

Angiogenin Induces Nitric Oxide Synthesis in Endothelial Cells through PI-3 and Akt Kinases[†]

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ABSTRACT: Angiogenesis, the formation of new blood vessels, is a critical but complex phenomenon modulated by numerous physicochemical conditions. Nitric oxide (NO) is a very well known biological mediator involved in vascular physiology. This study focuses on relationships between the effect of angiogenin, a major angiogenic factor, and extracellular NO release. NO concentration was sensed electrochemically using a fibronectin-coated multiple microelectrode array. Angiogenin was shown to increase NO levels, thus triggering nitric oxide synthase (NOS) activity. The effect of angiogenin on NOS was demonstrated using L-NAME, a competitive NOS inhibitor. Dose–time dependence was investigated, showing a stimulation threshold in the 250 ng/mL–1 µg/mL range and a maximal NO release after 30 min of exposure to angiogenin. To elucidate the very complex reactive pathway of angiogenin, we have used various selective inhibitors to investigate the mechanism leading to NO production. Neomycin, an antibiotic blocking nuclear translocation, inhibited the angiogenin effect on NOS. This result demonstrates that angiogenin activates NOS by interacting with the cell nucleus. Similarly, NOS activity was stopped by blocking the PI-3/Akt kinase signaling transduction cascade, showing the importance of this pathway.

Angiogenesis, the formation of new blood vessels, is currently an important topic for biochemical research because of its central role in cancer development (1). However, controlling angiogenesis is a rather challenging task as this complex biological phenomenon is known to depend on several parameters including interaction with the extracellular matrix (2), mechanical or shear stresses (3), and other biochemical factors. It has been reported that some growth factors, like vascular endothelial growth factor, increase endothelial growth and angiogenesis (4). Studying the biochemical interactions between endothelium and angiogenic factors is therefore essential for understanding and modulating angiogenesis for therapeutic purposes.

In this report, we focus on angiogenin, one of these angiogenic factors. Angiogenin is a 123 amino acid long single-chain protein of about 14.4 kDa (5). This molecule is essential for cell proliferation, even for angiogenesis induced by other angiogenic factors (6). In particular, it has been reported that nuclear translocation of angiogenin is a critical step for angiogenesis induced by acidic or basic fibroblast, epidermal, or vascular

endothelial growth factors (6). However, the cellular physiology of angiogenin is still quite unclear.

Nitric oxide (NO)¹ is a small molecule produced by nitric oxide synthase (NOS) from L-arginine (7). NO is an ubiquitous biological transmitter appearing in nervous and immune systems and in particular in vascular physiology (8). It has been demonstrated that NO plays a central role in angiogenesis (8) by triggering the synthesis of angiogenic factors and the differentiation of endothelial cells into vascular tube cells (9). Ziche et al. have also shown that, as with many other angiogenic factors, NO can lead to DNA synthesis and migration of coronary endothelial cells (10, 11). In addition, NO may also promote angiogenesis through its endothelial antiapoptotic properties (12): angiogenesis is mainly based on proliferation of endothelial cells and therefore on inhibition of their apoptosis.

As a consequence, NO is a key factor in angiogenesis. The purpose of this study is to investigate relationships between angiogenin and extracellular NO release. Human umbilical vein endothelial cells (HUVECs) were grown directly on the fibronectin-coated multiple microelectrode array, angiogenin was added, and NO levels were monitored. Sparse cell concentration (about 5×10^3 cells/cm² (13)) and endothelial growth medium were used to promote angiogenesis. NO was measured electrochemically using differential pulse voltammetry (DPV). Several inhibitors of angiogenin activities were used to investigate their role in NOS activity. Our results suggest that angiogenin induces NOS activity in a dose- and time-dependent manner. Furthermore, signal transduction through PI-3 and Akt kinases and nuclear translocation of angiogenin appear to be critical for NO synthesis.

EXPERIMENTAL PROCEDURES

Chemicals. All of the chemicals used in this study were purchased from Sigma, unless stated otherwise, and were used

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¹Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; MMA, multiple microelectrode array; HUVEC, human umbilical vein endothelial cell; L-NAME, N^ω-nitro-L-arginine methyl ester; DPV, differential pulse voltammetry.

without further purification. Deionized water purified through a Millipore system was used throughout all of the experiments. Angiogenin was purified as previously described (14).

Cell Culture. HUVECs were purchased from Clonetics (San Diego, CA) and cultivated in EGM-2 (endothelial growth media 2; Clonetics) on gelatin-coated 75 mL flasks, at 37 °C, 95% O₂, 5% CO₂. Cells from passages 6–9 were used.

MMA Design. Multiple microelectrode arrays (MMA) were used for the electrochemical sensing of NO. The MMA features six independent channels and was engineered using stereolithography. The chip was designed at Imperial College London (U.K.) and made by Innos Ltd. (Southampton, U.K.). Further details are available elsewhere (15).

Modification and Preparation of the Sensors. The MMA were modified using a Sylgard (Dow Corning, Midland, MI) custom-made reaction cell to allow deposition of a 500 μL volume on the sensor. A lid made from a Petri dish was also fitted on the cell to minimize evaporation.

Prior to any experiment, biological debris was removed using trypsin solution. Trypsin was deposited into the cell and incubated at 37 °C for at least 20 min. It was then rinsed with 70% ethanol followed by water. The gold electrodes were electrochemically cleaned by performing cyclic voltammograms between 1 and –0.8 V at 0.5 V/s in 0.5 M sulfuric acid until stability of the graphs. The electrode potential was then held at –0.6 V to reduce gold oxides. The sensor was then sterilized with 70% ethanol and rinsed with PBS (pH = 7.4). Human fibronectin (40 μL) (20 μg/mL, in DMEM) was deposited on the sensor and allowed to dry. Excess of fibronectin was removed by rinsing with PBS.

Electrochemical Measurements. Cells were harvested using trypsin and counted with a cytometer and Trypan blue. A total of 2000 cells were suspended in 500 μL of EGM-2 and deposited on the sensor. The chip was then incubated for at least 12 h (37 °C, 95% O₂, 5% CO₂). Culture media were replaced with fresh EGM-2, and after 3–4 h, DPV was recorded between 0.6 and 1.5 V vs Ag/AgCl (increment 0.004 V, amplitude 0.006 V, pulse width 0.06 s, pulse period 0.2 s). Measurements were stopped just after a complete peak was obtained to minimize its possible impact on HUVEC. In these conditions, a single DPV scan lasts less than 30 s. An adequate volume of angiogenin (1.14 mg/mL in deionized water) was added with the relevant inhibitor if required (protocol described below). The MMA was then placed back in the incubator, and another DPV was recorded after 30 min (or every 10 min for the time dependence study). In a set of experiments, angiogenin was replaced with 200 ng/mL VEGF dissolved in water.

Data Processing. The DPV results were processed by normalizing the peak current obtained after angiogenin injection by the peak current measured before injection. This normalization guaranteed consistency between the different measurements by minimizing the effect of variations in size, background species, and heterogeneity in cell population. The results obtained for each experimental condition were then averaged and the means compared using Student's *t* tests.

Inhibitors. For the *N*^ω-nitro-L-arginine methyl ester (L-NAME) study, the cells were maintained in culture medium containing 100 μM L-NAME for at least 1 h before any measurement. Similarly, in the neomycin setup, cells were cultivated for at least 1 h in medium containing 50 μM neomycin before any measurement. The PD 98,059 and LY 294,002 kinase inhibitors were dissolved in dimethyl sulfoxide and were added 1 h before measurements at a concentration of 10 μM.

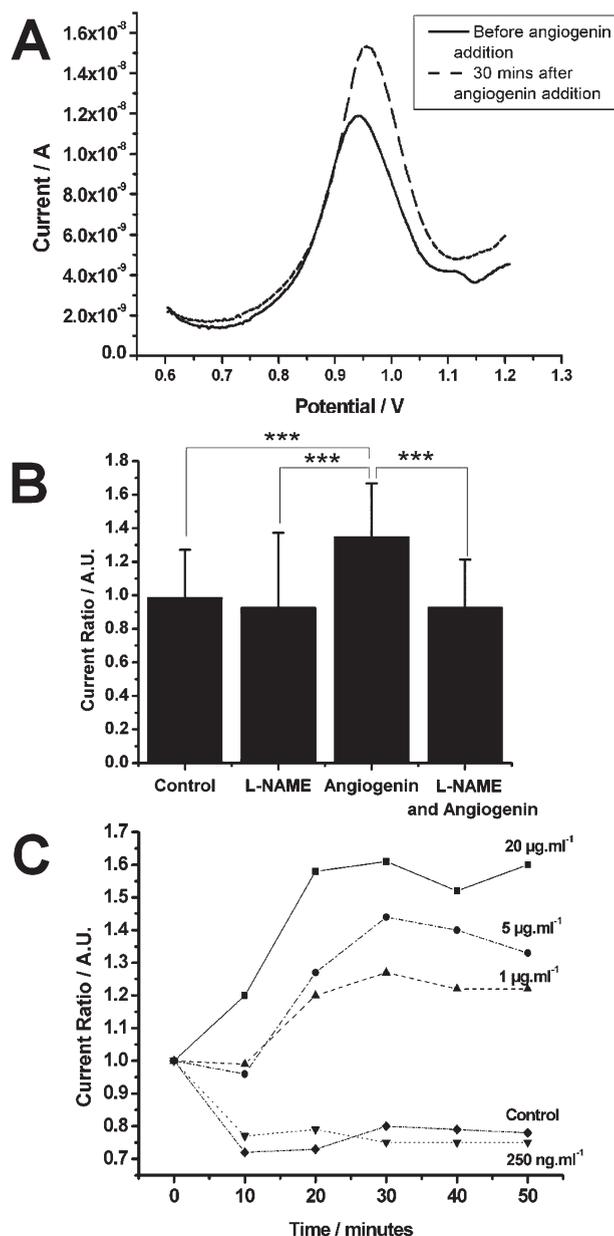


FIGURE 1: Effect of angiogenin on NOS activity. (A) Typical differential pulse voltammograms obtained in HUVEC culture before and 30 min after addition of 5 μg/mL angiogenin. Potentials were measured vs Ag/AgCl. (B) Summary of the current ratios obtained for differential pulse voltammograms performed in HUVEC culture before and 30 min after addition of 5 μg/mL angiogenin. Angiogenin was added in the presence or absence of 100 μM L-NAME, used as a NOS inhibitor. Controls with L-NAME or injection of 2 μL of water only has been performed. $n > 17$; ***, $p < 0.001$. (C) Current ratios obtained for differential pulse voltammograms obtained in HUVEC culture before and 10, 20, 30, 40, and 50 min after addition of various concentrations of angiogenin (20 μg/mL, 5 μg/mL, 1 μg/mL, 250 ng/mL, and control, i.e., injection of 9 μL of water). $n > 11$.

Laser Confocal Microscopic Analysis. Cells were plated sparsely ($5 \times 10^3/\text{cm}^2$) on 18 × 18 mm glass slides coated with fibronectin. The cultures were serum-deprived overnight with EBM-2 (endothelial basal media 2; Clonetics) supplemented with 1% FBS. After 30 min pretreatment with or without pretreatment of selective inhibitors (100 μM L-NAME, 10 μM LY 294,002, 10 μM PD 98,059, or 50 μM neomycin), angiogenin was added to a final concentration of 5 μg/mL, and incubation was continued for 30 min. Cells were fixed in methanol for 3 min

Table 1: Dose–Time Response

concn	$t = 10$ min	$t = 20$ min	$t = 30$ min	$t = 40$ min	$t = 50$ min	t test ^d
20 $\mu\text{g/mL}$	1.20 \pm 0.48 ^b	1.58 \pm 1.30	1.61 \pm 0.37	1.52 \pm 0.55	1.60 \pm 0.44	***
5 $\mu\text{g/mL}$	0.96 \pm 0.13	1.27 \pm 0.37	1.44 \pm 0.44	1.40 \pm 0.46	1.33 \pm 0.46	***
1 $\mu\text{g/mL}$	0.99 \pm 0.28	1.20 \pm 0.26	1.27 \pm 0.24	1.22 \pm 0.28	1.22 \pm 0.28	***
250 ng/mL	0.77 \pm 0.16	0.79 \pm 0.28	0.75 \pm 0.23	0.75 \pm 0.22	0.75 \pm 0.23	–
control	0.72 \pm 0.15	0.73 \pm 0.21	1.80 \pm 0.25	0.79 \pm 0.27	0.78 \pm 0.41	

^aStudent's t test was used to compare results obtained at $t = 30$ min with the control case. ***, $p < 0.001$; –, $p > 0.6$. ^bAverage \pm standard deviation for $n = 11$.

at -20°C and rinsed three times with cold PBS for 5 min at room temperature. The cells were then incubated with 1% bovine serum albumin in PBS at room temperature. Then mouse anti-endothelial NOS (eNOS) antibody or rabbit anti-phospho-eNOS (Ser1177) antibody (Cell Signaling Technology, Inc., Boston, MA) in PBS was added for 1 h at 37°C . After being rinsed with PBS, anti-mouse Cy5-labeled antibodies or anti-rabbit Cy3-labeled antibodies (Sigma-Aldrich, St. Louis, MO) were incubated for 1 h at room temperature and then rinsed five times for 5 min with PBS at room temperature. Slides were mounted using gel mount (Biomedica, Beaufort, SC) on microslides (Paul Marienfeld GmbH and Co. KG, Lauda-Königshofen, Germany), and the cells were observed with an inverted confocal imaging system (Carl Zeiss LSM 710). In the case of phospho-eNOS pictures, the data were processed by converting the image into gray scale and thresholding the image at a lightness of 45.

Effects of Selective Inhibitors on the Migration of HUVEC by Angiogenin in Vitro. The migration of HUVEC in response to angiogenin was determined using a scratch wound assay (16–18). Briefly, HUVECs were plated in a fibronectin-coated 35 mm dish at 1×10^6 cells/cm² and cultured in EGM-2 for 24 h. When the cells were confluent, they were washed with prewarmed PBS and then cultured in EBM-2 supplemented with 1% FBS for 12 h. The cell monolayer was scraped with a ultramicrotip to create a cell-free zone. After being washed three times with prewarmed EBM-2, 5 $\mu\text{g/mL}$ angiogenin was added with or without selective inhibitors (100 μM L-NAME, 10 μM LY 294,002, 10 μM PD 98,059, or 50 μM neomycin) in EBM-2 supplemented with 1% FBS. HUVEC migration was quantified by measuring the number of migrating cells using ImageJ software (v1.40) after 12 h under a Nikon TS100 inverted confocal imaging system.

RESULTS

Angiogenin Induces NOS Activity and NO Release. Figure 1A shows typical voltammograms obtained for DPV performed in HUVEC culture. The solid trace was obtained by scanning the potentials before angiogenin was added (to obtain a final concentration of 5 $\mu\text{g/mL}$); the dashed one was recorded 30 min after injection. Voltammetric peaks, reaching a maximum by 0.95 V vs Ag/AgCl, are observed. These results are consistent with data previously reported (15) for DPV performed with the MMA in dissolved NO solutions. Peak intensity increased by approximately 30% over 30 min following exposure to angiogenin. These preliminary results indicate a possible angiogenin-induced NO release.

To confirm that angiogenin induces NOS activity in HUVEC, we used L-NAME as a competitive NOS inhibitor (19). We repeated the previous protocol (i.e., running a DPV before and 30 min after injection of 5 $\mu\text{g/mL}$ angiogenin), and the current ratio (peak current at $t = 30$ min)/(peak current at $t = 0$ min) was

calculated for each of the six individual electrodes of the MMA. This method was used to obtain a dimensionless value, in order to decrease the impact of heterogeneity in cell division and sensor variability. The average and standard deviations for each experimental situations were obtained, and Student's t tests were performed.

The results for L-NAME inhibition are reported in Figure 1B. Controls for L-NAME only and for injection of 2 μL of water only gave no significant difference in the peak current ratio (0.93 and 0.99, respectively). In these setups, the peak current does not change significantly. As expected, the current ratio for angiogenin only is 1.35 because of the increase in peak current ($p < 0.001$ if compared to the control and L-NAME only situations). However, if 100 μM L-NAME is present when angiogenin is added, the current ratio is now 0.93. This means that L-NAME inhibits the peak increase induced by angiogenin ($p < 0.001$). As L-NAME is commonly used to inhibit NO synthesis (19), this result shows that angiogenin induces NO synthesis in HUVEC and that this resulting increase in extracellular NO concentration can be detected by calculating the peak current ratio.

NO Production Is Dose and Time Dependent. The dose and time dependence of this NO synthesis was then investigated. Different concentrations of angiogenin (20 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 250 ng/mL) and control (injection of 9 μL of water) were tested. DPVs were obtained every 10 min for 50 min, and peak current ratios were calculated by normalizing the peak current with the peak current measured at $t = 0$ min. The graphical results are presented in Figure 1C. However, for the sake of clarity, standard deviations were not added on the graph, but numerical values are reported in Table 1.

The 250 ng/mL angiogenin situation is not different from the control, both graphs stabilizing around 0.8. However, for higher angiogenin concentrations, we notice a strong increase in NO levels, starting almost immediately for 20 $\mu\text{g/mL}$ and after 10 min for 5 and 1 $\mu\text{g/mL}$. In the three cases, the maximum is reached 30 min after injection, and NO concentration starts to decrease slowly, in particular 5 and 1 $\mu\text{g/mL}$ angiogenin. The maximum NO concentration also increases with the concentration of angiogenin injected (1.61 for 20 $\mu\text{g/mL}$, 1.44 for 5 $\mu\text{g/mL}$, and 1.27 for 1 $\mu\text{g/mL}$ angiogenin).

NO Synthesis Depends on Nuclear Translocation of Angiogenin. To investigate the angiogenin pathways, we have again repeated the previous protocol but with L-NAME being replaced with 50 μM neomycin. Neomycin is an antibiotic frequently used to inhibit nuclear translocation (20).

The results obtained for angiogenin, for angiogenin and neomycin, and for neomycin alone are summarized in Figure 2A. Complete inhibition is observed in the presence of neomycin ($p < 0.001$). This could be due to inhibition of nuclear translocation or the effects of neomycin in Akt kinase

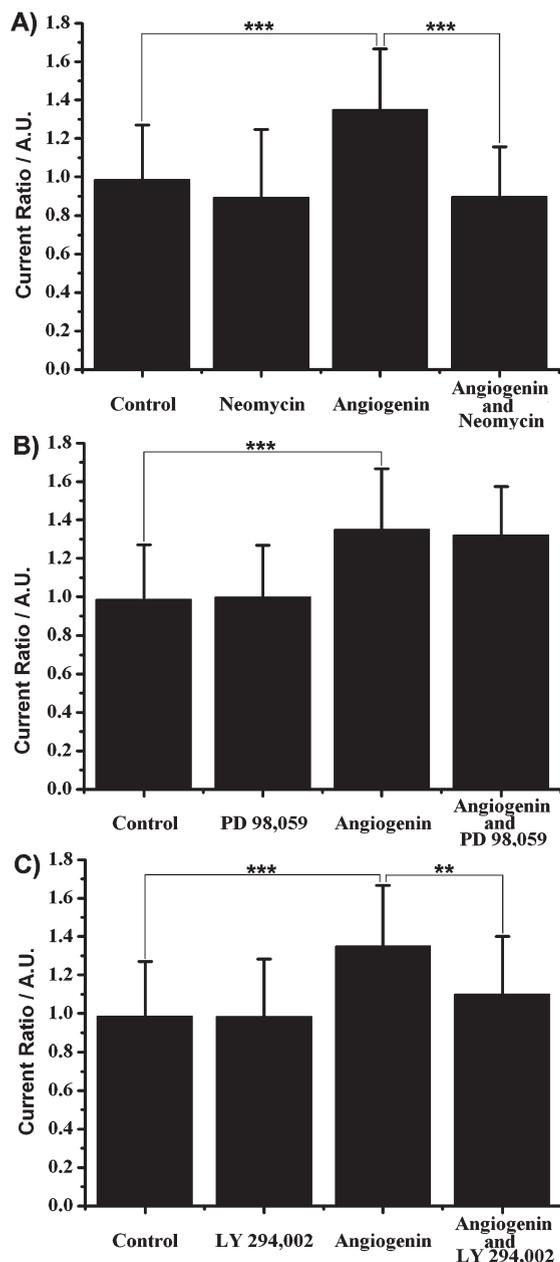


FIGURE 2: Effect of selective inhibitors of angiogenin physiological pathways. Summary of the current ratios obtained for differential pulse voltammograms performed in HUVEC culture before and 30 min after addition of 5 $\mu\text{g}/\text{mL}$ angiogenin. (A) Angiogenin was added in the presence or absence of 50 μM neomycin, used as a nuclear translocation inhibitor. Controls without and with neomycin have been performed. $n > 18$; ***, $p < 0.001$. (B) Angiogenin was added in the presence or absence of 10 μM PD98,059, used as an ERK kinase inhibitor. Controls without and with inhibitor have been performed. $n > 15$; ***, $p < 0.001$. (C) Angiogenin was added in the presence or absence of 10 μM LY 294,002, used as a Akt kinase inhibitor. Controls without and with inhibitor have been performed. $n > 18$; ***, $p < 0.001$; **, $p < 0.01$.

phosphorylation (21). The relative significance of these effects is investigated below by examining the effects of neomycin on NOS activation induced by VEGF. It is well established that the action of VEGF does not require nuclear translocation.

PI-3 Kinase Is Involved in Angiogenin-Induced NOS Activity but Not ERK 1/2. Kinase inhibitors PD 98,059 and LY 294,002, respectively inhibiting ERK 1/2 (22) and the phosphatidylinositol 3 (PI-3) (23) kinases, were then added instead of neomycin in separate experiments.

As shown in Figure 2B, PD 98,059 does not have any effect on NO release, indicating that ERK 1/2 is not involved in angiogenin-induced NOS activity. However, LY 294,002 leads to inhibition of NO synthesis ($p < 0.01$), thus indicating that PI-3 kinase mediates NOS activation (Figure 2C).

Angiogenin Regulates eNOS Activation and Intracellular Localization. We examined the effect of angiogenin on the intracellular localization of endogenous eNOS and phospho-eNOS by confocal microscopy (Figure 3). After angiogenin treatment, phospho-eNOS is enriched in the perinuclear region of cells in a punctate pattern (blue arrows) and at the periphery of cells (white arrows). In addition, phosphorylation of eNOS is increased by angiogenin. To demonstrate the exact cellular mechanism of angiogenin-regulated eNOS phosphorylation and intracellular location, LY 294,002 and PD 98,059 were tested. Pretreatment with LY 294,002, neomycin, or L-NAME for 30 min inhibited angiogenin-induced eNOS phosphorylation and intracellular localization. However, when cells were stimulated with PD 98,059, it did not affect eNOS activation and localization.

NOS Activity Is Involved in Angiogenin-Induced Endothelial Cell Migration. We then characterized the effect of the competitive NOS inhibitor L-NAME on angiogenin-induced endothelial cell migration in HUVEC. The NOS inhibitor reduced the cell migration induced by angiogenin, as shown in Figure 4. This results confirms the role of NO as a mediator in angiogenin physiology. Furthermore, LY 294,002, PD 98,059, and neomycin similarly inhibit cell migration.

Neomycin Inhibits VEGF-Induced NO Synthesis. Figure 5 shows that VEGF increased the magnitude of the NO peak ($p < 0.01$), as expected. The magnitude of this increase was 20%, somewhat lower than the increased NOS activation induced by angiogenin. Of particular relevance here is that the VEGF-induced NOS activation was inhibited by neomycin. This is strongly suggestive of a role for Akt kinase phosphorylation in NOS activation.

DISCUSSION

In this study, we have demonstrated that angiogenin induces NO synthesis through two pathways involving the PI-3 kinase signal transduction cascade and nuclear translocation.

Sensing of NO in biological conditions raises several problems due to the high reactivity of this molecule, leading to a very short lifetime. In addition, most of the commonly used methods, such as the Griess test, cannot be performed in real time and show high limits of detection and poor spatial and temporal resolution. For these reasons, measurement of NO was performed electrochemically, using a MMA (15). Electrochemical sensors are attractive devices for biomeasurements because of robustness, linear response to concentration, ease of production, and miniaturization. Furthermore, the MMA features six individual electrodes, thus enabling six simultaneous independent measurements. The utility of the MMA for NO sensing in fibroblast cells has already been demonstrated (15). To improve biocompatibility, the surface of the MMA was coated with fibronectin, an extracellular matrix protein. Fibronectin is known to promote adequate cell adhesion and growth, and we have shown that it does not interfere with electrochemical measurements (24, 25).

The dose dependence result shows two interesting features. First, NOS activity increases with angiogenin concentration. Second, there is a minimum concentration of angiogenin required

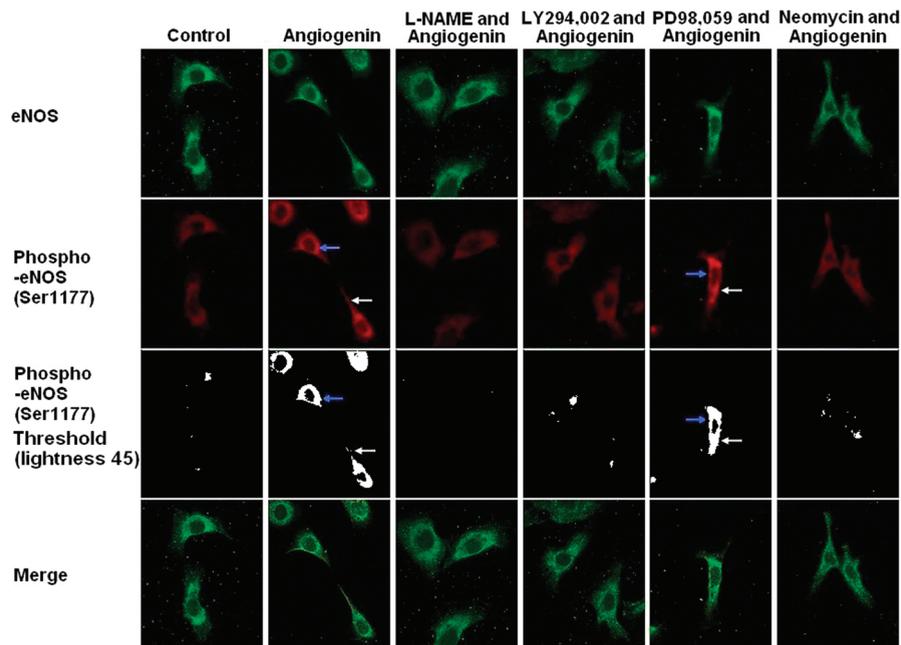


FIGURE 3: Cellular location of phospho-eNOS and eNOS in endothelial cells. HUVECs were stimulated with $5 \mu\text{g}/\text{mL}$ angiogenin in the presence or absence of $100 \mu\text{M}$ L-NAME, $10 \mu\text{M}$ LY 294,002, $10 \mu\text{M}$ PD 98,059, or $50 \mu\text{M}$ neomycin in EBM-2 and incubated for 30 min. Cells were immunolabeled with antibodies to total eNOS or phospho-eNOS (Ser1177). The upper panel shows the total eNOS, the middle panels show the phospho-eNOS (Ser1177) without and with thresholding, and the lower panel shows merged pictures.

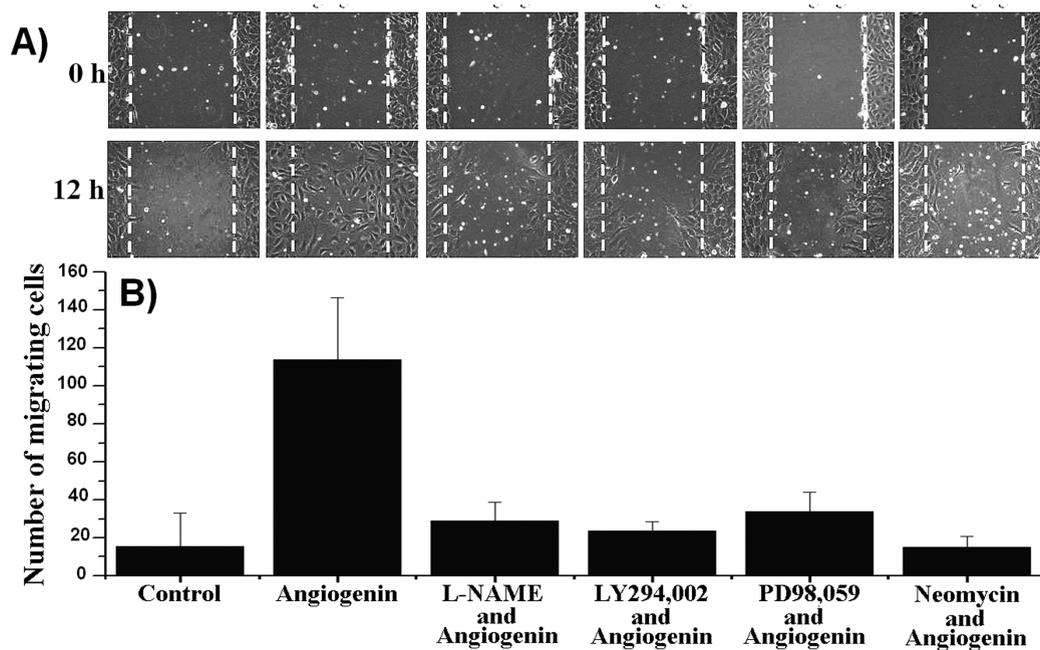


FIGURE 4: Effects of selective inhibitors on angiogenin-induced cell migration. HUVECs were plated in a 35 mm dish, and cell migration was quantified by the scratch method as described under Experimental Procedures. After the scratch, $5 \mu\text{g}/\text{mL}$ angiogenin was added in the presence or absence of $100 \mu\text{M}$ L-NAME, $10 \mu\text{M}$ LY 294,002, $10 \mu\text{M}$ PD 98,059, or $50 \mu\text{M}$ neomycin in EBM-2 supplemented with 1% FBS. (A) Pictures of each cell were taken at 0 h (upper lane) or 12 h (lower lane) after treatment with each sample. (B) Number of migrating cells was measured using ImageJ software (v1.40).

for NOS activation. The threshold for NOS activation, between $250 \text{ ng}/\text{mL}$ and $1 \mu\text{g}/\text{mL}$, can be related to angiogenin plasma concentration. In particular, the human plasma concentration of angiogenin is reported to be $359.0 \pm 59.9 \text{ ng}/\text{mL}$ (26). Therefore, adding $250 \text{ ng}/\text{mL}$ may be not enough to trigger NO production, as it is similar to basal concentrations of angiogenin.

Furthermore, the typical time scale of this NO synthesis is consistent with the behavior of other angiogenic factors.

For instance, vascular endothelial growth factor (VEGF) induces NOS activity, and a maximum is reached after 20 min (27). However, NO levels usually increase directly after addition of the growth factor, which is different from what we report for angiogenin, as the response is here delayed by typically 5 min.

This delay time in NO synthesis is consistent with the evidence that nuclear translocation is a critical step in the NOS activation pathway. Indeed, Xu et al. have shown that nuclear translocation

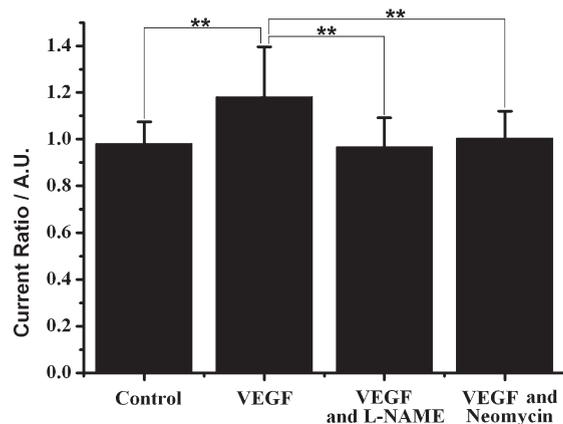


FIGURE 5: Effects of neomycin on VEGF-induced NO synthesis. Summary of the current ratios obtained for differential pulse voltammograms performed in HUVEC culture before and 2 h after addition of 200 ng/mL VEGF. VEGF was added in the presence or absence of 100 μ M L-NAME or of 50 μ M neomycin. $n > 18$; **, $p < 0.01$.

of angiogenin is dose–time dependent (20). For instance, at 300 ng/mL, angiogenin can be detected in the nucleus after 30 min. However, at 1 μ g/mL, angiogenin is localized into the nucleus after 10 min, which is consistent with the no-response time observed for NO synthesis and calcium influx at this concentration.

Furthermore, it has been reported by Kishimoto et al. that nuclear translocation of angiogenin is a general requirement for angiogenesis (6). This led to investigation of the effect of nuclear translocation, and of other angiogenin properties, on NO release to understand the biochemical cascade leading to NOS activation.

Indeed, angiogenin is known to have several physiological effects on cells (5): (1) angiogenin can bind to the actin receptors and cleave the extracellular matrix, thus allowing cell migration (28); (2) it has a RNase activity (29); (3) it can bind to cell membrane receptors and trigger an intracellular biochemical cascade (probably via signal-associated kinases ERK 1/2 (30) and protein kinase B/Akt (31) or via the phosphorylation of stress-associated kinase SAPK/JNK in smooth muscles (20)); (4) it can enter the cell nucleus and interact with ribonucleotides (nuclear translocation) (20). Nuclear translocation is time and dose dependent.

In this study, selective inhibitors for nuclear translocation (neomycin), for ERK 1/2 activity (PD 98,059), and for PI-3 kinase (LY 294,002) (thus inhibiting the downstream Akt kinase) have been used.

It is well established that the aminoglycoside antibiotic neomycin blocks nuclear translocation of angiogenin (32, 33) in endothelial cells and inhibits angiogenesis by angiogenin, acidic fibroblast growth factor, basic fibroblast growth factor, and epidermal growth factor, all of which undergo nuclear translocation, but does not have this effect on vascular endothelial growth factor which is not translocated. Relevant to this study is the observation that it blocks endogenous ATP-induced Ca^{2+} transients (34) and associated NO production in response to hypotonic stress. NO production *per se* is not inhibited by neomycin; rather it is the Ca^{2+} transients that are inhibited. These transients were observed some 100 s following hypotonic stress, a similar lag to that observed here following angiogenin administration, consistent with angiogenin involvement. Given the ability of neomycin to inhibit angiogenesis, it is unsurprising that it has been investigated as an antineoplastic agent (35) and that

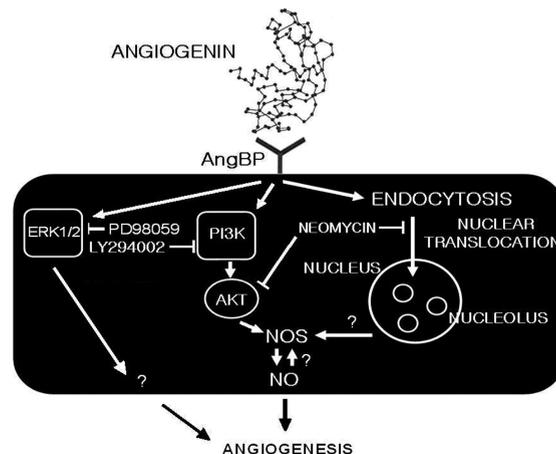


FIGURE 6: Proposed pathway for angiogenin-induced eNOS activation.

consequently its pharmacology has been widely studied. Other actions include inhibition of phospholipase C (36, 37). It is a calcium sensory receptor agonist (38) and can increase ERK 1/2 phosphorylation. Of these diverse pharmacological actions, it is neomycin's ability to block angiogenin nuclear translocation that is of major relevance here.

NO synthesis, eNOS activation and localization, and cell migration being all inhibited by neomycin, this would tend to show that nuclear translocation is involved in these phenomena. These results have also been related to the observed delayed response time reported above for angiogenin-induced NOS activity. Furthermore, this also supports the fact that angiogenin interaction with nucleus ribonucleotides is a critical step for angiogenesis. However, the VEGF experiments demonstrate that neomycin does not solely inhibit angiogenin nuclear translocation, as VEGF does not translocate into the nucleus. It has been reported by Miyazaki et al. that neomycin attenuates Akt kinase phosphorylation downstream of PI-3 kinase (21). Akt kinase being an important mediator for VEGF-induced NO synthesis (39, 40), this result demonstrates that other effects of neomycin have to be taken in account in angiogenic factor-induced angiogenesis. As a consequence, we cannot conclude yet over the role of nuclear translocation in the NO physiology.

Concerning the kinase pathways, the PI-3/Akt kinase pathway mediates NOS activation and eNOS cellular localization and phosphorylation, unlike ERK 1/2. This has again been related to the mechanisms of other angiogenic factors inducing NO release, such as VEGF. Indeed, VEGF also activates eNOS through PI-3 and Akt kinases (39, 40). However, in the case of the wound healing experiments, PD 98,059 totally inhibited cell migration, as well as LY 294,002. This indicates that the ERK 1/2 signal pathway is an important signal transduction cascade for cell growth and angiogenesis but does not mediate NOS activation. In addition, localization of NOS has been observed for other angiogenic factors, notably 17 β -estradiol (E2). Grasselli et al. (41) have demonstrated eNOS and estrogen 2 α receptor colocalization in the endothelial cell nucleus in response to E2 which is inhibited by L-NAME. Inhibition of eNOS localization by L-NAME, as noticed in our study and for E2 (41), is also an indication of a possible positive feedback loop of NO levels on eNOS.

Figure 6 summarizes the results presented here for angiogenin-induced eNOS activation. We suggest that angiogenin binds to a membrane receptor, thus transducing an intracellular signal

through PI-3 and Akt kinases. At the same time, endocytosis and nuclear translocation of angiogenin, and therefore possible interaction of angiogenin with ribonucleotides, may also have an effect on this phenomenon.

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