Sildenafil Citrate Induces Migration of Mouse Aortic Endothelial Cells and Proteinase Secretion

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Abstract Vascular endothelial cells release proteinases that degrade the extracellular matrix (ECM), thus enabling cell migration during angiogenesis and vasculogenesis. Sildenafil citrate stimulates the nitric oxide-cyclic guanosine monophosphate pathway through inhibition of phosphodiesterase type V (PDE5). In this report, we examined the mechanisms underlying sildenafil citrate-induced cell migration using cultured mouse aortic endothelial cells (MAECs). Sildenafil citrate induced migration and proteinase secretion by murine endothelial cells. Sildenafil citrate induced the secretion of matrix metalloproteinase-2 (MMP-2) and MMP-9, which is inhibited by NF-κB inhibitors. Sildenafil citrate also induced the secretion of plasmin, which is inhibited by PI 3'-kinase inhibitors. It is suggested that sildenafil citrate-induced migrating activity in endothelial cells may be accomplished by increased secretion of proteinases.

Keywords: sildenafil citrate, migration, MMPs, plasmin

INTRODUCTION

PDE5 is found in high concentrations in the lung and contributes to degradation of cyclic guanosine monophosphate (cGMP) within vascular smooth muscle [1]. cGMP plays a key role in many physiological systems including smooth muscle tone, neuronal excitability, epithelial electrolyte transport, phototransduction in the retina and cell adhesion [2]. PDE5 inhibitor, which has cGMP increasing activity, has proved to be effective in the treatment of penile dysfunction after oral administration in humans [3]. Sildenafil citrate is a selective inhibitor of PDE5, which has been shown to be a clinically effective treatment for erectile dysfunction. Its action results from increased levels of cGMP, which is normally degraded by PDE5 [4].

Angiogenesis is a complex physiological process consisting of several distinct steps leading to the development of new blood cells. This process begins when the surrounding basement membrane capillaries start to degrade [5,6]. The endothelial cells then migrate towards angiogenic stimuli and proliferate. New data suggest that MMPs play an important role in angiogenesis [7,8]. To encourage migration, endothelial cells must secrete protease to dissolve the adjacent extracellular matrix (ECM). One such family of enzymes is the MMPs. There are currently 24 known variants of MMPs [9]. MMPs are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs), which form 1:1 complexes with MMPs [10,11]. It is believed that the balance between the levels of MMPs and TIMPs is critical in regulating the breakdown of connective tissues by migrating cells. In the present study, the possible mechanisms underlying sildenafil citrate-induced cell migration were examined using cultured MAECs. Sildenafil citrate induced the migration and increased the secretion of MMPs and the plasmin of MAECs. Addition of MMPs and plasmin inhibitors inhibited sildenafil citrate-induced migrating activity.

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MATERIALS AND METHODS

Materials

Recombinant human MMP-2 and MMP-9 enzyme immunoassay kits were purchased from Fuji Chemical Industries (Toyama, Japan). Recombinant human plasmin enzyme immunoassay kits were purchased from Progen (Heidelberg, Germany). BB-94 and α_2-antiplasmin were purchased from British Biotechnology (Oxford, UK). Media and sera were obtained from Life Technology, Inc. (Gaithersburg, MD, USA). An MMP standard was purchased from Calbiochem (La Jolla, CA, USA). Sildenafil citrate (Viagra) was purchased from Pfizer Inc. (NY, USA). Other biochemical reagents, including gelatin, fibrinogen, Giemsa staining solutions, antibiotics, antimycotics, and trypsin-EDTA were purchased from Sigma, unless otherwise specified.

Cell Culture

MAECs were prepared from BALB/c mouse aortas by collagenase digestion, as previously described [12-14]. These endothelial cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with a 20% (v/v) heat-inactivated fetal bovine serum. The primary cultured cells used were taken between passages 2 and 4.

Migration Assays

A migration assay was performed using microcarrier beads as previously described [15]. MAECs were grown to confluency on microcarrier beads (diameter 175 μm; Sigma) and placed in a gelatinized 24-well plate (30 to 40 beads/well) in DMEM containing 2% serum with various reagents and incubated for 20 h. The wells were washed with PBS, fixed with ethanol, dried and stained with a Giemsa staining solution for 5 min. The cells that had migrated from the beads and attached to the wells were counted at 100 × magnification using an inverted phase-contrast microscope.

Enzyme Immunoassay of MMPs and Plasmin

MAECs were seeded to 24-well plates at a density of 5 × 10^4 cells/cm^2 and grown for 24 h in DMEM supplemented with 20% serum. Confluent MAECs were then incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with fresh medium, control buffer or the indicated reagents were applied for 12 h. The actual quantities of the MMPs and plasmin were assayed by enzyme immunoassay, performed according to the protocol of the manufacturer (Fuji Chemical Industries, Japan; Progen, Germany).

Zymography

MAECs were seeded in 24-well plates at a density of 5 × 10^4 cells/cm^2 and grown for 24 h in DMEM supplemented with 20% serum. Confluent MAECs were then incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with a fresh medium, a control buffer or the indicated reagents were applied for 12 h. The hydrolytic activities of MMPs were then evaluated by gelatin zymography [16]. Samples were mixed using a 5 × sample buffer (4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, and 0.1% bromophenol blue) and were applied to a 10% SDS-PAGE containing 0.1% gelatin. Reference standards were MMP-2 and MMP-9 (Chemicon, Temecula, CA, USA). After electrophoresis was run, the gels were incubated in 2.5% Triton X-100 for 1 h and incubated in enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) for 24 h at 37°C. The gels were stained with a 0.5% Coomassie brilliant blue 250 solution and destained with several changes of 30% methanol and 10% acetic acid. Next, the hydrolytic activity of the plasmin was measured by fibrin zymography. Thus, equal amounts of proteins (10 μg/lane) from supernatants were mixed with SDS sample buffer and eletrophoresed in 10% acrylamide gel containing 0.2% plasminogen (Athens Research and Technology, Athens, GA, USA). The gel was washed twice in 10 mM Tris-HCl (pH 8.0) containing 2.5% Triton X-100 for 30 min during agitation at room temperature. The gel was further rinsed in 10 mM Tris-HCl for 30 min, and was then incubated in a buffer containing 0.1 M glycine-NaOH (pH 8.3) at 37°C for 16 h. The gel was stained with 1% Coomassie brilliant blue 250 in 5% acetic acid and 10% methanol, and was then destained in the same mixture without dye.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from sildenafil citrate-treated MAECs, using Trizol reagent (Life Technologies, Rockville, MD, USA) according to the recommendations of manufacturer. Messenger RNA was isolated using a Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden) containing oligo (dT)-cellulose, according to the instructions of the manufacturer. Using the SuperScript II Reverse Transcriptase (Life Technologies), 100 ng of the isolated mRNA was reverse-transcribed into cDNA. A 3 μL aliquot of the reverse transcription reaction was utilized for PCR amplification of cDNA fragments for VEGF (sense primer, 5′-TGT ACC TCC ATG CCA AGT-3′; antisense primer, 5′-TTG GTC TGC ATT CAC ATC TGC-3′), HGF (sense primer, 5′-GCA TCA AAT GCC AGC CTT GGA-3′; antisense primer, 5′-ATT CAC AGC ACT GTG AGC GCA-3′), FGF-2 (sense primer, 5′-CCC ACA CGT CAA ACT ACA ACT-3′; antisense primer, 5′-TCA GCT CAA AGC AGA TGG-3′), and β-actin cDNA (sense primer, 5′-ATG TAC GTA GCC ATC CAG GCT-3′; antisense primer, 5′-ATG GAT GCC ACA GGA TTC CAT-3′) as internal standard. The conditions for PCR were the same for all the molecules; 94°C for 40 sec (denaturing), 53°C for 1 min (annealing), and 72°C for 1 min (extension). After 40 such cycles, PCR products were usually detectable by electrophoresis. Densitometric analysis was performed using a BAS 2500 System (Fuji Photo Film, Tokyo, Japan).

Data analysis

Data are expressed as mean ± standard deviation (SD). The statistical significance was tested using one-way ANOVA followed by the Newman-Keuls multiple comparison test. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Migration Assay of MAECs

Placing microcarrier beads onto a confluent monolayer of MAECs for 2 to 3 days produced beads covered by a confluent monolayer of cells with 25 to 30 cells per bead. When MAECs bearing microcarrier beads were placed

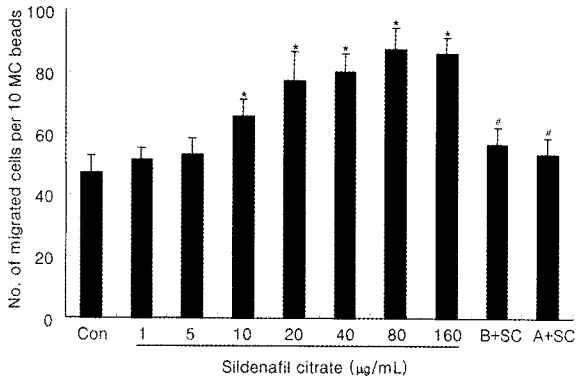

Fig. 1. Effects of sildenafil citrate, MMPs and plasmin inhibitors on migratory activity of MAECs. To measure migratory activity, the cells were grown to confluence on microcarrier beads, and the beads were placed into gelatinized 24-well plates in medium containing 2% serum and control buffer (Con) or various amounts of sildenafil citrate (SC), BB94 (20 ng/mL, B) plus sildenafil citrate (80 μg/mL) (B+SC), and α_2-antiplasmin (100 mU, A) plus sildenafil citrate (80 μg/mL) (A+SC), and were incubated for 20 h. The number of endothelial cells that migrated from the beads and attached to the well per 10 beads was counted. Bars represent mean ± SD from three independent experiments. Statistical significance was tested using one-way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p<0.05$ versus control buffer; $^{\#}p<0.05$ versus sildenafil citrate (80 μg/mL).

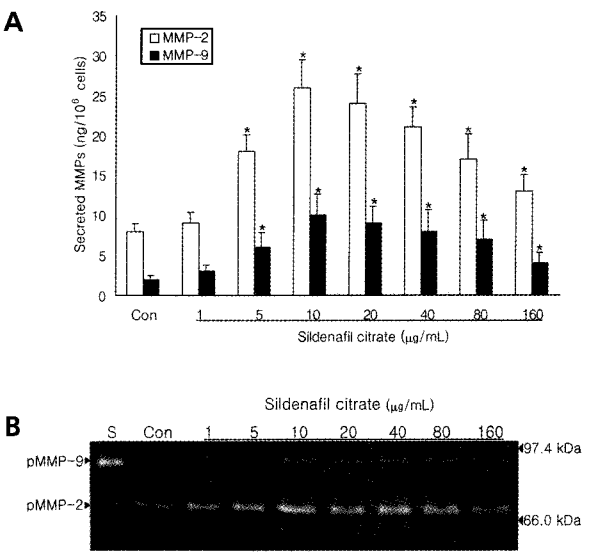

Fig. 2. Effects of sildenafil citrate on the secretion of MMP-2 and MMP-9 in MAECs. Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after addition of a control buffer (Con) and various amounts of sildenafil citrate, and media were quantitatively assayed by enzyme immunoassay (A) and gelatin zymography (B). The lane marked S contains standards of MMP-9 and MMP-2. Bars represent mean ± SD from three independent experiments. Statistical significance was tested using one-way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p<0.05$ versus control buffer.

onto gelatinized plastic dishes with a control buffer for 20 h, they yielded a basal level of nondirectional migration (45 to 50 cells per 10 beads, Fig. 1). With the addition of sildenafil citrate stimulation, the number of migrating cells increased in a dose-dependent manner (10~80 μg/mL). Also, the addition of BB94 (20 ng/mL), a broad spectrum MMP inhibitor, and α_2-antiplasmin (100 mU), a plasmin inhibitor, to cultured MAECs almost completely blocked the migratory effects of sildenafil citrate (80 μg/mL). To enhance angiogenesis, endothelial cells must increase their migratory activities. These data indicate that sildenafil citrate is a potent factor in migration of MAECs.

Sildenafil Citrate Induces MMP-2 and MMP-9 from MAECs

A crucial step in angiogenesis is the point at which proteases degrade the underlying basement membranes [17]. Endothelial cells release proteinases to degrade the ECM for their migration and proliferation *in vivo*. One family of such proteinases is the MMPs. The role of MMPs in angiogenesis has been demonstrated *in vitro* [9,11,18]. In culture, endothelial cells largely secrete MMP-2 and MMP-9, which can disrupt the ECM and enable migration and tube formation [19]. The step-wise activation processes of pro-MMPs suggest that MMP activities are controlled by endogenous inhibitors such as α_2-macroglobulin or TIMPs before MMPs are fully activated [20,21]. An enzyme immunoassay indicated that the culture media from MAECs contained easily detectable amounts of MMP-2 and MMP-9 (Fig. 2A). The addition of sildenafil citrate (10 μg/mL) on cultured MAECs for 12 h produced increases of approximately 3.3- and 5.0-fold in MMP-2 and MMP-9 secretion, respectively, compared with the addition of a control buffer. However, higher concentrations (40 to 160 μg/mL) of sildenafil citrate significantly suppressed the secretion of MMP-2 and MMP-9. Suitable stimulation in murine endothelial cells was achieved with 10 μg/mL of sildenafil citrate. The profiles of MMP-2 and MMP-9 in the media were quantitatively assayed by gelatin zymography. Consistent with the enzyme immunoassay, sildenafil citrate was capable of inducing MMP-2 secretion for 12 h compared to the addition of a control buffer. Additionally, sildenafil citrate was capable of inducing the secretion of MMP-9 for 12 h compared to the addition of a control buffer (Fig. 2B).

Sildenafil Citrate Induces Plasmin Secretion from MAECs

To migrate in response to sildenafil citrate stimulation in an *in vitro* fibrin gel, endothelial cells must secrete fibrinolytic enzymes. To date, the ability of endothelial cells to mediate fibrinolytic activity has been largely attributed to the fibrinolysin plasmin [22]. In the present study, plasmin secretion was confirmed by enzyme immunoas-

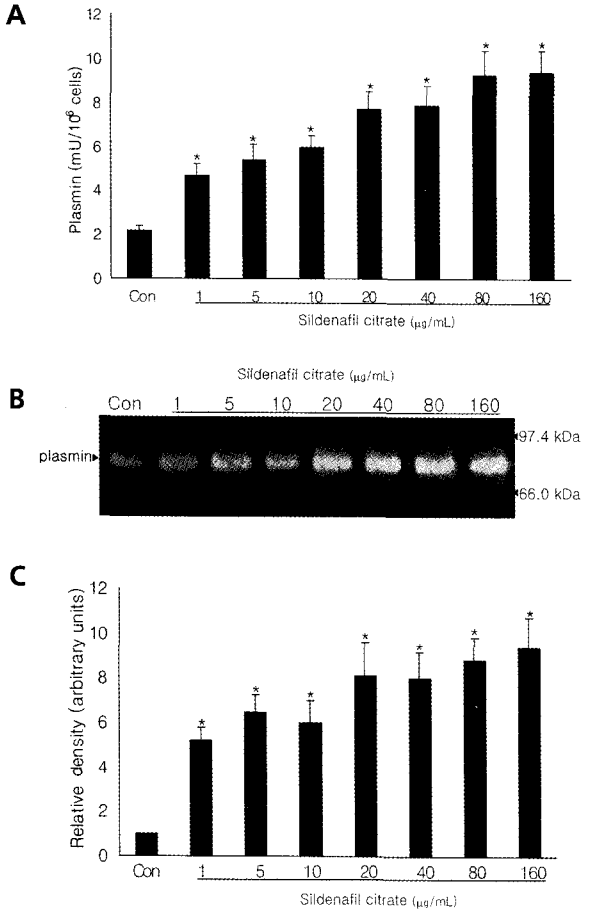

Fig. 3. Effects of sildenafil citrate on the secretion of plasmin in MAECs. Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after addition of a control buffer (Con) and various amounts of sildenafil citrate, and media were quantitatively assayed by enzyme immunoassay (A) and fibrin zymography (B). Densitometric analyses of the zymographs are presented as the relative ratio of induction of the plasmin by addition of sildenafil citrate. The plasmin secretion by addition of control buffer for 12 h is arbitrarily presented as 1 (C). Bars represent mean ± SD from three independent experiments. Statistical significance was tested using one-way ANOVA followed by the Newman-Keuls multiple comparison test. $^{*}p<0.05$ versus control buffer.

say and fibrin zymography. The addition of sildenafil citrate (10 μg/mL) to cultured MAECs for 12 h produced increases in plasmin secretion of approximately 4.2-fold as compared to the addition of a control buffer (Fig. 3A). The culture medium from sildenafil citrate-treated cells clearly had increased ~85 kDa fibrinolytic bands, compared with the cells treated with buffer alone (Fig. 3B). Plasmin secretion was increased in a dose-dependent manner. MMPs are generally secreted as zymogens that are extracellularly activated by several proteinases. MMPs, alone or in concert with the plasminogen/plasmin system, are involved in the degradation of components the ECM, a requirement for cell migration [23]. *In vitro*, plasmin directly activates MMPs [24], and activation of MMP-2

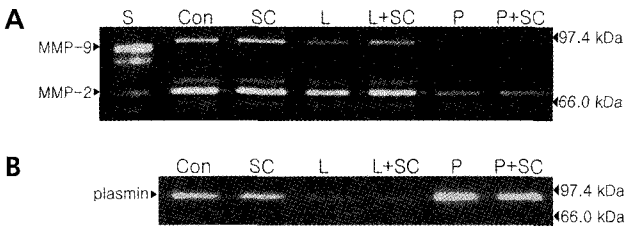

Fig. 4. Effects of PI 3′-kinase and NF-κB inhibitors on sildenafil citrate-induced proteinase secretion. Cells were incubated in serum- and phenol red-free DMEM for 12 h. Control buffer (Con), sildenafil citrate (80 μg/mL, SC), LY294002 (100 nM, L), and PDTC (50 μg/mL, P) were added to 0.5 mL of the same culture medium, cells were incubated for 12 h, and media were analyzed by gelatin zymography (A) and fibrin zymography (B). LY294002 (100 nM) and PDTC (50 μg/mL) were pretreated 60 min before sildenafil citrate (80 μg/mL) addition. Equal amounts of proteins (10 μg/lane) from supernatants were loaded into each lane. The lane marked S contains standards of MMP-9 and MMP-2. Results of three independent experiments were similar.

involves hydrolysis by MT1-MMP, yielding an intermediate that is activated by plasmin [25]. In this study, our fibrin zymography produced fibrinolytic bands where MMP-2 was active (Figs. 2 and 3). Activation of PI 3′-kinase has been demonstrated to play a role in the angiogenic factor-induced MMPs and plasmin secretions from endothelial cells [26]. To examine the involvement of PI 3′-kinase, we applied PI 3′-kinase inhibitor to MAECs. The preincubation of LY294002 (100 nM), a synthetic PI 3′-kinase specific inhibitor, did not show any changes in sildenafil citrate-induced MMP-2 and MMP-9 secretion from MAECs (Fig. 4A). We examined the effect of PI 3′-kinase inhibitors on the secretion of plasmin. As shown in Fig. 4B, PI 3′-kinase inhibitor, LY294002 (100 nM), completely suppressed sildenafil citrate-induced plasmin secretion. These results suggest that activation of PI 3′-kinase may be involved in the secretion of sildenafil citrate-induced plasmin.

Thus far, the NF-κB binding region has been found in the MMP-9 promoter, and this is one characteristic determining the unique expression pattern of this gene [27]. Therefore, targeted inhibition of NF-κB might be a logical step in modulating the MMP-9 activity. To examine the involvement of NF-κB activation, cells were preincubated with NF-κB inhibitors. A broad spectrum NF-κB inhibitor, PDTC (50 μg/mL), completely suppressed sildenafil citrate-induced secretion of MMP-2 and MMP-9. Thus, these results indicate that sildenafil citrate-induced MMP-9 secretion is an NF-κB-dependent process in murine endothelial cells. The mechanisms by which NF-κB is involved in sildenafil citrate-induced secretion of MMP-2 and MMP-9 will be examined in the future studies.

Sildenafil Citrate-Induced Migration is Inhibited by MMPs and Plasmin Inhibitors

Endothelial cell migration is an initial step in angio-

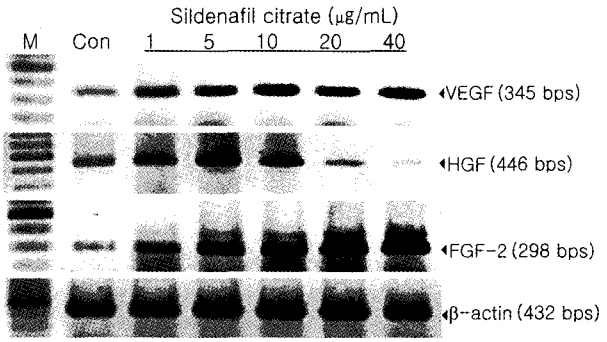

Fig. 5. Effect of sildenafil citrate on expression of angiogenic factors in MAECs. MAECs were incubated with control buffer (Con) or increasing concentrations of sildenafil citrate, and total RNA was extracted 12 h later. VEGF, HGF, and FGF-2 mRNA expression was determined by RT-PCR as described in Materials and Methods. β-Actin was used as a normalization control. The lane marked M represent size marker. The results shown correspond to a representative experiment of three independent experiments.

genesis and neovascularization [23]. This process requires cell migration and invasion into the ECM beneath the basement membrane. Since migratory activities were measured in gelatinized plates, sildenafil citrate-induced MMPs and plasmin secretion could be a determinant for migration (Figs. 2 and 3). To test whether the increased MMPs and plasmin were responsible for migration, the effect of the MMPs and plasmin inhibitors on migration was examined. Consistent with this idea, the addition of BB94 (20 ng/mL), a broad spectrum MMP inhibitor and α_2-antiplasmin (100 mU), a plasmin inhibitor, on cultured MAECs almost totally blocked the migratory effects of sildenafil citrate (80 μg/mL) (Fig. 1). Therefore, it is likely that, sildenafil citrate induces the migration of cultured endothelial cells through increased secretion of MMPs and plasmin.

Sildenafil Citrate Upregulates VEGF, HGF, and FGF-2 in MAECs

During the initial processes of angiogenesis and vasculogenesis, a variety of growth factors and cytokines are upregulated and exert their functions through autocrine and paracrine actions [6,28]. Of these, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2) may be the key molecules, since their receptors are selectively located in endothelial cells [29,30]. Therefore, MAECs were evaluated for their capacity to increase the production of angiogenic factor in response to sildenafil citrate. As expected, sildenafil citrate upregulates several angiogenic factors such as VEGF, HGF, and FGF-2 in MAECs (Fig. 5). Thus, sildenafil citrate is a reasonable potent candidate for therapeutic angiogenesis. The mechanisms by which sildenafil citrate-induced protease production and upregulation of angiogenic factors will be examined in future studies.

CONCLUSION

Angiogenesis is a physiological process involving the formation of new capillary structures. In this study, we examined the effect of sildenafil citrate on cell migration and secretion of proteinases from MAECs. Upon stimulation by sildenafil citrate, the number of migrating cells increased in a dose-dependent manner. The addition of sildenafil citrate (10 μg/mL) on cultured MAECs for 12 h produced increases of approximately 3.3-, 5.0-, and 4.2-fold in MMP-2, MMP-9 and plasmin secretion, respectively, compared with the addition of a control buffer. Sildenafil citrate also upregulates several angiogenic factors such as VEGF, HGF, and FGF-2 in murine endothelial cells. These data indicate that sildenafil citrate is a potent inducer of angiogenic factor of MAECs.

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