D, BB3103 pretreatment did not block MKP-1 induction by TRAP-1 or EGF individually or in combination. We verified the inhibitory activity of BB3103 independently by inhibiting matrix metalloproteinase enzyme activity in thrombin-treated cell extracts using in-gel collagen degradation (data not shown). These results suggest that an intracellular link between the PAR-1 and EGFR signaling pathways is responsible for thrombin plus EGF-mediated synergy in MKP-1 induction.

Two Distinctive PAR-1 Pathways Are Required for Synergy in MKP-1 Induction by Thrombin Plus EGF

PAR-1 and EGFR are known to activate Src family kinases and downstream MAP kinases.2,17 It was previously demonstrated that the Src family of kinases and MAP kinases are critical for thrombin and VEGF induction of MKP-1 in ECs.1,2 The inhibitor of the Src family of kinases, PP1, blocked both TRAP-1 and TRAP-1 plus EGF–induced MKP-1 in ECs (Figure 3A). Then, we determined the involvement of specific Src kinase family members in the synergistic induction of MKP-1. As shown in Figure 3B, the depletion of c-Src considerably diminished the synergy portion of the induction, without blocking TRAP-1–induced MKP-1; depletion of Fyn significantly reduced both...
TRAP-1– and TRAP-1 plus EGF–mediated MKP-1 induction. ERK inhibitor PD98059 prevented TRAP-1 induction of MKP-1; it only partially inhibited TRAP-1 plus EGF–induced MKP-1, and it did not at all inhibit the induction of MKP-1 by EGF (Figure 3C). In the TRAP-1 plus EGF group, the reduction in the amount of MKP-1 mRNA by PD98059 (an approximate 12-fold induction was reduced to an approximate 9-fold induction in the presence of PD98059) was similar to the level of MKP-1 mRNA induced by TRAP-1 alone (approximately 3.5-fold) (Figure 3D). These results suggest that 2 distinct signals (1 ERK dependent and 1 ERK independent) from the PAR-1 receptor were required for the synergy in MKP-1 induction.

Synergy in MKP-1 Induction by Thrombin Plus EGF Is at the Transcriptional Level

It was shown earlier that thrombin induction of MKP-1 is at the transcriptional level. The synergistic induction of MKP-1 mRNA in the presence of EGF could be due to mRNA stabilization or increased transcription. We tested for a change in mRNA stability by treating ECs with vehicle, TRAP-1, EGF, or TRAP-1 plus EGF for 1 hour, followed by treatment with actinomycin D. As shown in Figure 4A, compared with the control condition, TRAP-1, EGF, or TRAP-1 plus EGF caused no significant change in the half-life of MKP-1 mRNA. In a complementary approach, we measured the unspliced form of the MKP-1 transcript (MKP-1 premRNA) by quantitative real-time PCR using an intron-specific forward primer and an exon-specific reverse primer. At 1 hour, TRAP-1 and EGF upregulated MKP-1 premRNA by approximately 5- and 3-fold, respectively; TRAP-1 plus EGF showed an approximate 12-fold increase in MKP-1 pre-RNA (Figure 4B). This relative increase in the abundance of premRNA by TRAP-1 plus EGF points to an increase in the transcription rate.

Synergistic Induction of MKP-1 by Thrombin Plus EGF Requires VEGFR-2

It was previously reported that VEGF induces MKP-1 more effectively than thrombin in ECs. Herein, we determined the effect of VEGF on TRAP-1, LPA, and/or EGF on MKP-1 induction. We identified an additive effect in MKP-1 induction when cells were treated with TRAP-1 or LPA in the presence of VEGF (Figure 5A). TRAP-1 or LPA induction of MKP-1 was approximately 5-fold, and VEGF induction alone was approximately 12-fold. Thrombin or LPA plus VEGF induction was approximately 17-fold; furthermore, this additive effect was similar in quantity to the synergistic effect induced by TRAP-1 plus EGF (17-fold). VEGF did not augment TRAP-1 plus EGF induction of MKP-1. TRAP-1 plus LPA induction was similar to either of them alone, suggesting overlapping signaling pathways from the thrombin and LPA receptors in MKP-1 induction.

JNK, not ERK, activation was critical for VEGF induction of MKP-1. Because VEGF induction was similar to ERK-

**Figure 3.** The Src family of kinases and an ERK-dependent and a second ERK-independent signal from the PAR-1 are required for thrombin plus EGF-mediated synergy in MKP-1 induction. A, ECs were pretreated with the Src kinase inhibitor PP1, 10 μmol/L, for 30 minutes before TRAP-1 or EGF treatment; Northern blot analysis was performed using the MKP-1 probe. B, Fold change of MKP-1 mRNA (quantified by quantitative [Q]-PCR) in the presence of c-Src– or Fyn-specific small interfering RNA (siRNA). The efficiency of the siRNAs is shown by Western blot using C-Src– or Fyn-specific antibodies. C, The effect of ERK activity on MKP-1 induction measured by Northern blot analysis. D, Quantitative analysis of 3 independent experiments performed as described in C and normalized with the RPL-32 RNA signal.

**Figure 4.** Thrombin plus EGF did not stabilize MKP-1 mRNA but increased the MKP-1 premRNA abundance. A, ECs treated with vehicle, TRAP-1, EGF, or TRAP-1 plus EGF for 1 hour and then treated with actinomycin D, 10 μg/mL, for an additional 3 hours; the MKP-1 mRNA level was measured after Northern blot analysis. B, Relative MKP-1 premRNA abundance was determined by real-time PCR, as described in the “Methods” section.
independent thrombin plus EGF-mediated MKP-1 induction (approximately 12-fold), we next determined the role of JNK in thrombin plus EGF–induced MKP-1. As shown in Figure 5B, a JNK inhibitor blocked most of the observed synergy, and the uninhibited portion was equivalent to ERK-dependent thrombin induction. Simultaneous inhibition of both the ERK and JNK pathways blocked the synergistic effect almost completely. In a corollary experiment, TRAP-1 plus VEGF–induced MKP-1 was blocked completely by ERK and JNK inhibitors. ERK or JNK inhibitors brought the level of MKP-1 induction down to the thrombin or VEGF alone levels, respectively.

By using specific neutralizing antibodies to various VEGFRs, it was previously demonstrated that VEGFR-2 is responsible for VEGF-induced MKP-1 induction. Therefore, we next tested the role of VEGFR-2, if any, in MKP-1 induction by thrombin plus EGF. The synergistic induction of MKP-1 by TRAP-1 plus EGF was substantially reduced in the VEGFR-2–depleted cells, using VEGFR-2–specific small interfering RNA (Figure 5C). VEGFR-2 depletion did not alter MKP-1 induction by TRAP-1 alone. Furthermore, the VEGFR-2 kinase inhibitor ZM323881 showed similar results because it blocked the synergistic MKP-1 induction but not the TRAP-1 alone induction (Figure 5D). These results indicate that VEGFR activation is one of the downstream events to our identified ERK-independent pathway. Previous studies in other cell types have demonstrated VEGF gene expression by EGF and that the secreted VEGF activated VEGFR in an autocrine fashion. It was previously shown that, among the various VEGF isotypes, only VEGF-A induces MKP-1. In Figure 5E, a neutralizing antibody specific to VEGF-A did not block thrombin plus EGF-induced MKP-1, suggesting that the mechanism of VEGFR activation is not due to secreted VEGF-A and the subsequent autocrine activation of VEGFR. We failed to communoprecipitate EGFR and VEGFR-2 in the presence or absence of agonists, although we believe our conditions were appropriate because we successfully communoprecipitated a different heterologous membrane receptor pair (EGFR and human epidermal growth factor receptor [HER]-2) (data not shown). These results suggest that the synergistic induction of MKP-1 was not the result of EGFR and VEGFR-2 heterodimerization.

Figure 5. Synergistic induction of MKP-1 by thrombin (Thro) plus EGF requires VEGFR-2. A and B, ECs were treated with TRAP-1; EGF; LPA, 50 μmol/L; or VEGF-A165, 10 ng/mL, alone or in combinations without (A) or with (B) inhibitors of ERK or JNK activation. C, ECs were transfected with VEGFR-2–specific small interfering RNA (siRNA) or scrambled siRNA (control siRNA); 36 hours later, cells were treated with agonists. The inset shows the efficacy of VEGFR-2 depletion by a VEGFR-2–specific siRNA immunoblot. D, ECs were pretreated with VEGFR-2 inhibitor ZM323881 (Tocris Bioscience, St. Louis, Mo). E, ECs were pretreated with control goat IgG or anti-VEGF antibody, 0.2 μg/mL, 30 minutes before agonist treatment, and MKP-1 mRNA was measured by real-time PCR.
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MKP-1 and VEGF-induced EC Migration and Angiogenesis

Recent reports describe the induction of MKP-1 by thrombin or VEGF by distinct pathways and the role of this dual-specificity phosphatase in EC gene expression and migration. Herein, we report that thrombin or LPA treatment with EGF resulted in the synergistic induction of MKP-1 in ECs. This strong induction of MKP-1 has significant implications in vascular homeostasis and diseases because MKP-1 and agonists such as thrombin, LPA, VEGF, or EGF and their cognate receptors are known to be present in the vessel wall during development and under various injury or chronic disease conditions, such as atherosclerosis.

The receptors for thrombin, LPA, LPC, and endothelin-1 are GPCRs and have induced both common and distinct signaling pathways and elicit sometimes similar and sometimes distinctive biological end points. Herein, we demonstrated that MKP-1 induction was restricted to thrombin and LPA and not to LPC or endothelin-1. Furthermore, signaling pathways to induce MKP-1 by thrombin or LPA and either 1 of them with EGF were similar (supplemental Table II), suggesting that MKP-1 is a prototypical example of common signaling end points of the thrombin and LPA receptors.

Two mechanisms have been hypothesized to explain the signaling cross-talk between GPCRs and receptor tyrosine kinases. Prenzel et al showed evidence that GPCR activation led to metalloproteinase-mediated EGF ligand release from the EC surface, leading to activation of EGFR in an autocrine or paracrine manner. Alternatively, on GPCR activation, the Src family of proteins or G-protein subunits may physically interact with the cytosolic domain of EGFR, resulting in the triggering of the downstream EGFR signaling cascade. In this study, we have shown a novel metalloproteinase activity-independent, but EGF-dependent, signaling interaction between the thrombin or LPA receptor and EGFR, resulting in MKP-1 induction in ECs. We identified a requirement for both ERK-dependent and ERK-independent pathways originating from the activated GPCR for the full induction of MKP-1 when ECs are treated with thrombin or LPA plus EGF. The synergy in MKP-1 induction by thrombin or LPA plus EGF is because of the net effect of such converging signals. In the classic MAP kinase pathway, the Src family of kinases is upstream of Raf, Ras, MAPK/ERK kinase (MEK), and ERK; our inhibitor studies suggest that this pathway is responsible for the thrombin or LPA induction of MKP-1. The ERK-independent pathway responsible for the synergy is more complex. We determined that JNK activity and VEGFR-2 are involved in the ERK-independent pathway. It was previously shown that JNK is a downstream signaling intermediate in VEGF-induced MKP-1 induction. EGFR activation was shown to induce VEGF gene expression and secretion. However, a neutralizing antibody to VEGF-A, the only VEGF ligand capable of inducing MKP-1 in ECs, did not prevent thrombin plus EGF–induced MKP-1. Furthermore, the ERK-independent pathway from approximately 8- to approximately 4-fold, \( P<0.01 \). Thus, PAR-1 or PAR-1 plus EGFR–mediated EC migration is critically dependent on MKP-1 activity; EGF alone–induced migration is MKP-1 independent.
the thrombin receptor alone is not sufficient to induce MKP-1; a signaling component from EGF is also required. We believe that the ERK-independent pathway induced by thrombin plus EGF converges at VEGFR intracellularly, resulting in MKP-1 induction. Signaling interactions between GPCRs and various receptor tyrosine kinases are well-known. Recently, Greenberg et al. have demonstrated that activated VEGFR blocks PDGFB receptor activity and PDGF-induced neovascularization. To our knowledge, signaling interactions between GPCR, EGFR, and VEGFR, resulting in the transcriptional activation of a gene, have not been reported. Furthermore, our results emphasize the significance of interplay among the bioactive molecules in the vascular microenvironment.

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Disclosures
None.

References
Synergistic Induction of Mitogen-Activated Protein Kinase Phosphatase-1 by Thrombin and Epidermal Growth Factor Requires Vascular Endothelial Growth Factor Receptor-2
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Table I
Real-time PCR (Q-PCR) assay on RNA (1μg) isolated from treated or untreated EC in the presence or absence of PD98059 (a specific ERK inhibitor) using gene specific primers. Each reaction is performed in triplicate for all samples. Comparative quantification, based on cycle threshold of the gene of interest was normalized to RPL-32.

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(a) Confluent cultures of wild type (WT) or MKP-1-null mouse aortic EC were treated with thrombin (5 U/ml), EGF (16 ng/ml) or thrombin plus EGF at 37°C for 1 hour. Cells were lysed by Lammelli buffer and the cell extract from equal number of cells were subjected to SDS-PAGE, followed by immunoblot using anti-MKP-1 antibody. Absence of a band corresponding to MKP-1 protein in MKP-1-null cells treated with thrombin plus EGF demonstrated the authenticity of the anti-MKP-1 antibody. (b) Quantitative analysis of three independent experiments performed as described in (a) and normalized with the GAPDH signal. Quantification was performed using ImageQuant software. MKP-1 null mice were generated by Bristol Myers Squibb Pharmaceutical Institute that is maintained and genotyped as described in our previous paper³.
Confluent human umbilical vein EC were incubated at 37°C with EGF (16 ng/ml) alone or in presence of thrombin receptor activating peptide, TRAP-1 (100 µM), LPA (50 µM), LPC (20 µM) or endothelin-1 (100 µM) for 1 hour. Total RNA (10 µg) was isolated and subjected to Northern blot analysis using MKP-1 or RPL-32 probe.

Methods

Cell culture: Human Endothelial cells (EC) were isolated by trypsin digestion of human umbilical veins as described before1. Isolated EC were maintained in MCDB/F12 medium (Sigma) containing 15% FBS, 0.009% heparin, 0.015% ECGS and were used between passages three and five.

Mouse aortic EC (MAEC) isolation. EC from the thoracic aorta were isolated by an explant-technique as described previously2. EC authenticity was verified by staining for von Willebrand factor and endothelial specific gene expression profiling by real-time PCR.
Western blot analysis: Cell extracts from $10^5$ EC were electrophoresed in appropriate SDS/PAGE gels. The gel was soaked in protein transfer buffer (39 mM glycine/48 mM Tris base/0.037% SDS/20% methanol) for 20 min at room temperature and then transferred to nitrocellulose using a Trans-Blot semidry transfer cell (Bio-Rad). The blot was blocked using TBST (150 mM NaCl/50 mM Tris/HCl, pH7.5/0.1% Triton X-100) with 5% non-fat dry milk and then incubated with antibodies in TBST with 5% milk overnight at 4°C. The blot was washed three times with TBST, and incubated 1 h with horseradish peroxidase conjugated second antibody (Jackson Immunoresearch, PA) at room temperature. Signal was detected using an ECL western blotting kit (Amersham Biosciences, UK).

Northern analysis: EC were grown to confluence in 100 mm tissue-culture dishes coated with fibronectin. Total RNA from agonist-treated or untreated EC was isolated using Trizol reagent (Invitrogen); was subjected to electrophoresis in a 1% agarose gel; transferred to Nytran SuPerCharge membrane (Schleicher&Schuell, NH) and hybridized with random-primed (using the random-priming kit from Amersham Pharmacia Biotech) [$^{32}$P]-labeled MKP-1cDNA probes. Hybridized filters were washed multiple times and exposed to a phosphor screen (Molecular Dynamics)) for 24 to 48 h and imaged using a Storm phosphor imaging system (Molecular Dynamics) at 100-µ resolution. Quantification of the image was performed using ImageQuant software (Amersham Biosciences).
**RNA isolation and Real-time PCR assay.** First strand cDNA was synthesized from total RNA (1 µg) using Taqman Reverse Transcription Reagents (Roche, Branchburg, New Jersey) according to the manufacturer’s instructions. The reaction mixture (2µl) was used to perform PCR reactions with MKP-1-specific primer pairs. RPL-32 served as an internal control to ensure the quality and efficiency of the PCR. All the Real-time PCR data reported was verified to be derived from a single amplicon, and authenticity of each amplicon was verified by DNA sequencing. Experiments were performed in triplicate for all conditions and values are expressed as mean ± SD. The fold induction was quantified relative to the untreated control. Real-time PCR was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems, California.) and a Perkin Elmer ABI PRISM 7700 Sequence Detector, according to the manufacturer’s instructions.

**Real-time PCR assay to determine pre-mRNA of MKP-1:** First strand cDNA was synthesized from DNA-free total RNA (2 µg) isolated from agonist-treated or untreated EC using a random hexamer and SuperScript First-Strand Synthesis system for RT-PCR (Life Technologies). Reaction mixture was made up to 50 µl. 2 µl of this reaction mixture was used to perform a PCR reaction using an intron-specific forward primer (5’-AGT ACA TTT ATC TCT GGA AC-3’) and an exon-specific reverse primer (5’-CGT AGA GTG GGG TAC TGC AG-3’) of the MKP-1 gene. Real-time PCR was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems, UK) and a Perkin Elmer ABI PRISM 7700 Sequence Detector according to the manufacturer’s instructions.

**RNA interference:** VEGFR-2-specific small interfering RNA (siRNA) or a control siRNA
were purchased from Dharmacon RNAi Technologies, Thermo Scientific, CO. c-Src-specific siRNA is purchased from Dharmacon, Thermo Scientific and Fyn-specific siRNA was purchased from Invitrogen. Transfection experiments with siRNA were performed using Targefect F-2 and peptide enhancer (Targeting Systems, CA) according to the manufacturer’s instructions with a minor modification. Targefect F-2 (5 µl/ml), peptide enhancer (5 µl/ml), and 50 nM siRNA were successively added to Dulbecco modified Eagle medium, thoroughly mixed, and kept at 37°C for 30 min. Human EC (>90% confluent) were incubated with the transfection mixture for 4 h. Cells were replenished with fresh EC culture medium and, following a 24-h incubation period, treated with agonists. EC were harvested for subsequent RNA or protein analysis to verify the target protein or RNA depletion.

**EC migration assay**: In vitro migration of EC was studied using a transmigration assay. Human EC were seeded at (10⁴ cells/membrane) on the upper chamber of an 8.0-micron pore-sized diameter membrane chamber (Becton Dickinson Labware, Franklin Lake, NJ). Agonists were added to the bottom well of the appropriate chambers overnight. The cells were removed from the upper chamber of each well and membranes were fixed with ice-cold methanol then stained with hematoxylin (Sigma). The membranes were mounted on cover slides and cells were counted in random fields in a blinded manner. The assays were performed in triplicate wells for each condition, and each experiment was repeated at least 3 times.

**Statistical analysis**: Data are expressed as means ± SD. Means of two groups were compared using Student’s t test. P values of <0.05 were considered statistically significant.
