MATERIAL AND METHODS

Reagents

Recombinant SDF-1α, FGF, TNFα, IL-1β and VEGF proteins were obtained from R&D Systems (Minneapolis, MN). For Western blotting, NOX5 and p22phox antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the JNK3 antibody from Millipore (Billerica, MA). The MKP7 antibody was purchased from Novus Biologicals (Littleton, CO). Anti-pJNK, JNK, pERK1/2, ERK1/2, HO-1 antibodies were purchased from Cell Signaling (Danvers, MA) and used for Western blotting experiments. The oxidative stress indicators Dihydroethidium (DHE) and CM-H_2DCFDA were obtained from Life Technologies (Carlsbad, CA). Antioxidants (butylated hydroxyanisole (BHA), N-acetyl-L-cysteine (NAC), ebselen (Ebs)) and NOX inhibitors (diphenylene iodoniu (DPI), apocynin (Apo)) were purchased from Sigma (St. Louis, MO). The growth factor bFGF was obtained from R&D systems (Minneapolis, MN), and cytokines TNFα and IL1β were obtained from Sigma.

Cell culture and transient transfection

BAECs (bovine aortic endothelial cells) were purchased from Genlantis (San Diego, CA) and cultured in bovine aortic endothelial cell medium (Genlantis). HUVEC (Human umbilical vein endothelial cells) were purchased from Lonza (Allendale, NJ) and cultured in EGM-2 medium (Lonza). The cells from passages 4-8 were used for experiments. ~70% confluent cells in 6-cm dishes were transfected for 3 hours with 2 μg of plasmids using 8 μl of Lipofectamine LTX and 8 μl of Plus reagent (Life Technologies). Two days later, cells were treated with SDF-1α in the spent media. For transient expression experiments with 293 cells, 90-100% confluent 293 cells in 10-cm dishes were transfected 24 hours after plating with 2 μg of plasmid using 20 μl of Lipofectamine 2000 (Life Technologies).

siRNA design and transient transfection

The stealth siRNA duplexes were obtained from Life Technologies. The siRNA against bovine p22phox is a duplex of 5'- GCGUAUUGGUCUGCCUGCUGGAAUA-3'. The siRNAs against bovine NOX5 are a mixture of duplexes of 5'-CACCAGUUCUGUAACAUCAAGUGCU-3' and 5'-UGCCUCAACUUUGACUGCAGCUUCA-3'. eNOS siRNAs are a mixture of duplexes of 5'-CGGUGAAGAUCUCUGCCU CACUCAU-3', 5'-UGUUGCUGGACUCCUUUCUCUUCCG-3' and 5'-UACGUAUACGGCUUGUCACCUCCUG-3'. The control siRNA is the Stealth™ RNAi negative control duplex (Cat. No. 12935-300) and was purchased from Life Technologies. The siRNAs were transfected into BAECs according to our previous published protocol[1]. Briefly, for each sample, $2x10^5$ BAECs were transfected with 200 pmol siRNA. The experiments with p22phox, Nox5 or eNOS siRNA-transfected BAECs were performed three days later.

RT-PCR and PCR

The RNA was reverse transcribed into cDNAs with iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The specific pairs of primers used for the end-point PCR and real-time PCR are the following: nox1 (forward primer: 5'- tccctttaccctgacctctg-'3 and reverse primer: 5'-cccactgctcggatatgaat-'3), nox2 (forward primer: 5'-ctgccagtgaggacgtgtt-'3 and reverse primer: 5'-gtaaccccgatccctgct-'3), nox3 (forward primer: 5'- aggacagcccctgaagga-'3 and reverse primer: 5'-agtcagtgcagctccgaac-'3), nox4

(forward primer: 5'- ccgtttgcgtcaatcctc-'3 and reverse primer: 5'-tcttctaagcttgtatggtttcca-'3), nox5 (forward primer: 5'- ggtctccgagatgtcctctg-'3 and reverse primer: 5'-gccatcgatgtagcacttga-'3), p22phox (forward primer: 5'- cgcaagaagccgagtgag-'3 and reverse primer: 5'-cactggcatggggttttc-'3; designed by Universal ProbeLibrary Assay Design Center tool from Roche, Indianapolis, IN). The end-point PCR was performed with Platinum™ PCR supermix (Life technologies) in a Mastercycler EP PCR machine (Eppendorf, Hamburg, Germany) with the following program: 3 min 94°C to activate the polymerase followed by 40 cycles of 45 s 94°C, 45 s 55°C, and 45 s 72°C. GAPDH was the housekeeping gene used for internal control. The real-time PCR was performed with FastStart Universal Probe Master mix and specific primers and probes for each gene (Universal ProbeLibrary Single Probes #6 for nox1, #9 for nox2, #1 for nox3, #42 for nox4, #131 for nox5 and #70 for p22phox) in Roche Lightcycler 480 PCR machine. The reaction mixtures were incubated at 95°C for 10 min followed by 55 cycles at 95°C for 10 sec and 60°C for 30 sec. 18S was used as the housekeeping gene.

Immunoprecipitation and immunoblotting analysis
Cells were harvested in lysis buffer (1% Triton X-100, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L Na_3VO_4 and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 15,000 *g*. Equal amounts of proteins were incubated with a specific antibody overnight at 4 °C with gentle rotation. Protein A/G Plus-agarose beads (Santa Cruz Biotechnology) were used to pull down the antibody complexes. Immune complexes were then separated by SDS-PAGE and analyzed by Western blot.

Immunofluorescence
Immunofluorescence histochemistry was performed following our previously published protocol[2]. Cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature. After 3 washes with PBS, the cells were sequentially treated with 0.2% Triton X-100 for 5 min (for permeabilization), with 5% boiled serum for 1 hour (for blocking), then with the primary antibody overnight in the blocking solution. After 3 washes, cells were incubated in the dark with secondary antibody conjugated with Alexa Fluor® 568 (Life Technologies) in blocking solution for 90 min at 37°C. After 3 washes in PBS, the slides were mounted and the fluorescent signal was visualized by confocal laser scanning microscopy (Zeiss Pascal, Zeiss, Germany).

Detection of ROS generation
ROS were detected by staining BAECs with CM-H_2DCFDA or DHE. CM-H_2DCFDA is oxidized to green fluorescent DCF (dichlorofluorescein) by H_2O_2, and DHE is oxidized to red fluorescent ethidium by $O_2^{\cdot -}$. Cells were loaded with 5 μM CM-H_2DCFDA or 10 μM DHE for 0.5 hour followed by SDF-1α treatment for the indicated time periods. Cells were then washed with PBS and the mean fluorescence intensity was determined as ROS generation by flow cytometry CyAn™ ADP analyzer (for CM-H_2DCFDA, excitation: 485 nm and emission: 530 nm; Beckman-Coulter, Indianapolis, IN) or a Tecan microplate reader (for DHE, excitation: 520 nm and emission: 620 nm).

Boyden chamber assay
Boyden chamber assays were performed as previously described[3].

Wound healing assay
For detection of cell migration, a wound healing assay was performed as previously described[3].

In vitro matrigel angiogenesis assay
Endothelial cell tube formation was analyzed with the Matrigel-based tube formation assay as previously described[3].

Spheroid angiogenesis assay
BAECs were transfected with p22phox, NOX5 or control siRNAs. Two days later, BAEC spheroids were generated as previously described[4, 5]. Spheroids were cultured in the polymerized gel of neutralized collagen and carboxymethylcellulose at a ratio of 1:1, and containing 100 ng/ml SDF-1α. Images of the sprouts were taken at 4x magnification. The number of sprouts per spheroid was counted. For each experimental condition, at least 10 spheroids were analyzed.

In vitro phosphatase assay
In vitro phosphatase reactions were carried out on Flag-MKP7 protein immunoprecipitated from transfected cells and results visualized using Western blot analysis with phospho-specific JNK and JNK3 antibodies following our previous protocol[1].

Statistical analysis
Data are shown as the mean ± SEM for 3 to 4 separate experiments. Differences were analyzed with Student's *t*-test or ANOVA and followed by a post hoc test with Bonferroni correction when needed. Values of $P \leq 0.05$ were considered statistically significant.

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