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Antiangiogenesis Efficacy of Nitric Oxide Donors

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Abstract Angiogenesis is a complex process involving endothelial cell migration, proliferation, invasion, and tube formation. Inhibition of these processes might have implications in various angiogenesis-mediated disorders. Because nitric oxide (NO) is known to play a key role in various vascular diseases, the present study was undertaken to determine the role of NO in angiogenesis-mediated processes using the NO donor, *S*-nitroso *N*-acetyl penicillamine (SNAP) and *S*-nitroso *N*-acetyl glutathione (SNAG). The antiangiogenic efficacy of these NO donors was examined using in vivo and in vitro model systems. The in vitro studies demonstrated the ability of SNAP to inhibit cytokine fibroblast growth factor (FGF2)-stimulated tube formation and serum-induced cell proliferation. The inhibitory effect on cell proliferation by SNAP concentrations above the millimolar range was associated with significant shifts in the concentration of NO metabolites. Furthermore, using the mouse Matrigel implant model and the chick chorioallantoic membrane (CAM) models, SNAP demonstrated maximal inhibitory efficacy (85–95% inhibition) of cytokine (FGF2)-induced neovascularization in both in vivo models. SNAP and SNAG resulted in 85% inhibition of FGF2-induced neovascularization in the mouse Matrigel model when given at 5 mg/kg/day infusion in minipumps during 14 days and 87% inhibition of angiogenesis induced by FGF2 in the CAM when administered a single dose of 50 μg. Thus, NO donors might be a useful tool for the inhibition of angiogenesis associated with human tumor growth, or neovascular, ocular, and inflammatory diseases. J. Cell. Biochem. 80:104–114, 2000.

Key words: nitric oxide donors; angiogenesis; SNAP; SNAG

Angiogenesis is the development of new blood vessels from preexisting blood vessels. Physiologically, angiogenesis prepares the womb for egg implantation, ensures proper development of mature organisms, and plays a key role in wound healing. By contrast, it supports the pathological conditions associated with a number of disease states such as cancer, inflammation, and ocular diseases. Angiogenesis or neovascularization is a multistep process controlled by the balance of pro- and antiangiogenic factors. The latter stages of this process involve proliferation and the organization of endothelial cells (ECs) into tubelike structures. Mitogenic cytokines such as cytokine fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF) are thought to be key players in promoting EC growth and differentiation.

The endothelial cell produces a number of factors that regulate vascular tone and growth. Nitric oxide (NO), the free-radical gas, is one of the most important regulators and is formed as a byproduct of the conversion of L-arginine to L-citriculline [Ignarro et al., 1987; Palmer et al., 1987, 1988]. NO is synthesized by one of three enzyme isoforms of nitric oxide synthase (NOS). Endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) are constituitively expressed, but inducible nitric oxide synthase (iNOS) is expressed in response to various cellular stimuli. Hence, the iNOS isoform is thought to be the key regulator of NO formation in response to cytokine stimulation. In addition to the well-characterized vasodilatory and antiplatelet effects, NO also regulates the proliferation and migration of vascular smooth-muscle cells and fibroblasts [Garg and Hassid, 1989; Dubey et al., 1995; Cui et al., 1994]. Recently, it has been demonstrated that increased expression of iNOS and nitrite production induced by lipopolysaccharide are correlated with the progression angiogenesis [Pipili-Synetos et al., 2000].

The EC is the pivotal cellular component of the angiogenic process and responds to many cytokines through its cells surface receptors

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and intracellular signaling mechanisms. ECs in culture are capable of forming tubelike structures with a lumen. Therefore, ECs are not only a prerequisite for neovascularization, but the basal structural requirement as well. Because of the potent effects of NO as both a vasodilator and regulator of endothelium permeability, the idea that NO plays a pivotal role in angiogenesis is understandable. In fact, the data generated thus far are conflicting and demonstrate both proangiogenic and antiangiogenic effects. The arginine analog, N-nitro-L-arginine methyl ester (L-NAME) enhances FGF2-mediated angiogenesis, but not VEGF-mediated angiogenesis in an in vivo model of angiogenesis [Norbby, 1998]. Both EC differentiation and proliferation are altered in a dose-dependent manner by NO modulating compounds [RayChaudhury et al., 1996; Babaei et al., 1998].

We hypothesize that NO generated by high concentrations (greater than millimolar) of NO donors abrogates cytokine-induced angiogenic responses in vitro and in vivo. The purpose of this study was to determine whether alteration of NO levels plays an important role in the inhibition of angiogenesis and should be considered as a therapeutic target.

MATERIALS AND METHODS

Reagents

NO donor, *S*-nitroso *N*-acetyl penicillamine (SNAP) and *S*-nitroso *N*-acetyl glutathione (SNAG) were obtained from Research Biochemical International, (Natick, MA). The basic form of FGF2 was purchased from Gibco BRL (Gaithersburg, MD) and R&D Systems (Minneapolis, MN). Endothelial Basal Medium (EBM) (modified MCDB 131) was purchased from Clonetics, Inc. (Walkersville, MD). Endothelial Growth Medium (EGM), which was purchased from Clonetics, Inc., also contained human recombinant endothelial growth factor (hEGF) 10 ng/ml, hydrocortisone 1 mg/ml, gentamicin 50 mg/ml, amphotericin-B 50 μg/ml, blood brain extract 12 μg/ml, and 2% fetal bovine serum and was formulated by supplementing EBM. The matrix Matrigel basement membrane was purchased from Becton Dickinson (Bedford, MA).

Animals

Animal studies were conducted in a facility fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Female C57B1/6 mice, 6–8 weeks old (Charles River Labs, Wilmington, MA) were housed 10 per cage in a room maintained at 20 ± 2°C with a humidity of 50 ± 10% and a 12-h light–dark cycle. The animals were fed a standard pelleted mouse chow (Agway Inc., Waverly, NY) and water was available ad libitum.

Surgical Minipump Implantation

Minipumps (no. 2002) were purchased from Alza Co. (Palo Alto, CA). Three hours before implantation, pumps were filled with saline or test compounds. Pumps were then placed in approximately 5 ml of phosphate-buffered saline (PBS) and incubated at 37°C to initiate the flow.

Animals were anesthetized with a 25 mg/kg and 5 mg/kg xylazine (Rompun) mix. Using a scalpel, a small 2-cm incision was made on the dorsal skin near the scapula of the mouse. A small tunnel, 1-inch long, was made subcutaneously. The pump was inserted and the incision was closed with a clip.

Matrigel Preparation and Assay

The murine Matrigel model was conducted according to previously described methods [Kibbey et al., 1992]. Matrigel (Becton Dickinson) was thawed overnight at 4°C and placed on ice. Aliquots of Matrigel were placed into cold polypropylene tubes and FGF2 (R & D Systems) was added to a final concentration of 500 ng/ml. Tubes were mixed for a minimum of 3 h by rotating end over end at 4°C.

Matrigel with FGF2 500 μg/ml or without FGF2, as in the case of negative controls, was subcutaneously injected into the ventral midline of the mice. At day 14, the mice were killed and the solidified gels were resected. Gels were then analyzed for the presence of new vessels.

Hemoglobin Assay

The resected gels (as mentioned above) were analyzed for neovascularization by a method previously described by Okada et al. [1995]. Control and experimental gel implants were placed in a microcentrifuge tube containing 0.5 ml of cell lysis solution (Sigma, St. Louis, MO) and were crushed with a pestle. Subsequently, the tubes were allowed to incubate overnight at 4°C and centrifuged at 1,500*g* for

15 min on the following day. A 200-μl aliquot of cell lysate was added to 1.3 ml of Drabkin's reagent solution (Sigma) for each sample. The solution was then analyzed on a spectrophotometer at a 540-nm wavelength. The absorption of light is proportional to the amount of hemoglobin contained in the sample.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics. HUVECs were grown to 80–90% confluence in EGM containing hEGF 10 ng/ml, hydrocortisone 1 mg/ml, gentamicin 50 mg/ml, amphotercin-B 50 μg/ml, Blood Brain Extract 0.012 mg/ml, and 2% fetal bovine serum equilibrated with 95% air/5% CO^2 at 37°C. HUVEC cells were serially passaged and maintained in endothelial growth medium in cell culture flasks with coated with gelatin 0.2% (Sigma). Confluent cultures of ECs between the third and sixth passages were washes with Hanks' balanced salt solution and harvested using 0.025% trypsin /0.01% EDTA and counted by hemacytometer. ECs were resuspended in 24-well plates coated with Matrigel matrices or directly on 96-well cell culture plates. ECs were incubated 72 h in basal medium or growth medium in the presence or absence of SNAP with a concentration range of 0.1 nM to 10 mM.

Endothelial Cell Differentiation

Differentiation by ECs was examined using a method developed by Grant et al. [1991]. Matrigel growth factor reduced (GFR) (Becton Dickinson) was thawed overnight at 4°C. Using cold pipette tips, 250 μl of Matrigel GFR was placed in a cold 24-well multiwell plate (Falcon). Matrigel GFR was allowed to polymerize during incubation at 37°C for 30 min. Cells were trypsinized and centrifuged and subsequently washed twice in PBS. After counting, HUVE cells were plated at 40,000 cells/well in EBM containing FGF2 50 ng/ml at an initial volume of 125 μl in a 24-well plate (Corning). After 1–2 h of incubation at 37°C with 5% CO_2 and 95% humidity to allow cell attachment, 125-μl samples containing varied concentrations of SNAP dissolved in EBM medium were added. Plates were incubated overnight at 37°C with 5% CO_2 and 95% humidity. Subsequently, the medium was decanted and cells were fixed and stained using a modified Hema 3 Stain kit (Fisher, Swedesboro, NJ).

Microscopic Analysis of Endothelial Cell Differentiation

CAM tissue directly beneath an FGF2-saturated filter disk was resected from embryos treated 48 h previously with compounds or controls. Tissues were washed three times with PBS. Sections were placed in a 35-mm Petri dish (Nalge Nunc, Rochester, NY) and examined under a SV6 stereomicroscope (Karl Zeiss, Thornwood, NY) at 400× magnification. Digital images of microtiter well sections were collected using a DKC5000 3-CCD color video camera system (Sony Corporation, Inc., Japan) and analyzed with the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The area and major axis length of stained cells having a tubular morphology on the Matrigel surface counted from five images/well. Area data are expressed in units of 10^4 μm^2 and length data are expressed as length/area in units of mm/mm^2. Percent inhibition data are expressed as the quotient of the experimental value minus the negative control value (EBM medium) divided by the difference between the positive control value and the negative control value.

Cell Proliferation Methods

EC proliferation was assessed by a colorimetric method, the Cell Titer AQ Assay kit (Promega, Madison, WI), which is based on the cellular conversion of a tetrazolium compound to a formazan product [Denizot and Lang, 1986]. Cells were trypsinized, centrifuged, and subsequently washed twice in PBS. After counting, HUVE cells were plated at 5,000 cells/well in EBM or EGM medium (Clonetics) at an initial volume of 50 μl in a 96-well plate (Corning). After 1–2 h of incubation at 5% CO_2 and 95% humidity to allow cell attachment, 50-μl samples containing varied concentrations of SNAP dissolved in the appropriate medium were added. After incubating 72 h, 20 μl of the tetrazolium compound and phenazine methosulfate (MTS/PMS) solution was added to each well of the 96-well assay plate containing the cells. The plate was incubated for 1–4 h at 37°C in a humidified 5% CO_2 atmosphere. The absorbance was read at 490 nm using an enzyme-linked immunosorbent assay plate reader.

EC proliferation was also determined by a fluorometric method using the CYQUANT Cell Proliferation Assay kit (Molecular Probes, Eugene, OR), which is based on the fluorescent labeling of cellular DNA [Betel et al., 1979]. Cells were trypsinized, centrifuged, and subsequently washed twice in PBS. After counting, HUVE cells were plated at 10,000 cells/well in EBM or EGM defined medium at an initial volume of 150 μl in a 48-well plate (Corning). After 1–2 h of incubation at 37°C with 5% CO_2 and 95% humidity to allow cell attachment, 150-μl samples containing varied concentrations of SNAP dissolved in the appropriate medium were added. After incubating for 72 h, the medium was removed and the microtiter plate was blotted on to paper towels. Then 200 μl of lysis buffer was added and cells were subsequently frozen thawed. After plates returned to room temperature, 200 μl of 4x CYQUANT GR dye was added. Cells were incubated for 5 min at room temperature. The sample fluorescence was measured with a CytoFluor plate reader (Perseptive Biosystems, Framingham, MA) with filters of 495 nm and 530 nm for emission and excitation, respectively.

Nitrate/Nitrite Measurement

NO in the culture medium was measured by using a Nitrate/Nitrite Fluorometric Assay kit (Alexis Corp., San Diego, CA) based on the methods of Miles et al. [1996]. Aliquots (usually 80 μl) of filtered conditioned cell medium (EBM or EGM) collected from SNAP-treated cells and control cells were place in a 96-well microtiter plate. Nitrate was reduced to nitrite by the addition of 10 μl of nitrate reductase solution to each well according to the protocol. Then 50 μl each of Greiss Reagent I (sulfanilamide) and Greiss Reagent II (*N*-1-naphthylethylenediamine) were added to the supernatant fluid and incubated for 10 min. Fluorescence was measured with filters of 365 nm and 450 nm for emission and excitation, respectively, using a SpectraMax reader (Molecular Devices, Sunnyvale, CA).

Neovascularization on the Chick Chorioallantoic Membrane

In vivo neovascularization was examined by the method previously described by Auerbach et al. [1974]. Ten-day-old embryos were purchased from Spafas, Inc. (Preston, CT) and were incubated at 37°C with 55% relative humidity. A small hole was punctured with a hypodermic needle in the shell concealing the air sac. A second hole was punctured in the shell on the broadside of the egg directly over an avascular portion of the embryonic membrane, as observed during candling. A false air sac was created beneath the second hole by the application of negative pressure to the first hole, which caused the chorioallantoic membrane (CAM) to separate from the shell. A window, approximately 1.0 cm^2, was cut the in the shell over the dropped CAM with the use of a small crafts grinding wheel (Dremel, Division of Emerson Electric Company, Racine, WI), which allowed direct access to the underlying CAM.

Filter disks of no. 1 filter paper (Whatman International, United Kingdom) were soaked in 3 mg/ml cortisone acetate (Sigma, St. Louis, MO) in a solution of 95% ethanol and water and subsequently air dried under sterile conditions. FGF2 (Life Technologies, Gaithersburg, MD) was used to grow vessels on the CAMs of 10-day-old chick embryos. Sterile filter disks adsorbed with FGF2 dissolved in PBS at 1 μg/ml were placed on growing CAMs. At 24 h, test compounds or control vehicle were added directly to CAMs topically.

Microscopic Analysis of CAM Sections

CAM tissue directly beneath FGF2-saturated filter disk was resected from embryos treated 48 h previously with compound or control. Tissues were washed three times with PBS. Sections were placed in a 35-mm Petri dish (Nalge Nunc) and were examined under a SV6 stereomicroscope (Karl Zeiss) at 50× magnification. Digital images of CAM sections adjacent to filters were collected using a 3-CCD color video camera system (Toshiba America, New York, NY) and analyzed with the Image-Pro Plus software (Media Cybernetics). The number of vessel branch points contained in a circular region equal to the area of a filter disk was counted for each section. Percent inhibition data are expressed as the quotient of the experimental value minus the negative control value divided by the difference between the positive control value and the negative control value.

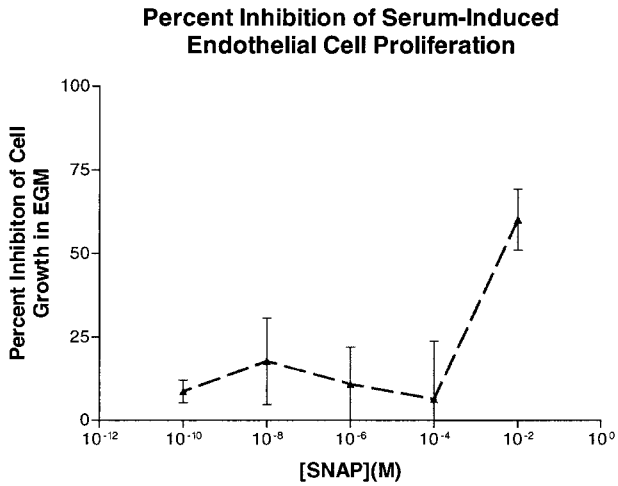

Fig. 1. Measurement of HUVE cell proliferation/survival after exposure to SNAP at various concentrations. HUVE cells were washed and plated at 5,000 cells/well in EBM (basal) or EGM well-defined medium. After 72 h, MTS/PMS solution was added and the plate was incubated for 1–4 h at 37°C. The absorbance was read using an enzyme-linked immunosorbent assay plate reader. Each point represents mean ± SEM, n = 6.

TABLE I. Nitric Oxide Metabolite Concentrations in Cell Medium[a]

	Nitrate + Nitrite (μM)	Nitrite (μM)	Nitrate (μM)
EGM	29.8 ± 3.4	13.8 ± 3.6	16.0
EGM + SNAP (1 mM)	677.1 ± 30.2	172.3 ± 2.7	504.9
EBM	35.6 ± 4.2	14.2 ± 2.7	21.3
EBM + SNAP (1 mM)	596.1 ± 59.3	156.5 ± 3.1	439.6

[a]Concentrations of NO metabolites were measured in the medium of HUVE cell after exposure to SNAP at various concentrations for 72 h. Medium samples were processed and subjected to a fluorometric assay. Values of the combined nitrate + nitrite concentration and the nitrite concentration represent the mean ± SEM, N = 3. The nitrate value is derived form the difference of the means of the two measured values.

Statistical Analysis

Statistical significance for data presented in these experiments was analyzed by the Student's *t*-test or analysis of variance (ANOVA).

RESULTS

Effects of SNAP on Endothelial Cell Proliferation

Treatment of HUVE cells with varied concentrations of SNAP abrogated cell proliferation. In cultures, growing in basal conditions, SNAP exhibited a dose-dependent stimulation of survival or growth (data not shown). In cultures undergoing serum-induced cell proliferation, 10 mM SNAP significantly inhibited cell proliferation by 60% (Fig. 1). Lower concentrations of the NO donor did not significantly decrease cell proliferation. These results were equivalent regardless of method used to analyze the cell population. Determination of nitrate and nitrite concentrations by Greiss reaction revealed that serum-induced cultures contain NO metabolites at an almost 1:1 ratio (Table I). The concentration of nitrate and nitrite in proliferating cultures was lower than basal cultures, although not significantly. However, more nitrates were detectable in both conditions. The addition of the NO donor, SNAP, at 10 mM raised the concentration of the NO metabolites nitrite and nitrate by greater than 10-fold and 20-fold, respectively (Table I). However, perhaps more significantly, the metabolite ratio, nitrate to nitrite, changed from nearly equimolar to greater than 3:1.

Inhibition of Angiogenesis Through Dosing of NO Donors

We examined the level of cytokine-induced angiogenesis that developed in a Matrigel plug containing FGF2 when exposed to NO donors. As shown in Table II, FGF2 containing Matri-

TABLE II. Inhibition of Neovascularization in the Murine Matrix Implant Model

Group	Dose[a] (mg/kg)	Hemoglobin[b] (g/dl)	SEM	% Inhibition
Negative control		0.45	0.10	
Positive control		1.45	0.16	
SNAP	5	0.63	0.07	85
SNAG	5	0.63[c]	0.14	85

[a]Subcutaneous minipump.
[b]Mean, n = 5.
[c]$P < 0.05$ versus positive control.

gel induces about a twofold increase in the amount of hemoglobin found in the excised Matrigel. Both NO donors, SNAP and SNAG, abrogated the neovascularization of the Matrigel significantly (Student's *t*-test, $P < 0.05$; n = 5). Treatment of the mice with 5 mg/kg of SNAP or SNAG after surgical implantation of a subcutaneous minipump resulted in 85% decrease in the angiogenesis index (Table II).

Dose-Dependent Inhibition of Angiogenesis in the CAM Model

Our previous experiment demonstrated that NO donors are capable of inhibiting angiogenesis. To determine whether this effect of NO on FGF2- induced angiogenesis is dose dependent, we treated 10-day-old fertilized chick embryos with filters saturated in PBS, filters saturated in FGF2 (1 μg/ml), or filters saturated with FGF2 (1 μg/ml) + SNAP (0.5 μg, 5.0 μg, and 50 μg). In the presence of FGF2 alone, adjacent CAM membranes undergo morphological changes and become vascularized. The extent of neovascularization in FGF2 treated CAMs is approximately 1.7-fold greater than control CAMs. A dose-dependent relationship existed between the amount of SNAP and the level of inhibition observed (Fig. 2A,B and Fig. 3). The level of inhibition observed (87%) at the highest concentration is significant (ANOVA; $P < 0.05$). SNAP proved to be an effective inhibitor of cytokine-induced angiogenesis with factors other than FGF2 (e.g., hepatocyte growth factor (HGF)) as well (data not shown). The concentration of NO metabolites in the CAM fluid was assayed by the Greiss reaction. The addition of 5 μg and 50 μg of SNAP to the fertilized chicken embryos was capable of raising the concentration of NO metabolites in the CAM fluid. This aqueous fluid, which is adjacent to the CAM membrane, had a nitrate concentration range of 270–370 nM and a nitrite concentration range of 72–101 nM (data not shown).

Inhibition by an NO Donor in an In Vitro Model of Angiogenesis

The later phases of neovascularization require ECs to undergo morphological changes that give rise to tubular structures possessing lumens. We further investigated the angiogenesis phenomena in an in vitro setting by examining EC differentiation on Matrigel matrices. Cultures of HUVECs were plated at a subconfluent level on solidified Matrigel in 24-well plates and incubated in the presence of FGF2 (50 ng/ml) with and without SNAP at various concentration ranging from 1 μM to 1 mM. HUVECs cultured in this assay form tubelike structures on the surface of the matrix. By contrast, HUVECs treated with 1 μM SNAP and higher failed to develop the level of tubes associated with control cultures. Quantitative measurements of tube formation revealed that SNAP inhibited the cytokine-stimulated tube formation by 116% (Fig. 4A,B). The results demonstrate that the inhibitory effects of NO donors may lie in their ability to prevent this critical step in the angiogenic process.

DISCUSSION

Previous studies have investigated the effect of NO-modulating compounds on angiogenesis using inhibitors and donors of NO. The in vivo and in vitro systems used for studying the modulation of angiogenesis by NO have demonstrated some interesting and often contradictory results [Pipili-Synetos et al., 1995; RayChaudhury et al., 1996; Norbby, 1998; Babaei et al., 1998]. In adult organisms, angiogenesis remains quiescent for the most part with a few exceptions—uterine preparation for fertilized egg implantation, wound healing, and that associated with pathological states. Using FGF2 with a synthetic matrix and two NO donors, we have identified NO as a potent inhibitor of the angiogenic process in vitro and in vivo. The angiogenic index provided an indirect measure of the extent of vascularization that invades the subcutaneous Matrigel plug containing the cytokine. Efforts to block FGF2-induced neovascularization with L-NAME

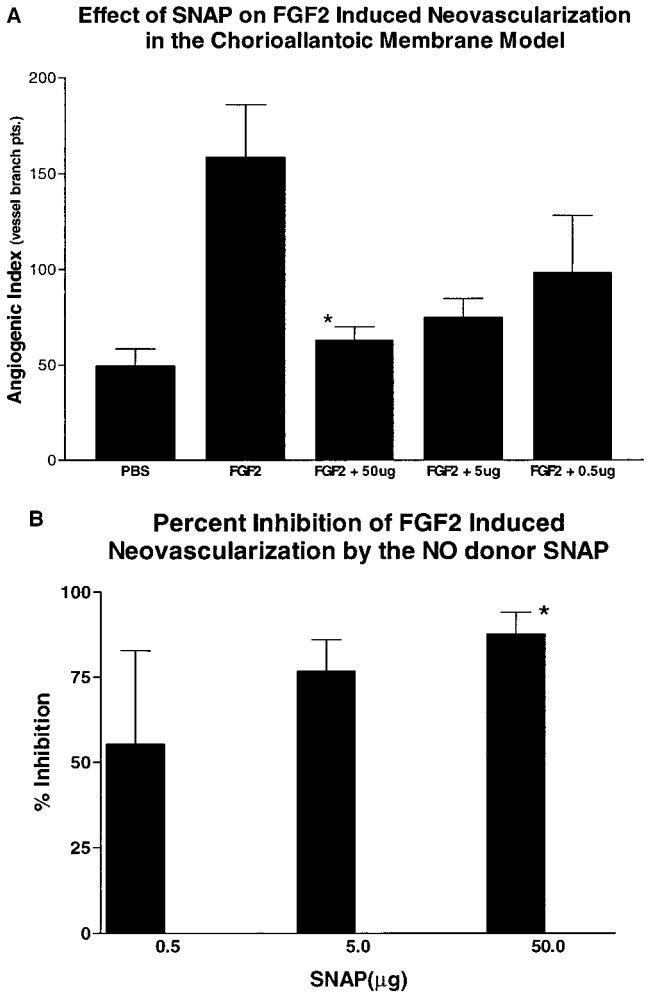

Fig. 2. NO donors dose-dependently inhibit neovascularization induced by FGF2 in vivo. CAMs of 10-day-old chicken embryos were treated with FGF2- or PBS-saturated filters. After 24 h, various amounts of SNAP were applied to FGF2-treated CAMs. **A:** After 48 h, CAMs were resected, photographed, and measurements were made by image analysis. **B:** The percent inhibition of neovascularization observed at each dose of SNAP. Each bar represents mean ± SEM, n = 3. $*P < 0.05$, ANOVA for difference between FGF2 treated and FGF2 with SNAP.

proved to be less significant and may have actually been enhanced in some cases. Increases in angiogenesis in patients with metastatic or inflammatory diseases are due in part to a shift in the balance of angiogenic factors. This pathophysiological angiogenesis may disturb the integrity of normal tissue adjacent to site of disease. Additionally, the new blood vessels serve as a conduit to increase the presence of nutrients and inflammatory mediators and proteins. Because of the difficulty in measuring local NO metabolite concentrations, it is hard to say with complete certainty that overproduction of NO metabolites is the responsible mechanism, but data examining the subprocesses of angiogenesis would support that concept. Our results are consistent with recent reports by Norbby [1998], which identified NO as suppressor FGF2-mediated angiogenesis in a different in vivo model. Furthermore, our data support the notion put forth by Pilipi-Synetos et al. [2000] by using a direct pharmacological method for increasing NO, we have demonstrated the antiangiogenic effects of NO in vivo and in vitro.

Proliferation and differentiation of ECs is a key role in the angiogenic process—in the pathophysiological state, because this particular cell population undergoes phenotypic and morphological changes, as evidenced by increased growth and tube formation. This study has identified NO as both an inhibitor and a

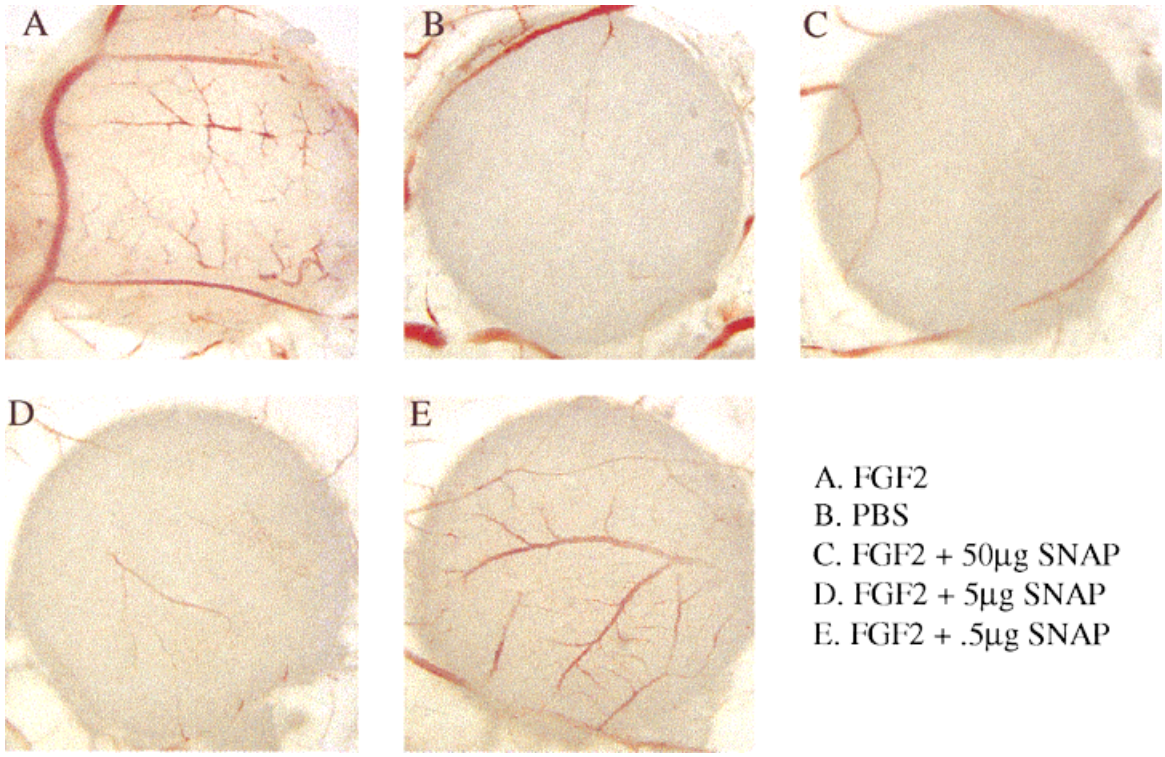

Fig. 3. NO donors dose-dependently inhibit neovascularization induced by FGF2 in vivo. CAMs of 10-day-old chicken embryos were treated with FGF2- or PBS-saturated filters. After 24 h, various amounts of SNAP were applied to FGF2-treated CAMs. **A–E:** Representative photographs of CAM sections taken at 72 h.

stimulator of EC proliferation. Our results are consistent with those of RayChaudhury et al. [1996] and Lopez-Farre et al. [1997]. It has been shown that treatment of proliferating EC cultures with high concentrations (>1 mM) of the NO donor, SNAP, inhibits serum-induced growth. The levels of nitrite and nitrate are not significantly higher in basal cultures than in serum-induced cultures. Under these conditions, both NO metabolites are found at nearly equimolar concentrations. Malinski et al. [1993] showed that after transient cerebral arterial occlusion, rat brain tissue was reported to have levels up to 4 μM. Thus, in situations of transient oxygen deprivation, antiproliferative concentrations of NO may develop in the microenvironment before entering apoptosis.

Shifting the NO metabolite concentration upward in excess of 10- and 20-fold for nitrite and nitrate, respectively, causes inhibition of the growth of serum-stimulated EC cultures by 60%. For instance, it may involve the turning on of the cGMP pathway to promote growth or the activation of other proteins such as heat shock, hemoxygenase-1, or cyclooxygenase-2 that attenuate apoptosis. Others have shown that NO may block apoptosis and certain toxic insults in ECs and that, specifically, tumor necrosis factor-α-induced apoptosis is abrogated via inhibition of caspases by NO donors.

The morphological changes of EC that occur during angiogenesis are not resistant to the effects of SNAP. We have shown that a low dose of SNAP, relative to that required to effect cell survival, has negative effects on these angiogenesis-associated changes. SNAP (1 μM) significantly interrupts the formation of tubelike structures on Matrigel matrices on stimulation by FGF2. This concentration is approximately 1,000-fold less than that observed to abrogate proliferation. Considering that NO has multiple pathways through which it may signal, the concentration of NO present in the intracellular compartment may dictate which pathways are activated. Thus, there is a possibility that the commonly used biochemical trigger *S*-nitrosylation or nitration may follow some hierarchical propensity for protein involved in cytoskeletal organization. Of course, there is also the added consideration that the integrins of ECs on Matrigel are more ac-

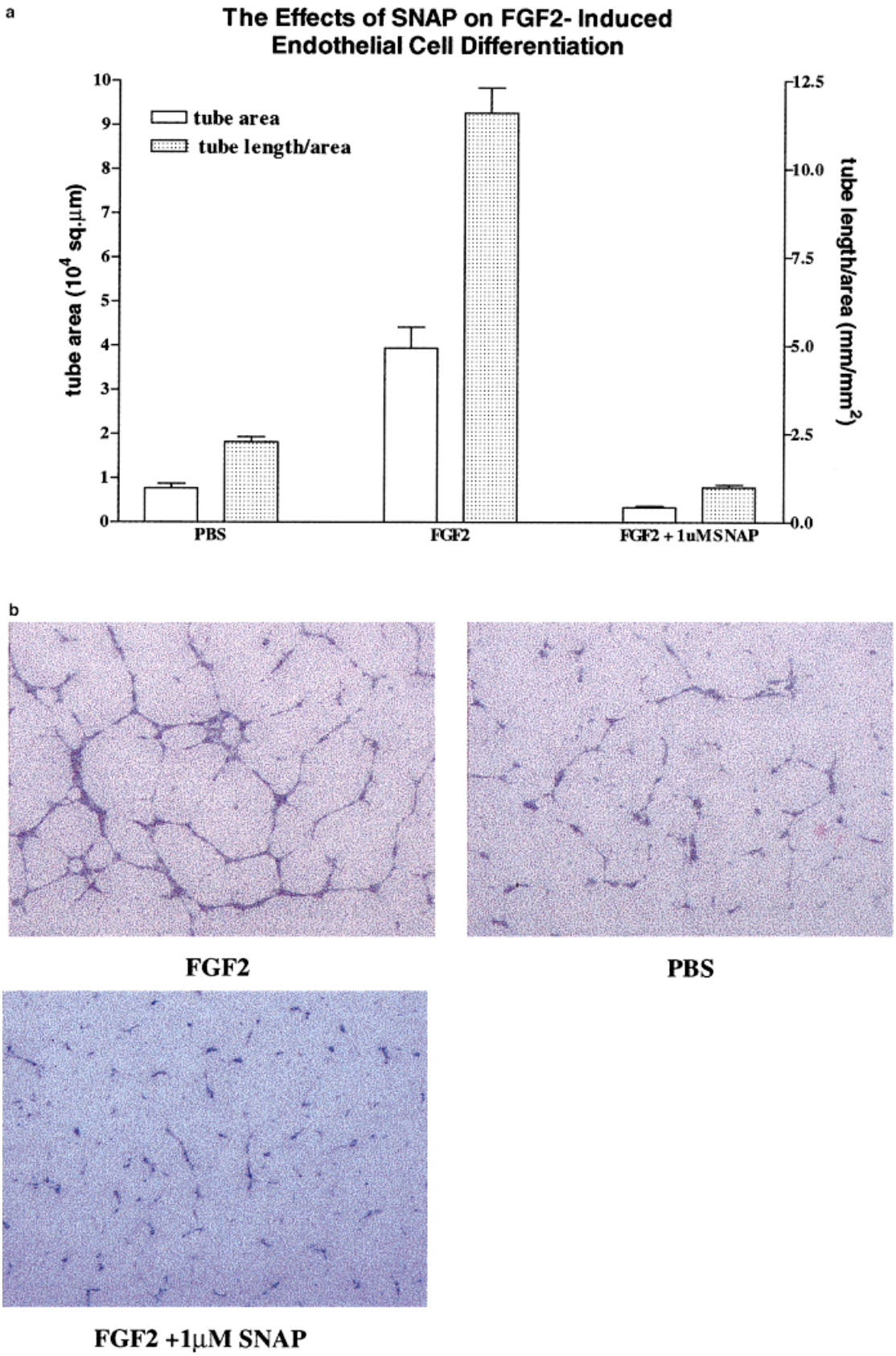

Fig. 4. NO donors inhibit differentiation of HUVE cells induced by FGF2 in vitro. Cells were plated at subconfluent levels on Matrigel matrices and incubated at 37°C in 5% CO_2 and 95% humidified atmosphere. **A:** After 16 h, cells were stained, photographed, and measurements were made by image analysis. Each bar represents mean ± SEM, n = 4. **B:** Representative photographs of endothelial cells taken at 16 h.

tively engaged in the processes occurring there as opposed to proliferation on tissue-culture-treated plastic.

Using FGF2 with a synthetic matrix and SNAP, we have identified SNAP as a dose-dependent inhibitor of the angiogenic process in vivo. The angiogenic index provided a direct measure of the extent of vascularization that is contained in an area of the CAM. Significant inhibition of the angiogenic response in this model was seen at only at the higher amounts of SNAP. This may suggest that there is some threshold level of NO required to activate an NO-dependent antiangiogenic response that is not being achieved by lower amounts of SNAP. Efforts to block cytokine-induced neovascularization with factors other than FGF2 (e.g., HGF) with SNAP proved to be effective as well (data not shown). From measurements of NO metabolite concentrations in the adjacent CAM fluid, we did not find significant differences between treated and control embryos. We believe that the volume of CAM fluid in the embryo reduces the magnitude of the change observed and does not reflect the change that occurs in the microenvironment. In experiments by RayChaudhury et al., they found that a single exposure to NO inhibits EC proliferation for up to 72 h, with recovery thereafter. Our vascular measurements in this paradigm are taken at 48 h after NO exposure.

Modulation of angiogenesis is context driven, depending on the particular stimuli that shift the balance and the integrin-mediated intracellular signaling pathways. It has been demonstrated that NO donors, SNAP and SNAG, are capable of significantly inhibiting (85%) FGF2-induced angiogenesis in the murine matrix implant model. Similarly, in the CAM model, SNAP is capable of inhibiting angiogenesis in a dose-dependent manner as examined by measurements of vascular density. Additionally, we have shown in vitro that SNAP is also capable of inhibiting tube formation. Thus, the notion that NO donor compounds may have a place in the therapeutic arsenal for the inhibition of angiogenesis associated with metastatic, neovascular ocular, and inflammatory diseases is supported by these findings.

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